MicroRNA-9a ensures the precise specification of sensory organ precursors in *Drosophila*

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MicroRNAs (miRNAs) have been implicated in regulating various aspects of animal development, but their functions in neurogenesis are largely unknown. Here we report that loss of *miR-9a* function in the *Drosophila* peripheral nervous system leads to ectopic production of sensory organ precursors (SOPs), whereas overexpression of *miR-9a* results in a severe loss of SOPs. We further demonstrate a strong genetic interaction between *miR-9a* and *senseless (sens)* in controlling the formation of SOPs in the adult wing imaginal disc. Moreover, *miR-9a* suppresses *Sens* expression through its 3' untranslated region. *miR-9a* is expressed in epithelial cells, including those adjacent to SOPs within proneural clusters, suggesting that *miR-9a* normally inhibits neuronal fate in non-SOP cells by down-regulating *Sens* expression. These results indicate that *miR-9a* ensures the generation of the precise number of neuronal precursor cells during development.

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The peripheral nervous system (PNS) is essential for animals to detect and relay environmental stimuli to central neurons for information processing. It is largely unknown what ensures the development of precise numbers of sensory organs for a particular external sensory cue. The *Drosophila* PNS has been used as an excellent model system for dissecting the genetic programs that control sensory organ formation (Ghysen and Dambly-Chaudière 1993; Jan and Jan 1993; Modolell 1997). In embryonic abdominal segments, external sensory (ES) organs and chordotonal (CH) organs contain single-dendrite neurons associated with support cells and function in receiving mechanical stimuli (Campos-Ortega and Hartenstein 1985; Ghysen et al. 1986). In contrast, multidendritic (MD) neurons elaborate highly branched dendrites underneath the epidermis (Bodmer and Jan 1987; Gao et al. 1999) that probably function as stretch, touch, or other sensory receptors (Ainsley et al. 2003; Liu et al. 2003; Tracey et al. 2003). In adult flies, most external sensory organs have one neuron, one hair cell, and a few support cells (Campos-Ortega and Hartenstein 1985).

Precise numbers of sensory organs are generated through similar developmental processes in embryos and adults (Campos-Ortega and Hartenstein 1985). For ex-

ample, in embryonic/larval abdominal segments, each dorsal cluster contains four ES organs and eight MD neurons. Some ES neurons are generated from sensory organ precursors (SOPs) that also produce MD neurons (ES–MD lineage), while other lineages produce ES but not MD neurons (Brewster and Bodmer 1995; Vervoort et al. 1997). Adult flies have four macrochaetes on the notum and a well-defined number of sensory bristles on the wing margin. All cells in each adult sensillum are generated from a single SOP in two or three rounds of asymmetric cell division (Hartenstein and Posakony 1989; Bardin et al. 2004).

The selection of SOPs from early ectoderm begins with the proneural cluster, which consists of a small number of cells that express proneural genes encoding the basic helix–loop–helix (bHLH) proteins (Achaete, Scute, Asense, Atonal, and Amos), rendering those cells competent to develop into SOPs (Romani et al. 1989; Cubas et al. 1991; Skeath and Carroll 1991; Jarman et al. 1993; Ruiz-Gomez and Ghysen 1993; Goulding et al. 2000; Huang et al. 2000). It is thought that the cells with the highest level of proneural proteins are selected as SOPs, which express a higher level of Delta and activate Notch in neighboring cells (Goriely et al. 1991; Artavanis-Tsakonas et al. 1999). Activation of Notch initiates a signaling cascade in which *Suppressor of hairless* [*Su(H)*] and *Enhancer of split* [*E(spl)*] complexes are involved to suppress neuronal fate in non-SOP cells (Knust et al. 1992; Schweisguth and Posakony 1992).

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One of the genes activated by bHLH proteins, *senseless* (*sens*), encodes a transcription factor with four zinc fingers whose expression is dynamically regulated within the proneural clusters (Nolo et al. 2000). Within the proneural cluster, a high level of *Sens* is required to up-regulate and maintain the proneural gene expression in SOPs (Nolo et al. 2000), while a low level of *Sens* represses the transcription of proneural genes in adjacent cells (Jafar-Nejad et al. 2003). This dual function of *Sens* as an activator or suppressor suggests that the proper level of *Sens* is required to control the formation of the precise number of SOPs. However, how differential expression levels of *Sens* in SOPs and adjacent cells are ensured remains unknown.

In recent years, post-transcriptional regulation by microRNAs (miRNAs) has emerged as an important mechanism for controlling gene expression in animal development (Carrington and Ambros 2003; Bartel 2004; He and Hannon 2004; Alvarez-Garcia and Miska 2005; Carthew 2006). miRNAs are endogenous noncoding small RNAs 21–23 nucleotides (nt) in length. Several miRNAs have been implicated in nervous system development, with roles ranging from regulating left/right neuronal asymmetry in *Caenorhabditis elegans*, photoreceptor formation in *Drosophila*, and brain morphogenesis in zebrafish, to neuronal differentiation in mammals (Johnston and Hobert 2003; Chang et al. 2004; Giraldez et al. 2005; Li and Carthew 2005; Vo et al. 2005; Krichevsky et al. 2006; Schratt et al. 2006). However, the precise roles of miRNAs in early neurogenesis have not been studied yet. Moreover, only a few miRNAs have been studied using loss-of-function approaches in *Drosophila* (e.g., Brennecke et al. 2003; Xu et al. 2003; Kwon et al. 2005; Li and Carthew 2005; Sokol and Ambros 2005; Teleman et al. 2006).

Here, through both loss-of-function and gain-of-function in vivo analyses, we found that *miR-9a* is required to ensure the generation of precise numbers of sensory organs in *Drosophila* embryos and adults. To accomplish this regulatory function, *miR-9a* down-regulates the expression of *Sens* through its 3' untranslated region (UTR) to ensure the differential expression of *Sens* in SOPs and adjacent epithelial cells. These findings provide new evidence that miRNAs function at the translational level to ensure the appropriate level of gene expression during different developmental processes.

Results

Generation of *miR-9a* mutant flies

To understand the roles of miRNAs in neural development, we chose *Drosophila miR-9a* for further analysis for the following reasons. First, the *Drosophila* homolog of the human fragile X mental retardation protein affects the formation of terminal dendritic branches (Lee et al. 2003) and is associated with miRNAs and the RNA-induced silencing complex, implicating miRNAs in human mental disorders (Caudy et al. 2002; Ishizuka et al. 2002). Second, *miR-9a* is one of the miRNAs that are

specifically and highly expressed in the mammalian brain, suggesting a unique role in neural development and/or function (Lagos-Quintana et al. 2002). Third, mature *miR-9a* is 100% conserved at the nucleotide level from flies to humans (Fig. 1A; Aravin et al. 2003), indicating an evolutionarily conserved function. Moreover, in contrast to the mouse genome, which has three *miR-9a* precursors located on different chromosomes (Lagos-Quintana et al. 2002), the *Drosophila* genome has only one (Aravin et al. 2003; Sempere et al. 2004), providing a good opportunity to study the function of this miRNA.

The *Drosophila miR-9a* precursor is located on the left arm of the third chromosome 76B7, 10 kb to the right of gene CG9300 and 6.4 kb to the left of gene CG9262 (Fig. 1B). To generate loss-of-function mutants of *miR-9a*, we used ends-out homologous recombination (Gong and Golic 2003) to replace the 78-nt long *miR-9a* precursor DNA sequence with the *white* gene (Fig. 1C). To screen for potential *miR-9a* mutants, we used PCR analysis with two primers flanking *miR-9a*. These primers produced a 0.5-kb fragment when wild-type genomic DNA was used as the template and a 4-kb fragment if *miR-9a* was replaced by *white* (Fig. 1D). From two independent gene-targeting experiments, we generated five mutant lines in which homologous recombination occurred at the *miR-9a* locus (Fig. 1D). To confirm that the expression of *miR-9a* was indeed abolished in mutants, we performed Northern blot analysis that showed the absence of *miR-9a* in all five mutant lines (Fig. 1E). Lines J22 and E39, generated from two independent targeting events, were selected for further analysis.

Loss of *miR-9a* function results in ectopic sensory neurons in *Drosophila* embryos and larvae

Both *miR-9a^{J22}* and *miR-9a^{E39}* mutant flies developed to adulthood at the expected Mendelian ratio and were fertile, suggesting that *miR-9a* activity is not essential for survival or fertility. However, when we examined the morphology of PNS neurons, we found unexpectedly that some of the MD neurons were duplicated. Normally, *Drosophila* embryos and larvae have 12 sensory neurons in each dorsal cluster in the abdominal segments (Fig. 2A), including eight MD neurons. At the larval stage, each MD neuron has a unique dendritic branching pattern that is fairly consistent from segment to segment and among different animals (Bodmer and Jan 1987; Grueber et al. 2002; Sweeney et al. 2002). Using a specific driver, *Gal4²²¹*, we examined two MD neurons, ddaE and ddaF (Sweeney et al. 2002), which extend smooth dendrites toward the posterior and anterior, respectively (Fig. 2B). Twenty-nine percent of *miR-9a^{J22}* ($n = 62$) and 37% of *miR-9a^{E39}* mutant third instar larvae ($n = 27$) contained an extra ddaE or ddaF neuron in one or two but not other hemisegments (Fig. 2C,D), while none of the wild-type larvae had this phenotype ($n = 24$). A similar phenotype was also seen in *vpda*, a ventral MD neuron that also extends simple and smooth dendritic branches (Supplementary Fig. S1).

The frequent observation of two-cell clones generated

Figure 1. Generation of *miR-9a* loss-of-function mutant flies. (A) Nucleotide sequence alignment of *miR-9a* among different species. (B) The cytological location of *miR-9a* in the *Drosophila* genome. (C) The ends-out recombination approach was used to replace the 78-nt long *miR-9a* precursor with the *white* gene. (D) The *miR-9a* mutant lines were identified by PCR analysis using primers flanking the *miR-9a* locus. (E) Northern blotting analysis confirmed that *miR-9a* was absent in the mutant lines. The same Northern membrane was probed with a 2s rRNA probe as the loading control.

by mosaic analysis with a repressible cell marker (MARCM) (Lee and Luo 1999) that contained an ES neuron and either a *ddaE* neuron or a *ddaF* neuron (Sweeney et al. 2002) suggests that *ddaE* and *ddaF* neurons are generated from the ES–MD lineages. To examine whether the duplicated neurons in *miR-9a* mutants resulted from cell fate transformation within the ES–MD lineage or from an ectopic SOP, we counted neurons and support cells of A5–A6 dorsal clusters in embryos. As shown by immunostaining, each wild-type dorsal cluster contained 12 *Elav*⁺ neurons (eight MD and four ES neurons) and eight *Cut*⁺/*ELAV*[−] cells (external cells, Fig. 2E). In the *miR-9a* mutant embryos, more cells were observed in some dorsal clusters. Among the four extra cells, there were two *Elav*⁺ neurons accompanied by two *Cut*⁺/*ELAV*[−] external cells (Fig. 2F), suggesting that an extra SOP was generated. These findings suggest that *miR-9a* affects the precise production of SOPs during *Drosophila* embryonic development.

To understand how *miR-9a* is involved in SOP specification, we performed *in situ* hybridization with a probe for the *miR-9a* precursor. The earliest expression of *miR-9a* was at stage 5 and was observed in all cells except most ventral cellular blastula (data not shown). After gastrulation at stage 7, *miR-9a* expression persisted in all epithelial cells except the ventral ectoderm (Fig. 2G). Ectodermal epithelial cells continued to express *miR-9a*, and at stage 10–13, strong expression of *miR-9a* was seen at the ectoderm of the segmental boundaries and stomodeum (Fig. 2H). During this stage, embryonic PNS SOPs are derived from the epithelial cells. Consistently, we observed a similar embryonic expression of *miR-9a* with

a probe made out of the mature *miR-9a* (data not shown), which was also reported independently during the course of our work by Stark et al. (2005).

miR-9a controls the generation of sensory organs in adult flies

The increased production of sensory neurons in *miR-9a* mutant embryos/larvae prompted us to further examine the sensory organ formation in adult flies. Wild-type anterior wing margins have a fixed number of regularly spaced sensory bristles (Fig. 3A'). About 40% of *miR-9a* mutants flies showed ectopic sensory bristles on anterior wing margins (Fig. 3B'), while flies overexpressing *miR-9a* had a marked decrease in the number of bristles (data not shown). *miR-9a* mutant flies also exhibited a posterior wing margin defect, which was 100% penetrant (Fig. 3B). To confirm that the wing phenotype was caused by the loss of *miR-9a* activity, we performed a genetic rescue experiment. Expressing the *miR-9a* precursor by *vg-Gal4*, which drives gene expression in the thin strip of dorsal–ventral boundary of wing imaginal discs, in the *miR-9a* mutant background completely rescued the defects on posterior wing margins ($n = 152$, Fig. 3C). The bristle defect on the anterior margin of *miR-9a* mutants was also largely rescued (Fig. 3C').

Besides the wing phenotype, *miR-9a* mutant flies also displayed an ectopic production of sensory organs on the notum. Each SOP generates five different cells in two to three rounds of division to form an entire sensory organ on the notum (Fig. 4A). Wild-type flies have two scutellar bristles (Fig. 4B) on each heminotum, but 14.3% of

Figure 2. Loss of *miR-9a* activity resulted in the formation of ectopic sensory neurons in *Drosophila* embryos and larvae. (A) Schematic representation of the dorsal cluster sensory neurons in each abdominal hemisegment. (B) Wild-type *ddaE* and *ddaF* neurons in third instar larvae are labeled by mCD8-GFP with *Gal4²²¹*. The white arrows in B, C, and D indicate axons of these sensory neurons. The red arrowheads indicate *ddaC* neurons that are faintly labeled by GFP with *Gal4²²¹*. In *miR-9a* mutant larvae, duplicated *ddaF* (C) and *ddaE* (D) neurons (yellow arrowheads) were observed with near normal dendritic branches (traced in C', D'). (E, F) All the neurons of one dorsal cluster of stage 15 embryos were double-labeled with anti-ELAV (green) and anti-Cut (red) antibodies. (E) In wild-type embryos, there are 12 ELAV⁺ cells, including MD neurons and ES neurons, and eight Cut⁺/ELAV⁻ support cells. (F) A *miR-9a²²* embryo with 14 ELAV⁺ cells and 10 Cut⁺/ELAV⁻ support cells. (G, H) *miR-9a* expression is detected in epithelial cells in wild-type embryos with in situ hybridization. (G) After gastrulation at stage 7, *miR-9a* expression persists in most epithelial cells except the ventral ectoderm. (H) At stage 12–13, *miR-9a* is expressed in the ectoderm, with higher levels at the segmental boundaries and stomodeum.

miR-9a mutant flies (14 out of 98) had three or four bristles (Fig. 4C) with accompanying shaft and socket cells (Fig. 4E). Conversely, flies overexpressing *miR-9a* in

the notum region by *ap-Gal4* had a severe loss of scutellar sensory organs (Fig. 4D). Like the wing margin phenotype, defects on the notum of *miR-9a* mutant could be

Figure 3. Wing margin defects in *miR-9a* mutant flies. (A) A wild-type wing. The rectangle indicates the areas enlarged into A' (same for those in B–D). (A') Sensory hairs on anterior wing margin are assembled in a regular pattern with four macrochaetes flanking one microchaete. (B) All *miR-9a* mutant flies lose the posterior wing margin, while the veins are normal in appearance. (B') Ectopic sensory hairs on the anterior wing margin in *miR-9a* mutants. (C,C') The wing margin defects in *miR-9a* were rescued by expression of the wild-type *miR-9a* precursor (line #8) with *vg-Gal4* ($n = 152$). (D,D') The wing margin defects of *miR-9a* mutants were suppressed by removing one copy of *sens*. *sens*^{E58} was recombined with *miR-9a*^{J22} onto the same chromosome.

rescued by expression of *miR-9a* precursors using *vg-Gal4* ($n = 152$). To further demonstrate the bristle phenotype was due to increased number of scutellar SOPs in *miR-9a* mutants, we stained the notum with Elav antibody. We found that each ectopic bristle was also associated with an extra neuron (Fig. 4E), supporting our hypothesis that *miR-9a* regulates the specification of SOPs during sensory organ development.

miR-9a genetically interacts with *sens*

The wing margin phenotypes of *miR-9a* mutant flies are remarkably similar to that seen in *Lyra*¹ mutant flies, a gain-of-function mutant of *sens*, and in flies with forced expression of *Sens* in the wing (Nolo et al. 2001). Moreover, the *sens* 3'UTR contains *miR-9a*-binding sites (Stark et al. 2003), raising the possibility that *Sens* might be misregulated in *miR-9a* mutant flies. To test this hypothesis, we performed genetic interaction studies. In a *miR-9a*^{J22} homozygous mutant background, removing one copy of *sens* (*sens*^{E58}/+) led to a substantial rescue of the wing margin defect. About 30% of these flies had wings indistinguishable from the wild type, while others lost only a smaller part of the posterior margin (Fig. 3D); the bristles on the anterior margin also resembled wild type (Fig. 3D'). In contrast, 100% of *miR-9a* mutant flies had posterior wing margin defects (Fig. 3). These findings suggest that up-regulation of *Sens* is a key downstream event caused by loss of *miR-9a* activity.

To provide further evidence that *miR-9a* regulates *Sens* during the sensory organ formation, we performed additional genetic interaction experiments on the notum. Overexpressing *Sens* with the *vg-Gal4* induced ectopic formation of numerous bristles on the notum (Fig. 4F). However, coexpressing of the *miR-9a* precursor and *Sens* largely repressed ectopic bristle formation (Fig. 4G), indicating that *miR-9a* can suppress *Sens* expression in vivo. In this experiment, the UAS-*sens* construct contains the *sens* 3'UTR. The bristle defect was rescued to different degrees with two different UAS-*pre-miR-9a* transgenic lines (lines #3 and #8) (Fig. 4H). As a control, overexpression of the mutant *miR-9a* precursor with a 7-nt deletion failed to rescue the *sens* overexpression phenotype (Fig. 4H). These data provide further evidence that *miR-9a* and *sens* function in the same genetic pathway in controlling SOP formation.

miR-9a regulates *Sens* expression in vivo

To provide mechanistic insights into the actions of *miR-9a* in controlling sensory organ formation, we set out to examine whether the *Sens* protein level could be affected by *miR-9a* in vivo. In the *Drosophila* imaginal wing disc, *sens* is expressed in proneural clusters and accumulates in presumptive SOPs (Nolo et al. 2000). We performed in situ hybridization on the wing discs of third instar larvae and found that *miR-9a* is widely expressed except for some cells in the wing margins in wild-type flies (Fig.

Figure 4. *miR-9a* mutant flies show a sensory bristle defect on the notum, and *miR-9a* genetically interacts with *sens*. (A) Schematic representation of the cell lineage of an adult sensory organ from a single SOP. (B) Wild-type flies have four scutellar bristles (asterisks) on the notum. (C) *miR-9a* mutant flies have four to seven scutellar bristles on the notum (asterisks). (D) Overexpression of *miR-9a* resulted in a loss of scutellar bristles (asterisk indicates the only remaining bristle). (E) In *miR-9a* mutant flies, all bristles (yellow arrowhead) have normal shaft and socket cells and are accompanied by ELAV⁺ sensory neurons (green arrowheads). Some bristles were lost during the staining process. (F) Overexpression of Sens dramatically increased the number of sensory bristles on the notum. (G) Coexpression of *miR-9a* suppressed the generation of ectopic bristles induced by Sens overexpression. (H) Statistical analysis of genetic interactions between *sens* and *miR-9a*. Genotypes are as follows: wild-type ($n = 50$) (bar 1); *vg-Gal4/+;UAS-sens/+* ($n = 20$) (bar 2); *vg-Gal4/+;UAS-sens/UAS-pre-miR-9a* (line #3) ($n = 20$) (bar 3); *vg-Gal4/UAS-pre-miR-9a; (line #8) UAS-sens/+* ($n = 20$) (bar 4); and *vg-Gal4/+;UAS-sens/UAS-mutant-pre-miR-9a* ($n = 20$) (bar 5). Values are means $\pm$ SEM. (***) $p < 0.001$.

5A), but not *miR-9a* mutants (Fig. 5B). To examine *miR-9a* expression at the cellular level, we performed in situ hybridization of *miR-9a* on a *A101-Gal4/UAS-mCD8-GFP* disc in which a subset of SOPs is labeled with GFP (Fig. 5C). Interestingly, *miR-9a* is not expressed in SOPs, but is expressed in most, if not all, epithelial cells, including the ones adjacent to SOPs (Fig. 5D,E). Sens is dynamically regulated within proneural clusters during SOP specification: It is up-regulated in SOPs and down-regulated in other cells (Nolo et al. 2000). The expression pattern of *miR-9a* is consistent with the “avoidance hypothesis” that *miR-9a* may control SOP formation through regulation of gene expression in non-SOP cells (Stark et al. 2005).

To provide direct evidence that *miR-9a* plays a role in SOP formation by regulating Sens expression, we examined Sens expression in the wing imaginal disc. As shown by immunostaining of wing imaginal discs of wild-type third instar larvae, Sens is first expressed in proneural cluster cells at an early stage and is concentrated in SOPs during SOP specification. Two rows of

Sens⁺ cells at the pouch region develop into SOPs in the late third instar stage and into sensory bristles on adult wing margins (Fig. 5F). In the region that develops into the notum, there are two Sens⁺ cells, which are the precursors for the two scutellar bristles on each hemi-notum (Fig. 5F, inset). In the *miR-9a* mutant, there were more SOPs in early third instar wing discs (Fig. 5G), and >60% of the discs contained three or four Sens⁺ SOPs in the notum region (Fig. 5G, inset). These findings are consistent with the ectopic bristles in adult *miR-9a* mutant flies (Fig. 4C).

Conversely, overexpression of *miR-9a* in the dorsal compartment of the wing disc by *ap-Gal4* (Calleja et al. 1996) significantly reduced the number of Sens⁺ cells, and the two SOPs in the notum region were absent (Fig. 5H), consistent with the balding phenotype in adult mutant flies (Fig. 4D). These data provide strong evidence that the ectopic sensory organs in *miR-9a* mutant flies result from ectopic SOPs during neurogenesis, and that *miR-9a* normally suppresses Sens expression, thereby inhibiting the SOP fate in surrounding epithelial cells.

Figure 5. *miR-9a* is expressed in epithelial cells, including cells adjacent to SOPs in wing imaginal discs. (A) *miR-9a* is ubiquitously expressed in wild-type wing imaginal discs except the wing margins, as shown with a Dig-labeled probe detected with an AP-conjugated anti-Dig antibody. (B) A *miR-9a*^{J22} mutant wing imaginal disc has no *miR-9a* expression. (C–E) High-resolution fluorescent in situ hybridization shows that *miR-9a* is expressed in the epithelial cells, including cells adjacent to the SOPs in the pouch region of a wild-type wing imaginal disc. (C) At the wing margins, SOPs were visualized by mCD8-GFP driven by *A101-Gal4* (yellow arrowheads indicate two of them). (D) A Dig-labeled *miR-9a* probe detected by Cy3-conjugated anti-Dig antibody (red) shows *miR-9a* is expressed in the epithelial cells, including ones adjacent to the SOPs. (E) Merged image of C and D showing *miR-9a* is excluded from the SOPs in the wing margins. (F–H) Immunostaining of Sens (red) in wing imaginal discs of early third instar larvae. (F) In the wild-type wing disc, Sens is highly expressed in SOPs and wing margins. *Inset* shows a magnified view of the notum region from which scutellar bristles are derived. (G) *miR-9a*^{J22} mutant imaginal wing disc has ectopic SOPs. *(Inset)* A high frequency of extra SOPs was observed in the notum region. (H) When *miR-9a* is overexpressed with *ap-Gal4* in the dorsal part of the wing disc, Sens expression was suppressed in most of the dorsal compartment. *Inset* shows the absence of Sens⁺ cells (SOPs) in the region that develops into the notum.

miR-9a directly suppresses *sens* expression through its 3'UTR

It was predicted that the *sens* 3'UTR contains three *miR-9a*-binding sites (Fig. 6A; Stark et al. 2003). To test whether *miR-9a* suppresses *Sens* expression directly through its 3'UTR, we performed luciferase assays. HEK293 cells were cotransfected with the wild-type *miR-9a* precursor and *sens* 3'UTR downstream from the luciferase coding region. The relative luciferase activity was 70% lower than in cells expressing the luciferase-*sens* 3'UTR alone (Fig. 6C). Two mutant constructs of *miR-9a* precursors were generated as controls, one with a 5-nt deletion in the mature *miR-9a* region (Mut-1) and one with a 7-nt deletion in the stem of the precursor (Mut-2) (Fig. 6B). Both of these mutant constructs failed to suppress the expression of luciferase with the *sens* 3'UTR (Fig. 6C). As an additional control, none of the *miR-9a* constructs had any effect on the expression of luciferase without the *sens* 3'UTR (data not shown). These findings provide strong evidence that *miR-9a* directly suppresses *sens* expression through its 3'UTR.

Discussion

Drosophila miR-9a suppresses neuronal precursor specification

miR-9a is one of the miRNAs that are highly expressed in the mammalian brain and 100% conserved at the

nucleotide sequence from flies to humans, suggesting an important role in brain development and/or function (Lagos-Quintana et al. 2002; Aravin et al. 2003; Sempere et al. 2004). We generated *miR-9a* loss-of-function alleles and found that homozygous mutant flies developed into adulthood at the expected Mendelian ratio. Adult mutant flies are grossly normal and fertile, indicating that *miR-9a* is not required for viability or fertility. This finding is different from the reported severe dorsal closure defects and embryonic lethal phenotype generated by antisense 2'-O-methyl oligoribonucleotide-mediated depletion of *miR-9a* (Leaman et al. 2005).

Interestingly, *Drosophila* *miR-9a* is not expressed in mature neurons, but is expressed in epithelial cells, including the proneural clusters that give rise to SOPs (Figs. 2, 5; Stark et al. 2005). Detailed analysis of embryonic PNS development revealed an unexpected finding that *miR-9a* mutants have an increased number of sensory neurons (Fig. 2) that elaborate extensive dendritic arbors underneath the epithelial cell layer, such as ddaE and ddaF neurons (Sweeney et al. 2002). The duplicated neurons occupy the same dendritic field and appear to have similar dendritic branching patterns (Fig. 2C,D). Indeed, the average numbers of dendritic ends of ddaE and ddaF neurons in abdominal segments 3–5 were similar in wild-type and *miR-9a* mutant larvae and MARCM clones (data not shown), indicating that loss of *miR-9a* activity affected the number of these sensory neurons only but had no cell-autonomous effect on their dendritic branching patterns.

The effect of *miR-9a* on the number of embryonic sen-

Figure 6. Luciferase assay showing the suppression by *miR-9a* through the *sens* 3'UTR. (A) The sequences and locations of three predicted *miR-9a*-binding sites in the *sens* 3'UTR and their alignment with *miR-9a* (red). (B) The predicted stem-loop-stem structure of the *miR-9a* precursor. (WT) Wild-type precursor; (Mut-1) mutant precursor 1 with a 5-nt deletion in mature *miR-9a*; (Mut-2) the mutant precursor with a 7-nt deletion in the stem region complementary to mature *miR-9a*. Mature *miR-9a* is highlighted in red, and strikethroughs indicate the deleted nucleotides. (C) Wild-type (WT) but not mutant *miR-9a* precursors (Mut-1 and Mut-2) could suppress the expression of luciferase with the *sens* 3'UTR. Bars indicate the average of luciferase activity in one triplicated experiment. (***) $p < 0.001$.

sory neurons has two major features. First, the ectopic ddaE or ddaF neurons were generated as a result of ectopic SOPs and not cell fate transformation within a cell lineage (Fig. 2), suggesting *miR-9a* affects an early step in neurogenesis, consistent with its embryonic expression pattern (Fig. 2; Stark et al. 2005). Second, both the expressivity and penetrance of this defect were relatively low. This finding supports the idea that miRNAs, at least in this particular case, are not developmental switches, but instead function as a fine-tuning mechanism to ensure the accuracy of a particular developmental process (e.g., Farh et al. 2005; Stark et al. 2005).

In this study, we focused on the formation of SOPs in adult flies. Like embryos, only ~14% of *miR-9a* mutant flies exhibited ectopic SOPs on the notum, again indicating a fine-tuning role for *miR-9a* in controlling SOP formation. However, our analysis of the *miR-9a* mutant phenotype in adult flies also indicates that miRNAs can have dramatic effects on some other developmental processes. For instance, *miR-9a* is widely expressed in the wing disc (Fig. 5), and 100% of *miR-9a* mutant flies exhibited a severe posterior wing margin defect (Fig. 3), suggesting that cell proliferation and/or survival are much more sensitive to changes in the expression levels of the proteins regulated by *miR-9a*.

MiR-9a inhibits Sens expression in non-SOP cells

How does *miR-9a* exert its effect on SOP formation? Sens is a zinc finger transcription factor required to maintain high-level expression of proneural gene in SOPs and to suppress their expression in non-SOP cells (Nolo et al. 2000; Jafar-Nejad et al. 2003). Several findings in this study demonstrate that Sens is a key target of *miR-9a* regulation and is essential for mediating *miR-9a* function in SOP formation. (1) The wing margin defects in *miR-9a* mutant flies were remarkably similar to that caused by overexpression of Sens by the UAS-Gal4 system or in *Lyra¹* mutants (Nolo et al. 2000, 2001). (2) *miR-9a* was expressed at a much lower level in SOPs than in adjacent epithelial cells, correlating with the high level of Sens expression in SOPs and the low level of Sens in non-SOP cells in proneural clusters. The inability to use immunostaining to detect subtle changes of Sens expression level in non-SOP cells due to *miR-9a* loss of function could be attributed to the following reasons: Sens expression is primarily down-regulated at the transcriptional level in the non-SOP proneural cells, and *miR-9a*'s function is limited to preventing translation of the leaky/residual *sens* mRNA, consistent with the model proposed by Stark et al. (2005). The alteration in

Figure 7. A model for the role of *miR-9a* in regulating SOP specification. Within one proneural cluster, the cell selected to become the SOP expresses a high level of Delta to activate Notch in the adjacent cells (non-SOPs). Upon activation, Notch releases the intracellular domain (N^{IC}), which translocates to nuclei and binds to Suppressor of Hairless, switching it to the active form [Su(H)^{Act}]. The N^{IC}/Su(H)^{Act} complex activates Enhancer of Split [E(spl)], and consequently represses *sens* transcription. In SOPs, without the activation of Notch, Su(H) functions as a repressor [Su(H)^{Rep}] to suppress the E(spl). Therefore, *sens* could be continuously transcribed, allowing high-level accumulation of Sens protein, which is required for further transcription of neuron-specific genes. *miR-9a* functions in non-SOP cells to further suppress Sens translation through its 3'UTR to ensure the precise production of SOPs.

Sens level, due to loss of *miR-9a* function in the non-SOP cells, is sufficient to initiate the production of ectopic SOPs, but it may not be dramatic enough to be detected by immunostaining. (3) The *sens* 3'UTR contains three *miR-9a*-binding sites and is the best predicted target of *miR-9a* (Stark et al. 2003). (4) Wild-type but not mutant *miR-9a* precursors down-regulated reporter gene expression through the *sens* 3'UTR in transfected cells. (5) Overexpression of *miR-9a* in vivo inhibited Sens expression. We observed that Sens expression along the wing margin in the dorsal compartment was lower than in the ventral compartment in some wing discs when *miR-9a* was expressed by *ap-Gal4* (data not shown). The failure to completely suppress Sens expression in the dorsal compartment (Fig. 5H) is probably due to the fact that, at this developmental stage, Sens expression in the wing margins is controlled by proneural genes, unlike SOPs in the notum region where Sens expression is maintained by itself (Jafar-Nejad et al. 2003). (6) *miR-9a* and *sens* showed strong genetic interactions in controlling SOP formation.

These findings provide an experimental example to support the notion that miRNAs and their mRNA targets are often expressed in cells adjacent to each other (Stark et al. 2005). The differential expression of Sens in SOPs and adjacent neuroepithelial cells is essential for the production of a precise number of SOPs during development (Nolo et al. 2000; Jafar-Nejad et al. 2003). We propose a model in which *miR-9a* functions in non-SOP cells to further suppress Sens expression at the translational level, as a complementary mechanism to the transcriptional inhibition of Sens expression by E(spl) (Fig. 7). Loss of *miR-9a* function increases Sens protein level, not so dramatically but just enough to convert Sens in some neuroepithelial cells from a transcription repressor into an activator of proneural genes, therefore resulting in the formation of a small number of ectopic SOPs. However, unlike many other genes essential for neurogenesis, such as Notch and Delta, *miR-9a* does not function as an absolute switch. Instead, it only ensures the accurate differential Sens expression and fine-tunes this developmental process.

Overexpression of *miR-9a* in the wing imaginal disc could dramatically inhibit the formation of sensory organs on the notum (Fig. 4D), suggesting that misregulation of *miR-9a* expression itself could potentially have severe developmental consequences. Since both *miR-9a* and E(spl) have similar functions in non-SOP cells, it is possible that both genes may be regulated by a similar transcriptional mechanism. Indeed, we noticed that binding sites for the Achaete–Scute complex and Su(H) are present in the regulatory region of *miR-9a*. Taken together, our studies presented here have uncovered another layer of gene regulation during early neurogenesis in the *Drosophila* PNS. *miR-9a* is 100% conserved at the nucleotide level from flies to humans. Moreover, the human *miR-9a* is highly expressed in fetal but not in adult brains (Nelson et al. 2006). Therefore, a similar mechanism of *miR-9a* function may operate during mammalian neurogenesis as well.

Materials and methods

Fly strains and genetics

All the flies were maintained at 22°C–25°C on standard medium. The *w¹¹¹⁸* strain was used as a wild-type control. *Gal4²²¹* combined with UAS-*mCD8::GFP* was used to visualize individual ddaE and ddaF neurons in the larval PNS. It was recombined with *miR-9a* mutants to generate the homozygous stock *Gal4²²¹*, UAS-*mCD8::GFP*, *miR-9a*. For studies of the wing and the notum, the following lines were used: *vg-Gal4*, *ap-Gal4* (Calleja et al. 1996), and UAS-*sens* (Nolo et al. 2000). The presence of *sens* 3'UTR in the UAS-*sens* construct was confirmed by PCR. *sens^{E58}* was used as the *sens* loss-of-function mutant allele in genetic interaction experiments.

Target homologous recombination constructs

miR-9a loss-of-function mutations were generated with the ends-out technology (Gong and Golic 2003). The targeting construct contains the *w⁺* gene flanked by two arms of genomic DNA fragments at the *miR-9a* locus. The two arms were prepared by PCR from the genomic DNA using primers 5'-AGC-GATCGTCGTCGGACTAC-3' and 5'-ACACTGCAGATGGT-TGAAAG-3' for the left arm, and primers 5'-ACTCGAGC-CAAAAACGAGGCCACACA-3' and 5'-AGGTACCGAGACAG CAAAATCGTAGAA-3' for the right arm. Two independent transgenic fly lines carrying the targeting construct were crossed to *hs-FLP*, *I-SceI* (Bloomington Stock Center), and the progenies at late second to third instar stages were heat-shocked for 45 min at 38°C. Virgin female flies with mosaic eyes were collected and crossed to *p70FLP/10* (Bloomington Stock Center), and at the next generation, individual males with solid-color eyes were crossed to *TM3 Ser/TM6 Tb* as candidate lines. For the replacement of the *miR-9a* by *w⁺*, a PCR screen was performed with genomic DNAs isolated from each line. Northern blot was used to confirm the absence of *miR-9a* expression in mutant flies.

Transgenic constructs

The 78-base-pair (bp) *miR-9a* precursor was cloned from wild-type genomic DNA with primers 5'-GAATTCTATACAGGGT-GCTATGTTG-3' and 5'-TCTAGACGCTGGGCAGACGC-TAATATTAACCTTCGG-3'. The mutant precursor with a 7-bp deletion on the stem was generated with primers 5'-GAATTC-TATACAGGGTGCTATGTTG-3' and 5'-TCTAGACGCTGGCAGACGCTAATATTAAGCTAGCTTTATGACGT-3'. Both fragments were subcloned into the pUAST vector and injected into embryos to generate transgenic fly stocks that express either wild-type or mutant *miR-9a* precursor under the control of UAS elements.

Northern blot

Total RNA was extracted from larvae with Trizol, according to the manufacturer's instructions (Invitrogen). For each sample, 20 µg of total RNA was run on a 12% polyacrylamide gel (Se-quagel, National Diagnostics) and transferred to a Nytran SuperCharge Signal membrane (Schleicher & Schuell). *miR-9a* was detected with a purified ³²P-end-labeled oligo probe, 5'-TCATACAGCTAGATAACCAAAGA-3', which is complementary to the mature *miR-9a* sequence.

In situ hybridization

Embryos and wing imaginal discs were fixed and stained according to standard procedures (Tautz and Pfeifle 1989). A genomic

fragment of 1 kb flanking the *miR-9a* precursor was obtained by PCR and cloned into the pBSK vector. A Digoxigenin (Dig)-labeled riboprobe was generated by in vitro transcription of this construct with T7 RNA polymerase. A *Drosophila miR-9a* locked nucleic acid (LNA) probe was obtained from Exiqon (Vedbaek), end-labeled with a Dig oligonucleotide 3'-end-labeling kit (Roche), and purified on a G-25 Microspin column (Amersham Biosciences). In situ hybridization with both probes was detected by AP-conjugated cy3-conjugated anti-Dig antibody according to standard procedures (Tautz and Pfeifle 1989) with the following modifications: After post-fixation and washes, the discs were washed with water and treated twice for 5 min each with 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine (pH 8.0). The tissues were prehybridized and hybridized at 50°C in hybridization buffer (pH 6.0, adjusted with citric acid).

Antibody staining

Embryos were fixed according to the standard protocol. Third instar larval wing imaginal discs were fixed in 4% formaldehyde in PBT (PBS and 0.1% Triton X-100) for 30 min at room temperature. Pupae were dissected 48 h after pupa formation and fixed in 4% formaldehyde in PBT overnight at 4°C. The following pairs of primary and secondary antibodies were used for all experiments: anti-Sens (1:1000) (Nolo et al. 2000) and anti-guinea pig-Cy3 (1:500; Molecular Probes); anti-ELAV (1:100; Developmental Hybridoma Study Bank) and anti-mouse-Cy3 or anti-mouse-FITC (1:300; Jackson Laboratory); anti-Cut (1:1000; Developmental Hybridoma Study Bank) and anti-rabbit-Cy3 (1:300; Jackson Laboratory).

Microscopy and imaging

All samples with fluorescent signals were imaged by confocal microscopy (Nikon, D-Eclipse C1), including GFP-labeled DA neurons in third instar larvae; immunostaining on embryos, wing discs, and the pupa notum; and fluorescence-labeled in situ hybridization on wing discs. Images of adult wing and notum and Dig-labeled in situ hybridization on wing discs were obtained with an Olympus BX60 microscope and AxioCam Hrc camera.

Luciferase reporter assays

The wild-type and two mutant *miR-9a* precursors were obtained by PCR and cloned into the pSUPER vector under the control of the H1 promoter (OligoEngine). A 600-bp fragment of the *sens* 3'UTR was cloned into the pGL3-promoter vector (Promega) downstream from the firefly *luciferase* gene. The *Renilla* luciferase plasmid served as the transfection control. HEK293 cells were cotransfected in 12-well plates with three vectors (0.5 µg of each vector per well): the luciferase-*sens* 3'UTR reporter plasmid, the *Renilla* luciferase plasmid, and the pSUPER vector containing *miR-9a* precursor (wild type, Mut-1, or Mut-2) or the vector only. Three independent transfections with triplicates each time were performed, and cell lysates were collected 28 h after transfection. Relative luciferase activity was measured with Dual-luciferase assays (Promega) according to the manufacturer's protocol.

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