Single cell visualisation of *mir-9a* and *Senseless* co-expression during *Drosophila melanogaster* embryonic and larval peripheral nervous system development

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25 **Dynamics of *mir-9a* and *Sens* expression**

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28 **Keywords**

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Abstract

The *Drosophila melanogaster* peripheral nervous system (PNS) comprises the sensory organs that allow the fly to detect environmental factors such as temperature and pressure. PNS development is a highly specified process where each sensilla originates from a single sensory organ precursor (SOP) cell. One of the major genetic orchestrators of PNS development is *Senseless,* which encodes a zinc finger transcription factor (Sens). Sens is both necessary and sufficient for SOP differentiation. *Senseless* expression and SOP number are regulated by the microRNA *miR-9a*. However, the reciprocal dynamics of *Senseless* and *miR-9a* are still obscure. By coupling smFISH with immunofluorescence, we are able to visualize transcription of the *mir-9a* locus and expression of Sens simultaneously. During embryogenesis, we show that the expression of *mir-9a* in SOP cells is rapidly lost as *Senseless* expression increases. However, this mutually exclusive expression pattern is not observed in the third instar imaginal wing disc, where some *Senseless*-expressing cells show active sites of *mir-9a* transcription. These data challenge and extend previous models of *Senseless* regulation, and show complex co-expression dynamics between *mir-9a* and *Senseless*. The differences in this dynamic relationship between embryonic and larval PNS development suggest a possible switch in *miR-9a* function. Our work brings single-cell resolution to the understanding of dynamic regulation of PNS development by *Senseless* and *miR-9a*.

Introduction

One of the most impressive demonstrations of developmental robustness is the specification of the *Drosophila melanogaster* peripheral nervous system (PNS), which comprises all the organs that allow the fly to detect movement, pressure, temperature, and more. *Drosophila* sensilla number and position exhibit little or no variation from individual to individual, even in diverse environmental conditions (Hartenstein 1988). The key early developmental step involves the selection and specification of sensory organ precursor (SOP) cells from a field of equipotent cells. During early embryogenesis (~5h from fertilization), groups of epidermal cells start to express Achete-Scute complex genes. These proneural genes impart the potential to become neurons (Jarman *et al.* 1993; Jan and Jan 1994; Goulding *et al.* 2000; Huang *et al.* 2000; Reeves and Posakony 2005). This potential is then constrained via Notch lateral inhibition to a single cell in the cluster, the SOP cell (Ghysen and Dambly-Chaudiere 1989; Artavanis-Tsakonas and Simpson 1991). The many different classes of sensory organs all originate from SOPs and develop via common shared rounds of cellular division (Lai and Orgogozo 2004). The eventual differences between sensory organs arise in part through subsequent changes in cell death and proliferation (Orgogozo *et al.* 2001; Orgogozo and Schweisguth 2004).

One of the major effectors of PNS development is a gene named *Senseless* (*Sens*) (Nolo *et al.* 2000). *Sens* encodes a transcription factor (Sens) whose expression is initially activated and subsequently maintained by the proneural genes *achete* and *scute* (Jafar-Nejad *et al.* 2006). *Sens* in turn maintains the expression of proneural genes to direct proper neuronal cell differentiation (Nolo *et al.* 2000; Acar *et al.* 2006). *Sens* expression is first detectable during

stage 10 of *Drosophila* embryogenesis, as isolated cells start to specify according to their SOP fate potential. As embryogenesis proceeds, these isolated *Sens*-expressing SOPs ultimately give rise to the entire sensory organ. *Sens* expression becomes repressed around stage 13, when SOPs are fully specified (Nolo *et al.* 2000). Since *Sens* maintains proneural gene activation, loss-of-function *Sens* mutant embryos exhibit a decreased number of SOPs, corresponding to a loss of sensory organs in the adult fly (Nolo *et al.* 2000). Gain of function mutations and ectopic expression of Sens cause an increased number of SOPs and consequently sensory organs (Jafar-Nejad *et al.* 2003; Li *et al.* 2006). Therefore, it is suggested that *Sens* is necessary and sufficient for SOP differentiation (Nolo *et al.* 2000). The robustness and reproducibility of sensory organ development between individuals implicates *Sens* as a keystone gene whose fine-scale regulation involves multiple feedback inputs.

Neurogenesis is extensively regulated by microRNAs (miRNAs) (Nolo *et al.* 2000; Hilgers *et al.* 2010; Caygill and Brand 2017). These small regulators of translation and mRNA stability contribute to the robustness of many biological processes. It has been shown that *miR-263a/b* stabilize sensory organ patterning in the retina by inhibiting SO cell apoptosis (Hilgers *et al.* 2010), and that *miR-7* stabilizes neuronal differentiation in the *Drosophila* larval brain by targeting the Notch pathway (Caygill and Brand 2017). In addition, Li and co-workers (2006) showed that *miR-9a* regulates Sens function through multiple target recognition sites in the Sens 3' UTR. When Sens' *miR-9a* binding sites are mutated, Sens levels are not only higher but more sensitive to temperature perturbations (Cassidy *et al.* 2013), resulting in an altered distribution of sensory organs in the wing margin (Cassidy *et al.* 2013; Giri *et al.* 2020). Loss-of-function and over-expression of *miR-9a* produce opposite

121 phenotypes with respect to *Senseless* in both embryos and larvae. Thus the phenotypic
122 consequences of *miR-9a* disruption mirror those of *Sens*, suggesting that *miR-9a* is
123 necessary to ensure appropriate Sens expression in the right cells and at the right level to
124 convey robustness to SOP specification (Li *et al.* 2006).
125
126 The *miR-9a* microRNA is a member of one of the ~30-40 families that are predicted to pre-
127 date the divergence of protostomes and deuterostomes, and therefore to be conserved in
128 essentially all bilaterian animals (Wheeler *et al.* 2009; Ninova *et al.* 2014). In every animal
129 where *miR-9* family members have been studied functionally, they are found to regulate
130 processes related to neurogenesis and neuronal progenitor proliferation (Sempere *et al.*
131 2004; Wheeler *et al.* 2009; Delaloy *et al.* 2010). For instance, over-expression of *miR-9* in
132 zebrafish embryo (Leucht *et al.* 2008), mouse embryonic cortex (Zhao *et al.* 2009) and
133 chicken spinal cord (Yoo *et al.* 2011) all lead to a reduction of the number of proliferating
134 neural progenitors and impairment of PNS development. These studies demonstrate clear
135 similarities between *miR-9* expression and function in *Drosophila* and vertebrates. Disrupted
136 *miR-9* function has also been linked with some human pathologies, including cancer
137 progression (Nowek *et al.* 2018) and neurodegenerative amyloid diseases (Packer *et al.*
138 2008). For instance tumorigenic cells from medulloblastoma appear to have decreased
139 expression of *miR-9*, while a subclass of glioblastoma tumour cells express *miR-9* at a higher
140 level (Ferretti *et al.* 2009; Kim *et al.* 2011). In addition, *miR-9* has been also found to have a
141 role as a proto-oncogene and/or as a tumour-suppressor gene during progression of cancers
142 not directly related with the nervous system (Coolen *et al.* 2013).
143

The current model of *miR-9a* function in *Drosophila* SOP specification suggests mutually exclusive reciprocal expression of *miR-9a* and *Sens* in SOPs (Li *et al.* 2006). This in turn suggests a role for *miR-9a* in ensuring that only one of the cells in the progenitor field takes on SOP identity. In this work, we use single-cell quantitative FISH and nascent transcript FISH to investigate the *miR-9a*/Sens/SOP regulatory model in hitherto unseen detail. This use of single-molecule *in situ* hybridization (smFISH) coupled with immunofluorescence (IF) allows us to simultaneously visualize active sites of *miR-9a* transcription and Sens protein in both embryos and larval wing disc at the single cell level. We use these data to analyse the dynamics of *miR-9a* transcription and Sens protein abundance. We find that *miR-9a* and Sens are initially co-expressed, but ultimately exhibit a dynamic reciprocal expression pattern. We observe that *miR-9a* transcription becomes rapidly repressed in high Sens expressing SOPs during embryogenesis, presumably as Sens protein accumulates in the cell nucleus. A subtly different co-expression dynamic was observed during wing disc development, where many SOPs also express *miR-9a*. These SOPs exhibit an inverse relationship between Sens abundance and *miR-9a* transcription. These new data refine and expand the previous model to provide key new insights into *miR-9a/Sens* regulation in PNS development (Li *et al.* 2006). In particular, we include for the first time a temporal element to the understanding of the dynamics of *miR-9a* regulation of SOP differentiation.

Methods

Fly stocks, embryo collection and fixing and larval dissection

Flies were grown at 25°C or 18°C. Embryos were collected after ~20h and fixed in 1V heptane + 1V 4% formaldehyde for 30 minutes shaking at 220 rpm. The embryos were then washed and shaken vigorously for one minute in 100% methanol. Fixed embryos were stored in methanol at -20°C. Larvae were dissected in 1XPBS, carcasses were fixed in 1V 1XPBS + 1V 10% formaldehyde for ~1h, washed with methanol and stored in methanol at -20°C.

Genotypes used for this study are: W [1118], Bloomington stock 3605 and 2XTY1-SGFP-V5-preTEV-BLRP-3XFLAG-Sens, VDRC stock ID 318017

Probe design, smFISH and immunofluorescence

We adapted the inexpensive version (Tsanov *et al.* 2016) of the conventional smFISH protocol in *Drosophila* (Trcek *et al.* 2017). Primary probes were designed against the mature *Senseless* and sfGFP mRNA and a genomic region flanking the *mir-9a* gene locus, all from FlyBase, using the Biosearch Technologies Stellaris probe Designer (version 4.2). To the 5' end of each probe was added the Flap sequence CCTCCTAAGTTTCGAGCTGGACTCAGTG. Multiple secondary probes that are complementary to the Flap sequence were tagged with fluorophores (CAL Fluor Orange 560, CAL Fluor Red 610, Quasar 670) to allow multiplexing.

Antibodies used were Anti Green Fluorescent Protein rabbit igG fraction (Invitrogen #A11122) at 1:500, anti-Sens (Nolo *et al.* 2000) at 1:1000, Goat anti-Guinea Pig IgG (H+L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor 555 (Invitrogen #A21435) at 1:500, and Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody Alexa Fluor 488 (Invitrogen #A11008) at 1:500.

Imaging and quantification

Imaging was performed using a Leica SP8 Inverted Tandem Head confocal microscope with LAS X v.3.5.1.18803 software (University of Manchester Bioimaging facility), using 20X, 40X and 100X magnifications. Deconvolution was performed using Huygens Pro v16.05 software. Protein fluorescence levels were measured using FIJI for Macintosh. From each picture, five measurements of background mean intensity were taken. Each single cell measurement was then adjusted using the formula: integrated density of nucleus – (area of nucleus x background mean).

Results

miR-9a is expressed in the dorsal ectoderm during embryogenesis and ubiquitously in the wing disc

In order to understand the interaction between *miR-9a* and its target *Senseless*, we first systematically describe the *mir-9a* expression pattern in the embryo and imaginal disc at the single cell level. Imaging mature miRNAs is difficult. Previously applied techniques require amplification and often have significant background noise problems (e.g. probes labelled with digoxigenin) (Biryukova *et al.* 2009). Many researchers have tried to overcome this (Lu and Tsourkas 2009), but these approaches still have very limited use for single cell analysis and quantification.

We have used a nascent transcript approach using single molecule FISH to detect expression in cells that are actively producing the miRNA primary transcript (pri-miRNA). To do so we have designed sets of ~48 probes (table 3) that hybridize to a ~1kb region in the primary miRNA transcript flanking the *mir-9a* locus. This allows the detection of active *mir-9a* transcription in the cell nuclei as previously described by Aboobaker and co-workers (2005). We find that expression of *mir-9a* initiates in early stage 5, at which point it is expressed throughout the dorsal ectoderm of the embryo (Fig. 1 A-A'). The pattern displays a precise

boundary between actively transcribing cells and non-expressing cells, which correlates with the mesoderm-ectoderm boundary similar to that described by Fu et al. (2014). There are also small domains at the posterior and anterior embryonic ends lacking *mir-9a* expression. Later, during *Drosophila* embryonic stages 6 and 7, *mir-9a* expression is maintained in this pattern throughout the ectoderm (Fig. 1 B-B' and Fig.1 C-C'), clearly marking the boundary between ectodermal cells and invaginating mesodermal cells. At stage 8, the mesoderm is almost completely invaginated and the *mir-9a* expression domain covers the embryo surface (Fig. D-D'), with the exclusion of the same regions at the anterior and posterior ends. At stages 11 and 14, *mir-9a* continues to be expressed throughout the ectoderm, except from a dorsal anterior region, and it is largely absent from the amnioserosa (Fig. 1 E-F).

It has previously been reported that *mir-9a* is expressed widely in the 3rd instar wing disc but not in cells expressing *Senseless* (Li *et al.* 2006; Biryukova *et al.* 2009). Similarly, we observed that *mir-9a* is actively transcribed everywhere in the wing disc, with the exception of a few randomly distributed cells (Fig. 1 G).

Immunodetection of *Sens*-sfGFP fusion protein allows the study of *Sens* expression in *Drosophila* embryos at single-cell resolution

The role of *miR-9a* in regulating the transcription factor *Senseless* (*Sens*) is well characterised genetically during SOP specification (Li *et al.* 2006; Cassidy *et al.* 2013). To investigate the dynamics of *miR-9a* regulation of *Sens* we developed a strategy to simultaneously observe *Sens* transcription and protein accumulation at the single-cell level

via confocal fluorescent microscopy. In principle, the efficient detection of proteins in fixed samples using IF relies on the availability of antibodies that can specifically detect the protein of interest. Alternatively, there have been developed reporter *Drosophila* lines that express the protein of interest fused to a reporter protein that can be detected enzymatically or by fluorescence (Timmons *et al.* 1997; Chatterjee and Bohmann 2012). We have therefore used a transgenic *Drosophila* reporter line that carries two additional C-terminally tagged Sens-sfGFP reporters that can be detected either directly (live imaging) or by immunofluorescence (Sarov *et al.* 2016). We were therefore able to use two methods in order to examine Sens dynamics: direct detection of Sens using antibodies against the endogenous protein, and indirectly using anti-GFP antibodies. To validate that the reporter accurately reflects endogenous Sens protein pattern and level we performed a double staining experiment against endogenous Sens using an anti-Sens antibody (obtained from H. Bellen lab; Nolo et al., 2000), and an anti-sfGFP antibody, in both embryos and wing discs (Fig.2). Under these conditions, we expect that anti-Sens antibodies tag protein products deriving from 4 *Senseless* genes (2 wild type and 2 sfGFP-tagged), while anti-sfGFP antibodies detect products from only the 2 sfGFP-tagged genes.

The co-localization of the WT and sfGFP-tagged signals in embryogenesis and wing disc development are shown in Fig. 2. The data clearly show that during both embryogenesis (Fig. 2 A-D) and wing disc development (Fig 2 E-H), the two signals co-localize in the same cells. We are therefore confident that the reporter accurately recapitulates the endogenous pattern of Sens expression during embryonic and wing imaginal disc development. We further measured the relative fluorescence from each of the two antibodies in single cells to ensure that the reporter gene reflects Sens abundance. As expected, the sfGFP reporter is

285 expressed proportionally to *Senseless* in both embryos (Fig. 2 D) and wing discs (Fig. 2 I).
286 This indicates that the fluorescence signal from antibodies against sfGFP provides reliable
287 information on endogenous *Senseless* localization and relative expression levels.
288
289 **_mir-9a expression pattern_ and _Senseless_ protein levels are inversely correlated during**
290 **embryogenesis**
291
292 In order to study the reciprocal dynamics of *mir-9a* and *Senseless* during embryogenesis and
293 wing disc development, we simultaneously tracked active sites of *mir-9a* transcription
294 (transcription sites – TS) using smFISH and Sens abundance via IF. We investigated these
295 patterns at three different stages of embryonic development: stage 10, 11 and 12 (Fig. 3 A-
296 C) after early *Senseless* expression and the initial stages of SOP specification. Interestingly
297 we found that *mir-9a* transcription levels were inversely corelated with *Senseless* protein
298 levels, and that *mir-9a* transcription is repressed rapidly after the initiation of Sens
299 expression. Intriguingly, we noticed that a very small number of *Senseless*-expressing cells
300 also displayed active *mir-9a* TSs (Fig. 3 A''-C''). Moreover, when we look at the fluorescence
301 levels of sfGFP and the size of *mir-9a* TSs in the subset of cells that express both, it is evident
302 that both miRNA active transcription and Sens level are lower in comparison to the rest of
303 the cells. We believe that *Senseless* has just started to be translated in these cells and *mir-*
304 *9a* transcription is stopping. Our interpretation is that the accumulation of Sens in the
305 nucleus is associated with repression of *mir-9a* transcription, either by direct repression, or
306 through an intermediary negative regulator.
307

To further investigate the dynamic relationship between expression of *mir-9a* and Sens, we developed a multi-channel experiment to simultaneously study the expression pattern of *mir-9a* TSs and Sens-sfGFP with *Senseless* and sfGFP mRNAs (Fig. 4). Intriguingly, we observe that cells that are transcribing *Senseless,* but that have not yet accumulated observable quantities of Sens protein (total lack of sfGFP signal), transcribe *mir-9a* (Fig. 4 B). *Senseless* expressing cells occupy several embryonic cellular layers. From an orthogonal projection that allows clear visualization of embryonic cell layers, we can see that cells containing Sens protein are located inwards, whereas cells that are transcribing both *mir-9a* and *Senseless* mRNA, but not yet translating Sens protein, are usually localized on the embryonic surface (Fig.4 C). We believe that the rapid dynamic changes in *mir-9a* expression are correlated with SOP differentiation, during which SOPs progressively migrate inwards as Sens protein accumulates and *mir-9a* is turned off.

mir-9a is actively transcribed in cells containing Sens during early SO specification in the third instar imaginal wing disc

Since *Senseless* regulates SOP differentiation during PNS development in the *Drosophila* larvae (Singhania and Grueber 2014), we applied the approach outlined above to study *mir-9a* expression pattern and Sens abundance in third instar imaginal wing discs at the single cell level. The adult *Drosophila* wing possesses a spatially organized series of bristles (a class of SO) located at the wing margin (Lu *et al.* 2011). Flies in which Sens is ectopically expressed in the wing disc exhibit an increased bristle number in that wing region (Jafar-Nejad *et al.* 2003). During larval development, *Senseless* starts to be expressed at around 15h after third instar ecdysis in single SOPs in the wing notum. At around 30h *Senseless* is

expressed in an increased number of isolated SOPs in the wing and notum area plus in 2 distinct stripes of cells in the wing disc pouch (Mirth *et al.* 2009). Cells belonging to these 2 rows of *Senseless*-expressing cells will give rise to the adult wing margin bristles (Jafar-Nejad *et al.* 2006).

By nascent transcript smFISH, we observed that *mir-9a* is expressed in nearly all cells in the wing disc. When we correlated *mir-9a* expression with that of *Senseless* in third instar discs we identified a small population of *Senseless* expressing cells with no *mir-9a* expression. These cells always had high levels of Sens protein, similar to our observations in the embryo. We also observed that many cells that have low or intermediate Sens protein levels are actively transcribing *mir-9a* (Fig. 5 A-B). It is interesting to note that *mir-9a* TSs size in *Senseless*-expressing cells do not differ from the size of TSs belonging to cells that are not expressing Sens protein. This indicates that these cells may not shut down *mir-9a* transcription, which might be kept active or modulated via transcriptional bursting. Nonetheless, at this stage only a minority of cells that contain Sens protein are not transcribing *mir-9a*. We therefore measured the intensity coming from sfGFP antibody (a proxy for Sens protein levels) at the single cell level to see if there was a difference in *Senseless* levels between *mir-9a*-expressing and non-expressing cells. The data clearly show that Sens is more abundant in cells that are not transcribing *mir-9a* (Fig. 5 C). However, our finding of concurrent expression of Sens and *mir-9a* contradicts aspects of the previously established model of triple row bristle specification (Li *et al.* 2006), which suggested a binary co-expression pattern.

Discussion

The stable and reproducible development of the *Drosophila* PNS is an extraordinary model of how the stereotyped stability of cellular differentiation is achieved (Jan and Jan 1994; Barad *et al.* 2011). In this study we focused on the role of *Drosophila miR-9a* in regulating the function of *Sens*, a crucial transcription factor that orchestrates SOP differentiation and PNS development in embryos and larvae. Dysregulated *miR-9a* expression results in disrupted *Sens* function leading to altered SOP differentiation and loss of stable stereotyped neural development (Li *et al.* 2006; Cassidy *et al.* 2013). It has been hypothesized that Notch signalling plays a key role in regulating *mir-9a* transcription in epithelial cells adjacent to potential SOPs thus preventing accumulation of Sens and unintended differentiation of additional SOPs (Li *et al.* 2006). Despite extensive study of *Sens* expression (Nolo *et al.* 2000; Mirth *et al.* 2009), there is little information regarding the developmental profile of *Sens* and *mir-9a* co-expression.

During embryonic development, we show that *mir-9a* is initially expressed throughout the neurogenic ectoderm, and a mutually exclusive expression pattern with *Sens* is established. Our single cell analysis shows that cells just initiating *Sens* expression, who therefore have not accumulated measurable Sens protein, actively transcribe *mir-9a*. However once Sens protein levels increase, *mir-9a* transcription is lost. The data suggest that *mir-9a* expression is turned off when the level of Sens protein reaches a specific threshold. Without *miR-9a* repression, Sens protein then accumulates rapidly, leading to SOP differentiation (Fig. 6 A-B). We suggest that this negative feedback loop involving Sens and *miR-9a* may be key in the

379 regulative establishment of the SOP pattern. It is currently unclear if *Senseless* directly
380 switches off *mir-9a* transcription or if it is an indirect effect of a multi-level genetic cascade.
381
382 Li and co-workers presented evidence that in the third instar wing imaginal disc cells that
383 express *Senseless* do not express *mir-9a*, which is otherwise widely expressed throughout
384 the disc (Li *et al.* 2006). We find that *mir-9a* and *Senseless* are often though not always co-
385 expressed in the wing disc. More specifically we find that among the *Senseless* expressing
386 cells, those that are also transcribing *mir-9a* present a lower level of nuclear Sens, similar to
387 that seen fleetingly prior to the establishment of the terminal and mutually exclusive
388 pattern of SOP specification in the early embryo. The main difference here is that this subset
389 of cells in the wing disc do not seem to be stopping *mir-9a* expression as it was happening in
390 the embryo. This suggests that *mir-9a* and *Senseless* have an intricated reciprocal dynamic
391 expression during embryogenesis and larval development.
392
393 Our findings complement the model (Fig. 6 D-E) presented by Li (Li *et al.* 2006) and show
394 that *mir-9a* and *Senseless* exhibit dynamic co-expression rather than a binary one. Our
395 findings are also in concordance with the suggestion by Jafar-Nejad (2006) that the genes
396 that orchestrate PNS development in embryos and larvae might be different, even though
397 the process follows a similar pattern. For instance, during embryogenesis *achete* and *scute*
398 are necessary for *Sens* activation, while during larval development this function is
399 accomplished by *Wingless* (Jafar-Nejad *et al.* 2006). *mir-9a* function might therefore differ
400 between embryonic and larval SOP development via the presence or absence of other *mir-*
401 *9a* targets. We propose that *mir-9a* repression during embryogenesis allows *Sens* to reach a
402 specific threshold in order to establish the correct number and pattern of embryonic SOPs.

403 During larval development, *Sens* might be expressed at different levels depending on the
404 subclass of SO and this in part involves regulatory feedback by *mir-9a*. The fly wing margin
405 possesses 2 different kinds of SO, the mechanoreceptors and chemoreceptors, and these
406 have a very precise pattern (Hartenstein 1993; Raad *et al.* 2016). *mir-9a* expression may be
407 involved in a regulatory loop with *Sens* to set different *Sens* levels and thereby control the
408 kind of SO that will develop from each particular SOP. Our data further suggest that *Sens*-
409 expressing cells that are not transcribing *mir-9a* will adopt chemoreceptor SOP fate:
410 chemoreceptors are lower in number than mechanoreceptors and their localization and
411 alternation resembles the pattern of cells with high Sens expression level. Therefore, we
412 believe that *mir-9a* serves to keep Sens expression low in mechanoreceptor precursor cells
413 to ensure they adopt the correct SOP cell fate. Temporally, our model suggests that *mir-9a*
414 is initially expressed in all *Sens*-expressing cells, delaying the adoption of a specific SOP fate
415 (Fig. 6 F-G). As SOPs adopt their specific cell fate, *mir-9a* is switched off as a consequence of
416 the transition from a multipotent precursor to a determined cell.
417
418 Our work suggests that *mir-9a* has a dynamic role in the specification of SOP differentiation,
419 tuning *Sens* expression to ensure that the correct number of cells adopt the appropriate
420 SOP fate. The molecular mechanism that dictates *mir-9a* transcription during PNS
421 development remains unknown; its elucidation is important for complete understanding of
422 this dynamic process. A fundamental question that needs to be answered is the mechanism
423 by which the mutual regulation of *mir-9a* and *Sens* act to establish the observed co-
424 expression dynamic, switching from mutually exclusive to co-expressed depending on the fly
425 developmental stage. This study demonstrates the importance of examining miRNA
426 regulation and miRNA-target gene expression dynamics at a single cell level in the

427 developing organism. We suggest that these dynamic co-regulatory processes are a general
428 feature of microRNA function during development.
429

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436

437 **Author contributions**
438 LG, MR and SGJ conceived the project. Experiments were designed by LG and MR and
439 performed by LG. The manuscript was written by all the authors.
440

441 **Competing interests**
442 The authors declare no competing interests.

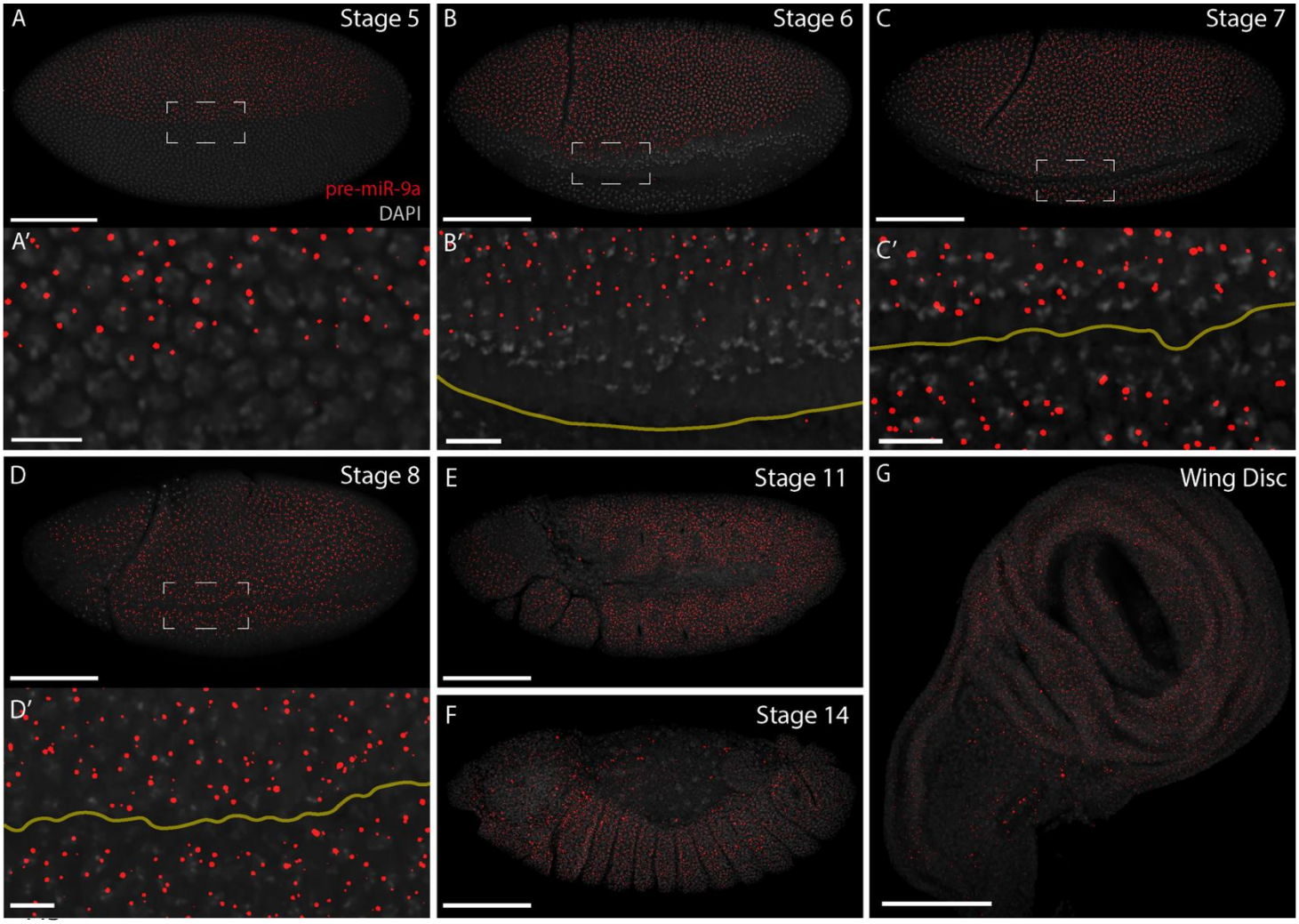

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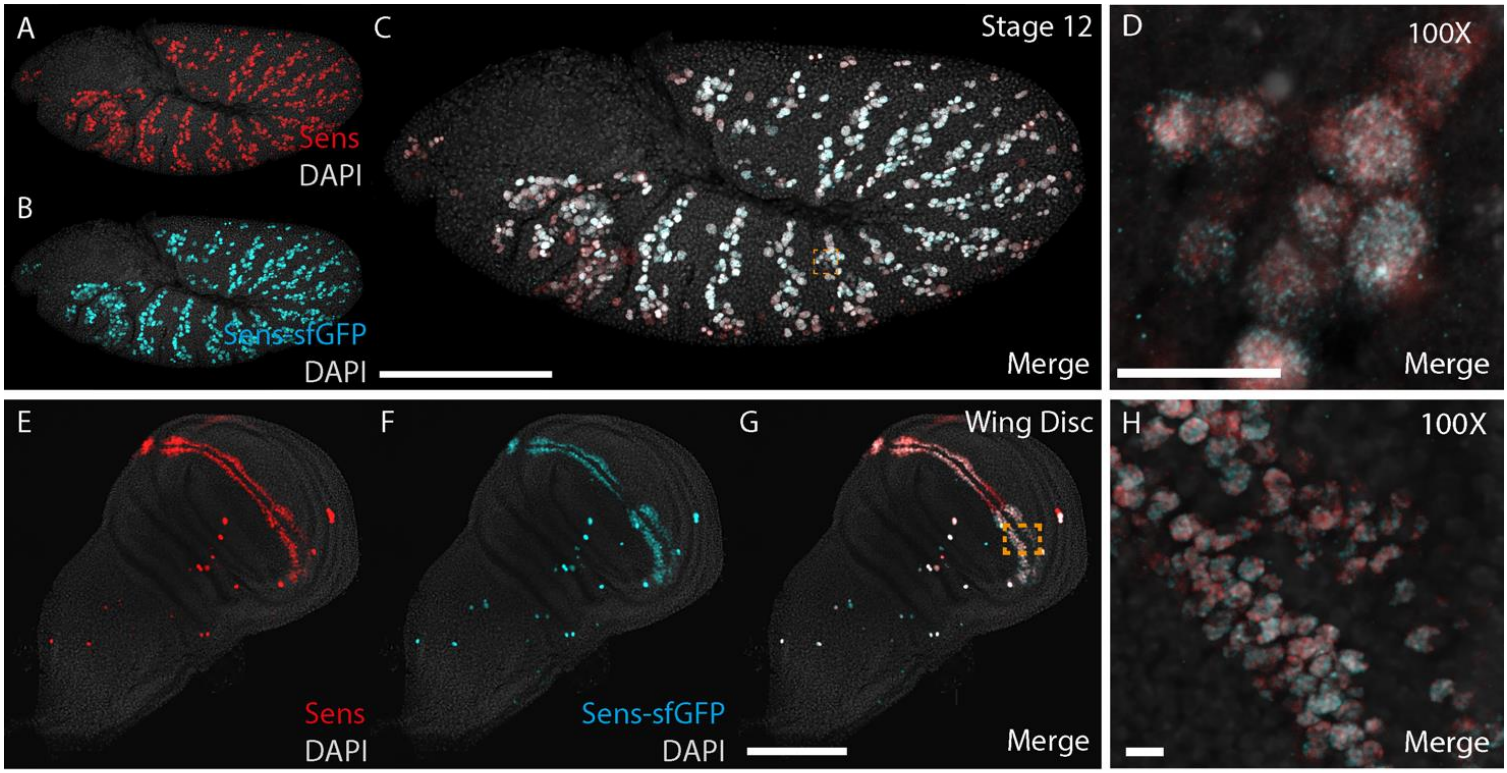
Figure 1. *mir-9a* expression pattern during embryogenesis and in the wing disc. Cells that are actively transcribing *mir-9a* present one or two puncta, indicating *mir-9a* active TSSs. (A-A') Stage 5 WT *Drosophila* embryo: *mir-9a* is expressed in the dorsal ectoderm, before the ventral furrow is evident. (B-B') Stage 6 WT: The *mir-9a* expression domain extends towards the forming ventral furrow (highlighted with a yellow line). (C-C') Stage 7 WT. (D-D') Stage 8 WT. (E-F) At later stages, *mir-9a* is expressed throughout the ectoderm. (G) WT *Drosophila* wing disc: *mir-9a* is expressed widely throughout the disc. Scalebars: (A) 100 μm, (A') 10 μm, (G) 100 μm.



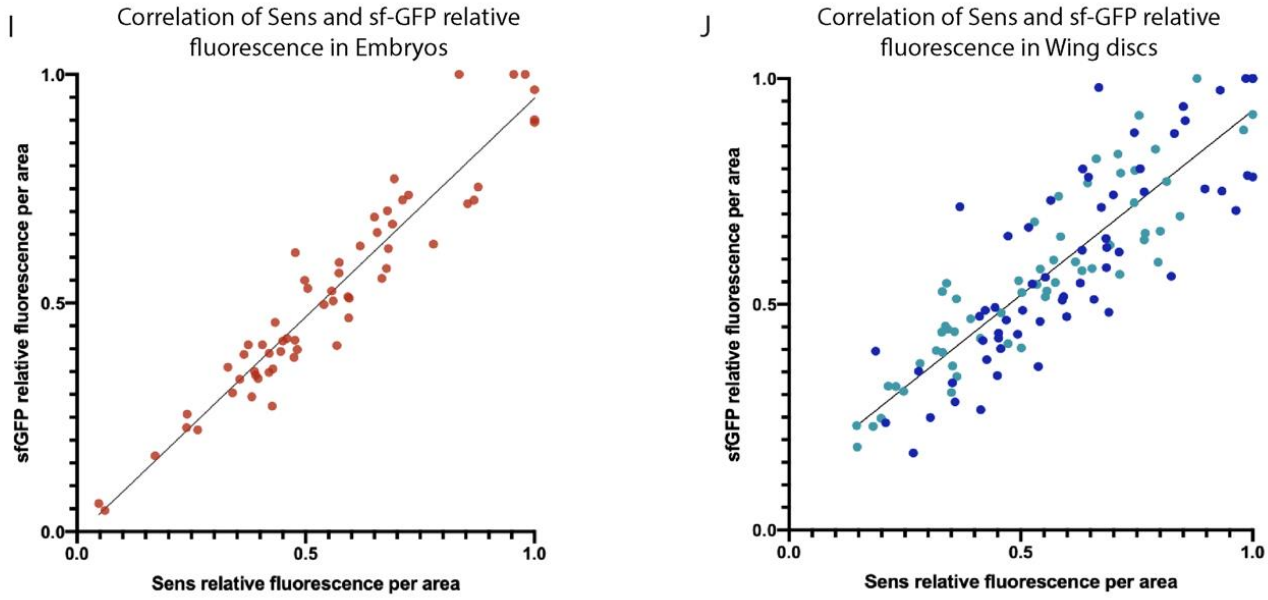


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Figure 2. Colocalization of sfGFP reporter with endogenous Sens. Transgenic stage 12 embryo stained with antibodies against Sens (A, red) and sfGFP (B, cyan). The two antibodies colocalize (merged in D,E), showing that the sfGFP reporter is expressed in the same cells that are expressing endogenous Sens. Transgenic third instar larval wing disc stained with antibodies against Sens (E, red) and sfGFP (F, cyan). Again, the two antibodies colocalize in the same cells (merged in G,H). Correlation between relative fluorescence of Sens antibody and sfGFP antibody in embryos (I) and wing disc (J). For each of 3 embryos, fluorescence measurements were performed in 10 cells, while for each of 3 wing disc, 20 cells were measured. For each replicate, antibody fluorescence was normalized using the maximum value measured in that replicate. Scalebars: (C) 100 μm, (D) 10 μm, (G) 100 μm, (H) 10 μm.

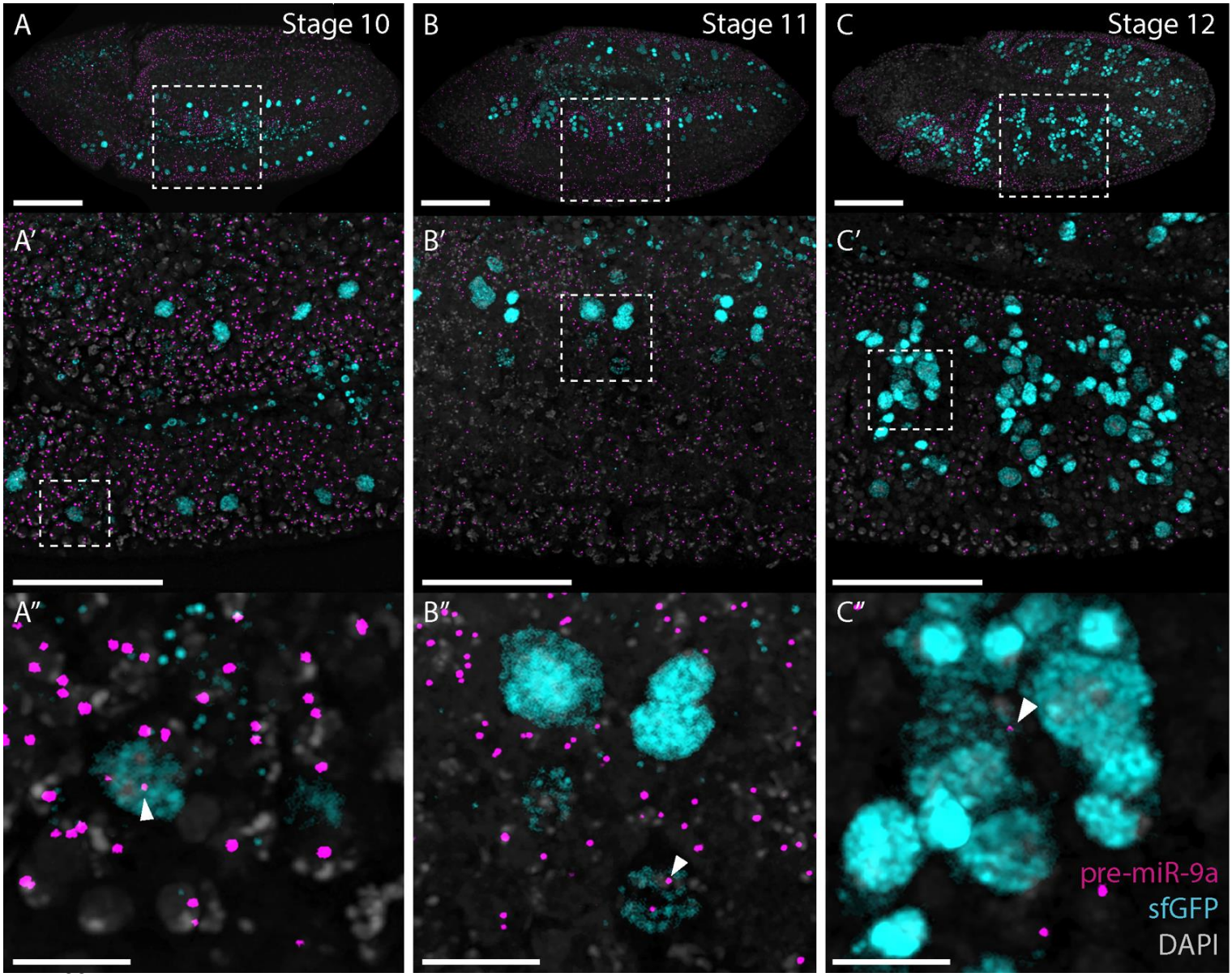

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Figure 3. *mir-9a* is generally not actively transcribed in *Senseless* expressing cells during embryogenesis. (A-C) Transgenic embryos stained with probes against pri-*mir-9a* (magenta) and antibodies against sfGFP (cyan). (A,A',A'') Stage 10 embryo. Sens is expressed in isolated cells. Some Sens-expressing cells have active sites of transcription for *mir-9a,* but these appear to be less intense. (B,B',B'') Stage 11 embryo. Sens is expressed in more cells, a few of which still transcribe *mir-9a*. (C,C',C'') Stage 12 embryo. Sens expression reaches its peak during embryogenesis and *mir-9a* is generally not transcribed in Sens-expressing cells. Scalebars: (A-C) 100 μm, (A'-C') 50 μm, (A''-C'') 10 μm.

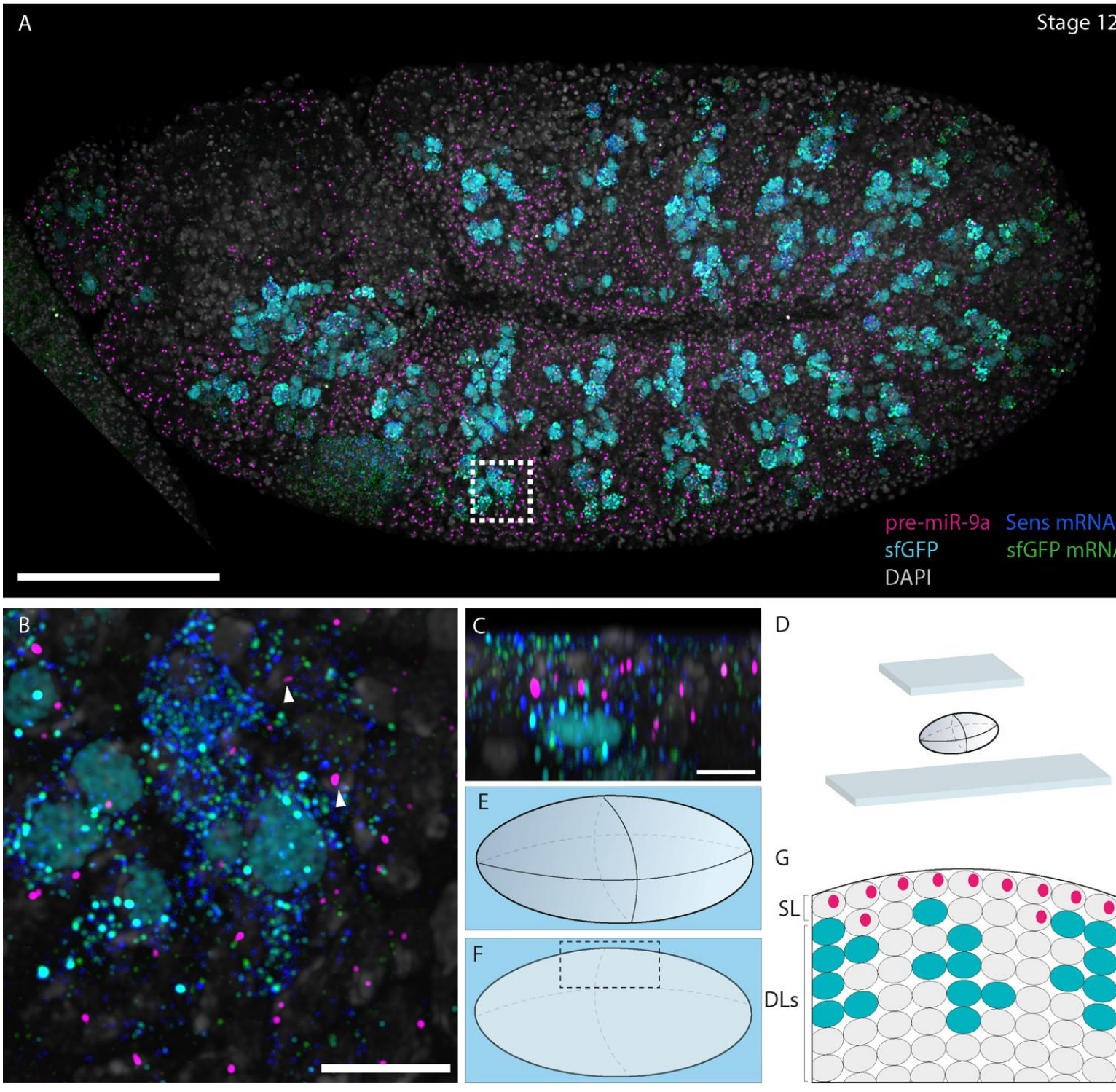

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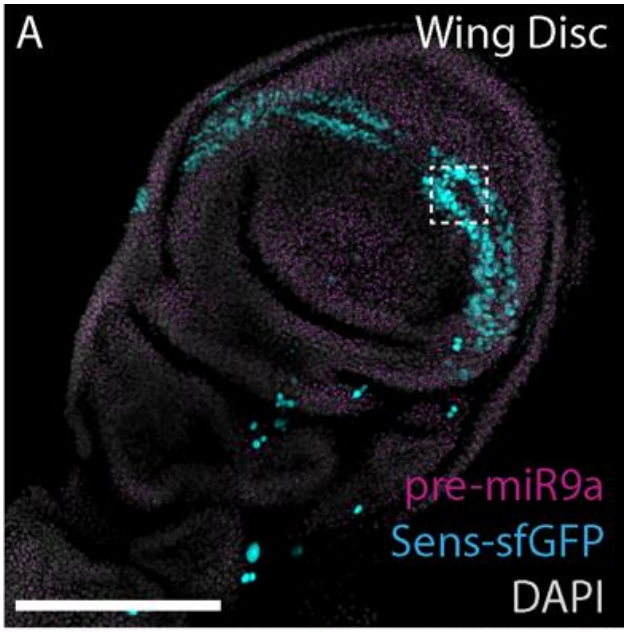
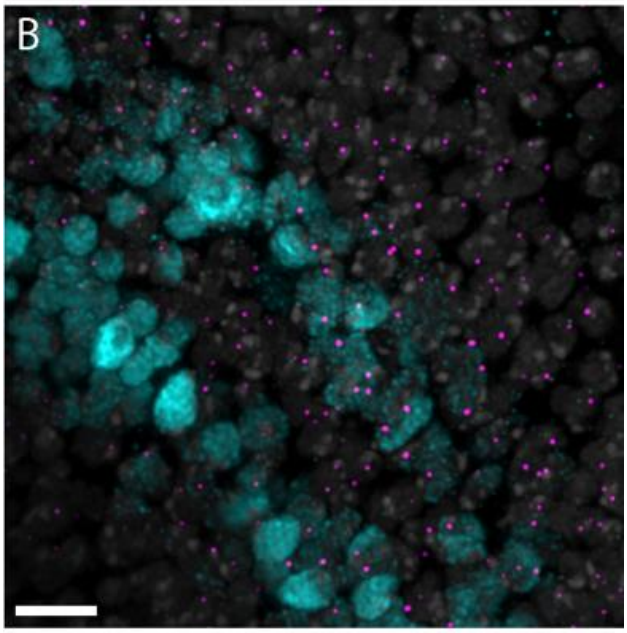
Figure 4. *miR-9a* is actively transcribed after *Senseless* transcription starts but before Sens protein is detectable. (A) Stage 12 transgenic embryo stained with probes against pri-*mir-9a*, sfGFP mRNA and *Senseless* mRNA and antibody against sfGFP. (B) zoom from different focal plane showing that some cells transcribing *Senseless* are still transcribing *mir-9a*. (C) Orthogonal view from (B) showing *mir-9a* is mostly expressed in cells at the embryo surface, some of which show detectable *Senseless* and GFP mRNA. Cells that already have Sens protein have migrated inwards. (D) Schematic representation of embryo mounting after smFISH and/or IF. (E) Lateral view of an embryo. (F) Lateral section of embryo in (E). (G) Schematic representation of what is reported in panel (C). SL = superficial cellular layer, DLs = deeper cellular layers. Scalebars: (A) 100 μm, (B, C) 10 μm.





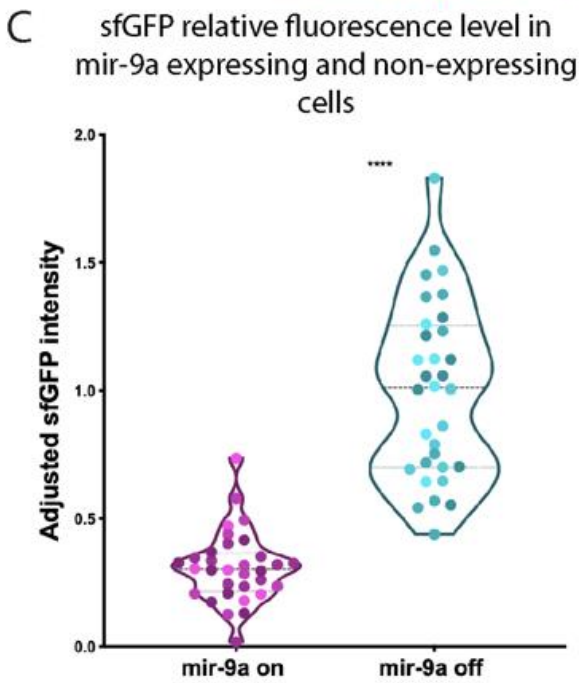


Figure 5. In the wing disc, Sens-expressing cells are generally actively transcribing *mir-9a*.

(A-B) Third instar larval transgenic wing disc stained with probes against pri-*mir-9a* and antibody against sfGFP. Many cells that are actively transcribing *mir-9a* are also expressing Sens. (C) Adjusted sfGFP intensity coming from Sens expressing cells that are and are not actively transcribing *mir-9a*. Different colours represent data coming from different discs (n=4). Data from each replicate was normalized using the maximum adjusted fluorescence value from the group *mir-9a* off from that replicate. Scalebars: (A) 100μm, (B) 10 μm.

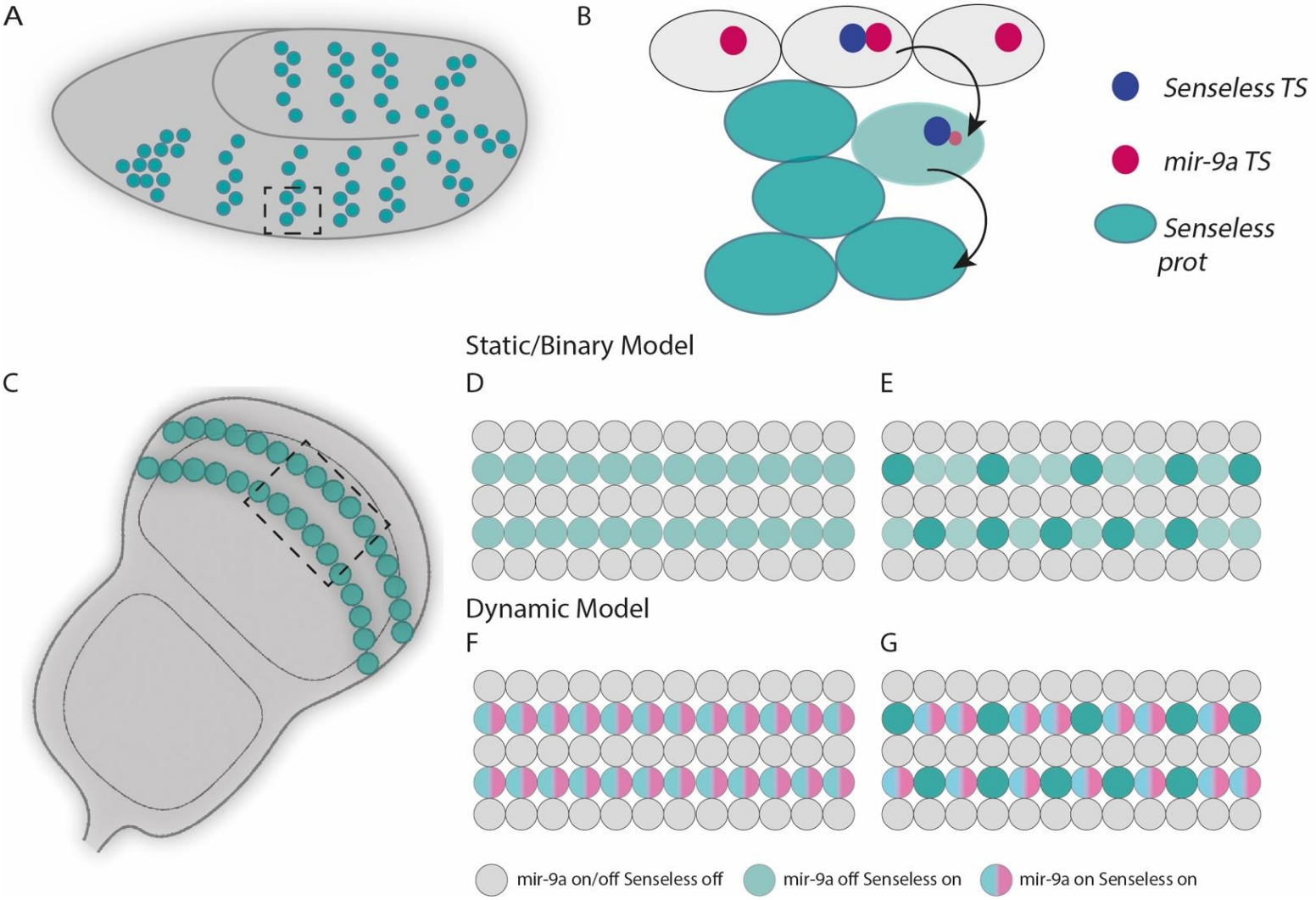


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Figure 6. Model of *mir-9a* and *Senseless* interaction during embryogenesis and wing disc development. In the embryo (A) *Senseless* is expressed in clusters of cells. The orthogonal view of one of these clusters (B) shows that *mir-9a* is transcribed in cells at the top, some of which are turning *Senseless* transcription on. As *Senseless* mRNA gets translated, these cells stop transcribing *mir-9a* and move inwards. (C) In the *Drosophila* wing disc, *Senseless* is expressed in 2 rows of cells (plus additional isolated cells not shown). (D-E) In a static model, *mir-9a* and *Senseless* are not co-expressed. (F-G) Dynamic model in which all the cells that contain Sens are transcribing mir-9a, which is then turned off in a specific class of SOPs.

505 **Supplemental Table 1**

506 **Table 1. Probes against pri-*mir-9a*.**

Probe #	Probe sequence (5'-> 3')
1	GTTGGTCAAGTGACTGTAAC
2	CCATCGCATTCTCAATGTTT
3	CCATTCTTACTCTACTCTTA
4	TTATGCCCACCAAAACGAGA
5	ATATGCACGCTACGACGATC
6	AAGCGAGGAGCTGCAATGAT
7	GAAAACTCGATGCCATGGGA
8	GTTCGTCCAGAACCAGAAAC
9	AGGTCAAAGTTGGACGAGCG
10	GCAACGAGATAATGGTCGGA
11	GTATGCATTTTGCATAGACT
12	GCTGCGAATGCATTGGGAAA
13	TACAAGTGTGTTTTTGCGCA
14	TTGGTTTTGGCTTTTTGTGT
15	TGTCGTCATTGTTGTTTTGG
16	GCACACAATCTAATTTCCCA
17	CTCGTTTCACCGACTTGTCG
18	CCCATGGACATTCACTCGAT
19	ATTTATGTTAAGTGCTCGCT
20	TTTTTTTTCGCTTTTGGTGC

21	ATGAACGCTTATTTCGCCTC
22	GCCTCCTCTTTTGTTAAATT
23	CTGCAGATGGTTGAAAGGGA
24	GTATATAGAGTCGATTGTGT
25	ACCAAAGACAACATAGCACC
26	TTATCACTCATACAGCTAGA
27	TCGGTAAGCTAGCTTTATGA
28	CTGGGCAGACGCTAATATTA
29	AAGTATACGCAATGTGGGCC
30	GCTTATTTTGATGTGTTTCC
31	ATGCATGGTGTACATATGGG
32	TTGGCCGTAAAGCCAAACTG
33	GGTTTTTTTGCTTGCCAAAG
34	AACTTACTCGTTTGTACGCG
35	CCATGCAAAGGTCGTATCTA
36	GTTCGCAGGGGGTAAAACAA
37	TGTCTTTTCCACCTCTTTTG
38	CAACGTCATTGACTGCTGTT
39	AATCAGTGTTCATCAGGTGC
40	GCCTGCAAATGATCTTTCAT
41	AGCTTGCCGTTATTATCTTG
42	GTTGTTGCTATCCGAAACTT
43	TTTCCTCTAAAGTTCCTAGC

44	GTTCGACGGCTTTAAGAGTG
45	GTTTATGGTGTTTACAAGTT
46	ATGTTATTTGCTTACTTTCC
47	ATAACTTATTGCACGCTATT
48	GCTTTCTATTAAGCTGATCA

507 **Table 2. Probes against *Senseless***

Probe #	Probe sequence (5' -> 3')
1	TCACTGGAGACGAACACGTC
2	TTCAGTGCGGTTTTATCTTG
3	GGATTCGAACGGATCTTCGA
4	AATCTCACTTTCTTGGCGTT
5	TCTCAATACTGTTCACTGCA
6	TGGAATCTCACTGGATCTGA
7	CGGCGATAGGTGATTCATTT
8	TATCACAGTGTTGAAGGCGG
9	GATCTCGACCATTGAGTTTC
10	GAGCAGCGGGTTGTACAAAA
11	AAAATTGGGGCCACAGTAGG
12	CTGACCCAAAACCACAGAGG
13	TCAAGGCAAAGTCACGATCC
14	TTCATTTGGAGTTCATGCTC
15	CTTGCTGTTCTCATTGTTAT
16	GTGCTTAGATTTAGTGGCAT

17	GTTGGAATCATCCGATGTGA
18	TAACGTTCAGCGAGGTCATC
19	TTTAGATTCACAGCGCTCAG
20	TTGCTGTGGTGTACTCGAAC
21	CGGTGACCAGATGATGTTAC
22	GCTCATACTTGAACTTTCGC
23	ATTGCAGCGAGGATATGGAT
24	ATGGGCATACAACTGCTGTT
25	GACGAGGAGGACGAACGACG
26	TTCGTTTTCTCCTTGGTAAG
27	GTTTGCACTGGAAATTCCTG
28	ACGAACGCTTGAAGCTCTTG
29	TGTGGATGAGCAGATGTGTG
30	CCACAATATTGGCAGGGATA
31	ATGTCCGACTTTTGGTGGAA
32	GTATGTATGTACGTGTGCTT
33	TCATATGCGTGATCAGGTTG
34	TGGCAACCGAAGGGTTTATA
35	CTGGAAGGACTGATCGCAAA
36	ATCTCCTGGATCAGAGATTG
37	GTCCTGCTTAGCTAATACTG
38	TAGACTTAGGGCCAACTGTG
39	ACTTTGGCTTCATAGCAACG

40	TCTTTGCGAAGGCCTAAACA
41	CTTAAGGCAGTATCTTAGCT
42	GAGTCTTGTTTGGGATAACT
43	TAAGCTAGGGTTTAACGGCT
44	CTGCCAATTTTGGGTTAAGT
45	TGAGGTTGTTCTTTGGTACG
46	TTAGACAAGTGGTCGCCAAG
47	GTGGGTGATGCCATCAATAA
48	TTGTTCTCTTTTAGCTTTCA

508 **Table 3. Probes against sfGFP.**

Probe #	Probe sequence (5'-> 3')
1	CGGTGAACAGCTCCTCGC
2	CACCAGGATGGGCACCAC
3	GTTCACGTCGCCATCCAG
4	CGCACGCTGAACTTGTGG
5	GTCAGCTTGCCGTTGGTG
6	CCGGTGGTGCAGATGAAC
7	GTAGGTCAGGGTGGTCAC
8	GTAGCGGCTGAAGCACTG
9	TCGTGCTGCTTCATGTGA
10	GCATGGCGCTCTTGAAGA
11	TGAAGCTGATGGTGCGCT
12	CTTGTAGGTGCCGTCATC

13	TCGAACTTCACCTCGGCG
14	CGGTTCACCAGGGTATCG
15	AAATCGATGCCCTTCAGC
16	CAGGATGTTGCCATCCTC
17	TTGTACTCCAGCTTGTGG
18	GTACACGTTGTGGCTGTT
19	TTCTGCTTATCGGCGGTG
20	GAAGTTGGCCTTGATGCC
21	TCCACATTGTGGCGGATC
22	TAGTGATCGGCCAGCTGC
23	CGATGGGGGTGTTCTGCT
24	TATCGGGCAGCAGCACTG
25	CTGGGTGCTCAGGTAGTG
26	GTTGGGGTCCTTGGACAG
27	ACCATGTGATCGCGCTTC
28	CGGTCACGAACTCCAGCA
29	TCCATGCCCAGGGTGATG
30	CCTCGAGCTTGTACAGCT

509 **References**

510 Aboobaker, A. A., P. Tomancak, N. Patel, G. M. Rubin, and E. C. Lai, 2005 Drosophila
511 microRNAs exhibit diverse spatial expression patterns during embryonic development.
512 Proc. Natl. Acad. Sci. U. S. A. 102: 18017–18022.

513 Acar, M., H. Jafar-Nejad, N. Giagtzoglou, S. Yallampalli, G. David *et al.*, 2006 Senseless
514 physically interacts with proneural proteins and functions as a transcriptional co-
515 activator. Development 133: 1979–1989.

516 Artavanis-Tsakonas, S., and P. Simpson, 1991 Choosing a cell fate: a view from the Notch
517 locus. Trends Genet. 7: 403–408.

518 Barad, O., E. Hornstein, and N. Barkai, 2011 Robust selection of sensory organ precursors by
519 the Notch-Delta pathway. Curr. Opin. Cell Biol. 23: 663–667.

520 Biryukova, I., J. Asmar, H. Abdesselem, and P. Heitzler, 2009 Drosophila mir-9a regulates
521 wing development via fine-tuning expression of the LIM only factor, dLMO. Dev. Biol.
522 327: 487–496.

523 Cassidy, J. J., A. R. Jha, D. M. Posadas, R. Giri, K. J. T. Venken *et al.*, 2013 MiR-9a minimizes
524 the phenotypic impact of genomic diversity by buffering a transcription factor. Cell 155:
525 1556–1567.

526 Caygill, E. E., and A. H. Brand, 2017 miR-7 Buffers Differentiation in the Developing
527 Drosophila Visual System. Cell Rep. 20: 1255–1261.

528 Chatterjee, N., and D. Bohmann, 2012 A versatile ϕC31 based reporter system for
529 measuring AP-1 and NRF2 signaling in Drosophila and in tissue culture. PLoS One 7:.

530 Coolen, M., S. Katz, and L. Bally-Cuif, 2013 miR-9: A versatile regulator of neurogenesis.
531 Front. Cell. Neurosci. 7:.

532 Delaloy, C., L. Liu, J. A. Lee, H. Su, F. Shen *et al.*, 2010 MicroRNA-9 Coordinates Proliferation

and Migration of Human Embryonic Stem Cell-Derived Neural Progenitors. Cell Stem Cell 6: 323–335.

Ferretti, E., E. De Smaele, A. Po, L. Di Marcotullio, E. Tosi *et al.*, 2009 MicroRNA profiling in human medulloblastoma. Int. J. Cancer 124: 568–577.

Fu, S., C. Y. Nien, H. L. Liang, and C. Rushlow, 2014 Co-activation of microRNAs by Zelda is essential for early drosophila development. Dev. 141: 2108–2118.

Ghysen, A., and C. Dambly-Chaudiere, 1989 Genesis of the Drosophila peripheral nervous system. Trends Genet. 5: 251–255.

Giri, R., D. K. Papadopoulos, D. M. Posadas, H. K. Potluri, P. Tomancak *et al.*, 2020 Ordered patterning of the sensory system is susceptible to stochastic features of gene expression. Elife 9:.

Goulding, S. E., P. zur Lage, and A. P. Jarman, 2000 amos, a proneural gene for Drosophila olfactory sense organs that is regulated by lozenge. Neuron 25: 69–78.

Hartenstein, V., 1993 Atlas of Drosophila development. Dev. Drosoph. melanogaster.

Hartenstein, V., 1988 Development of Drosophila larval sensory organs: Spatiotemporal pattern of sensory neurones, peripheral axonal pathways and sensilla differentiation. Development 102: 869–886.

Hilgers, V., N. Bushati, and S. M. Cohen, 2010 Drosophila microRNAs 263a/b confer robustness during development by protecting nascent sense organs from apoptosis. PLoS Biol. 8:.

Huang, M. L., C. H. Hsu, and C. T. Chien, 2000 The proneural gene amos promotes multiple dendritic neuron formation in the Drosophila peripheral nervous system. Neuron 25: 57–67.

Jafar-Nejad, H., M. Acar, R. Nolo, H. Lacin, H. Pan *et al.*, 2003 Senseless acts as a binary

switch during sensory organ precursor selection. Genes Dev. 17: 2966–2978.

Jafar-Nejad, H., A. C. Tien, M. Acar, and H. J. Bellen, 2006 Senseless and Daughterless confer neuronal identity to epithelial cells in the Drosophila wing margin. Development 133: 1683–1692.

Jan, Y. N., and L. Y. Jan, 1994 Genetic Control of Cell Fate Specification in Drosophila Peripheral Nervous System. Annu. Rev. Genet. 28: 373–393.

Jarman, A. P., Y. Grau, L. Y. Jan, and Y. N. Jan, 1993 atonal is a proneural gene that directs chordotonal organ formation in the Drosophila peripheral nervous system. Cell 73: 1307–1321.

Kim, T. M., W. Huang, R. Park, P. J. Park, and M. D. Johnson, 2011 A developmental taxonomy of glioblastoma defined and maintained by microRNAs. Cancer Res. 71: 3387–3399.

Lai, E. C., and V. Orgogozo, 2004 A hidden program in Drosophila peripheral neurogenesis revealed: Fundamental principles underlying sensory organ diversity. Dev. Biol. 269: 1–17.

Leucht, C., C. Stigloher, A. Wizenmann, R. Klafke, A. Folchert *et al.*, 2008 MicroRNA-9 directs late organizer activity of the midbrain-hindbrain boundary. Nat. Neurosci. 11: 641–648.

Li, Y., F. Wang, J. A. Lee, and F. B. Gao, 2006 MicroRNA-9a ensures the precise specification of sensory organ precursors in Drosophila. Genes Dev. 20: 2793–2805.

Lu, Q., T. F. Rau, V. Harris, M. Johnson, D. J. Poulsen *et al.*, 2011 Increased p38 mitogen-activated protein kinase signaling is involved in the oxidative stress associated with oxygen and glucose deprivation in neonatal hippocampal slice cultures. Eur. J. Neurosci. 34: 1093–1101.

Lu, J., and A. Tsourkas, 2009 Imaging individual microRNAs in single mammalian cells in situ.

581 Nucleic Acids Res. 37:.

582 Mirth, C. K., J. W. Truman, and L. M. Riddiford, 2009 The Ecdysone receptor controls the
583 post-critical weight switch to nutrition-independent differentiation in Drosophila wing
584 imaginal discs. Development 136: 2345–2353.

585 Ninova, M., M. Ronshaugen, and S. Griffiths-Jones, 2014 Fast-evolving microRNAs are highly
586 expressed in the early embryo of Drosophila virilis. Rna 20: 360–372.

587 Nolo, R., L. A. Abbott, and H. J. Bellen, 2000 Senseless, a Zn finger transcription factor, is
588 necessary and sufficient for sensory organ development in Drosophila. Cell 102: 349–
589 362.

590 Nowek, K., E. A. C. Wiemer, and M. Jongen-Lavrencic, 2018 The versatile nature of miR-9/9*
591 in human cancer. Oncotarget 9: 20838–20854.

592 Orgogozo, V., and F. Schweisguth, 2004 Evolution of the larval peripheral nervous system in
593 Drosophila species has involved a change in sensory cell lineage. Dev. Genes Evol. 214:
594 442–452.

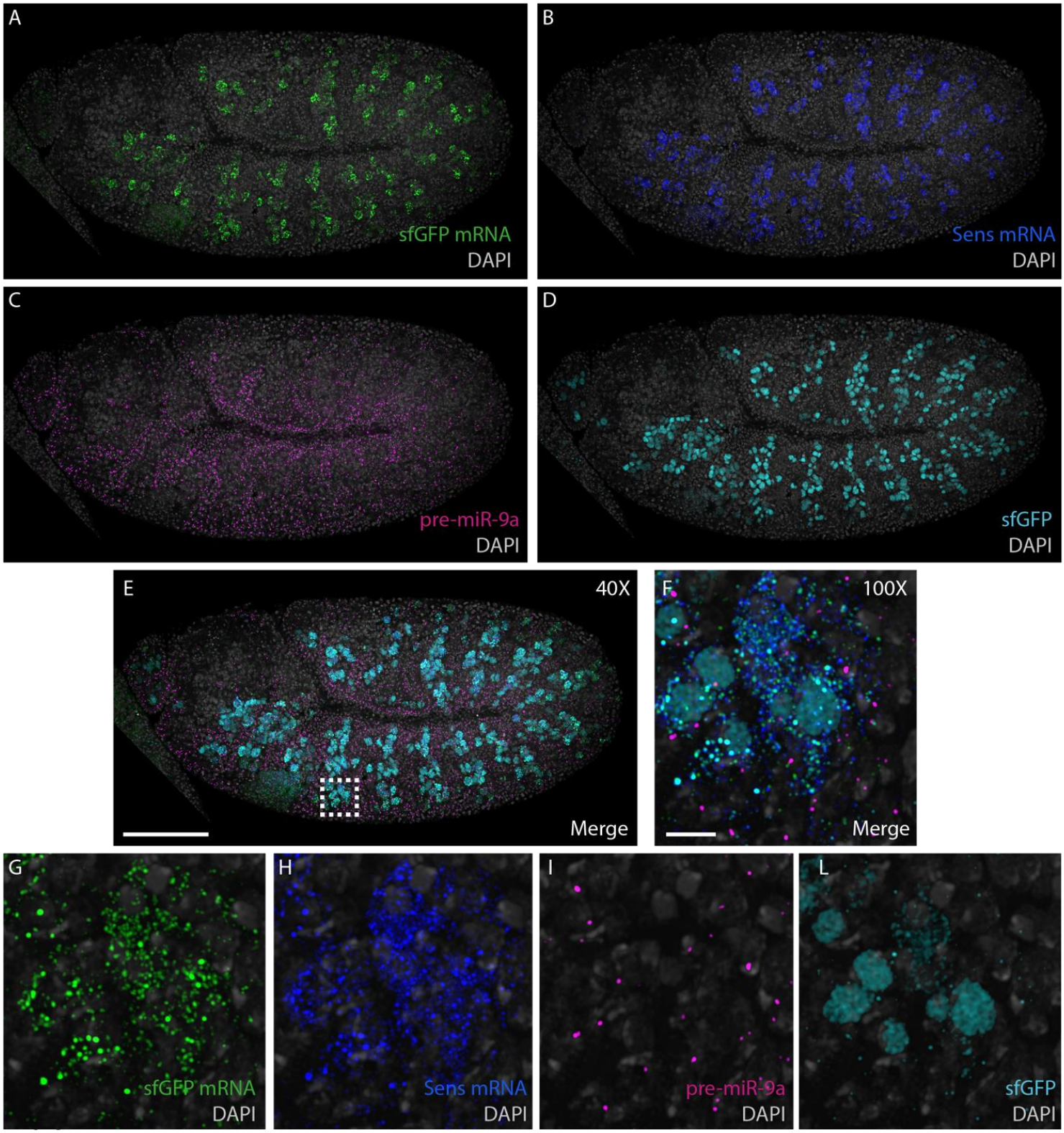
595 Orgogozo, V., F. Schweisguth, and Y. Bellaïche, 2001 Lineage, cell polarity and inscuteable
596 function in the peripheral nervous system of the Drosophila embryo. Development
597 128: 631–643.

598 Packer, A. N., Y. Xing, S. Q. Harper, L. Jones, and B. L. Davidson, 2008 The bifunctional
599 microRNA miR-9/miR-9* regulates REST and CoREST and is downregulated in
600 Huntington's disease. J. Neurosci. 28: 14341–14346.

601 Raad, H., J. F. Ferveur, N. Ledger, M. Capovilla, and A. Robichon, 2016 Functional Gustatory
602 Role of Chemoreceptors in Drosophila Wings. Cell Rep. 15: 1442–1454.

603 Reeves, N., and J. W. Posakony, 2005 Genetic programs activated by proneural proteins in
604 the developing Drosophila PNS. Dev. Cell 8: 413–425.

605 Sarov, M., C. Barz, H. Jambor, M. Y. Hein, C. Schmied *et al.*, 2016 A genome-wide resource
606 for the analysis of protein localisation in Drosophila. Elife 5:.
607 Sempere, L. F., S. Freemantle, I. Pitha-Rowe, E. Moss, E. Dmitrovsky *et al.*, 2004 Expression
608 profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs
609 with possible roles in murine and human neuronal differentiation. Genome Biol. 5:.
610 Singhania, A., and W. B. Grueber, 2014 Development of the embryonic and larval peripheral
611 nervous system of Drosophila. Wiley Interdiscip. Rev. Dev. Biol. 3: 193–210.
612 Timmons, L., J. Becker, P. Barthmaier, C. Fyrberg, A. Shearn *et al.*, 1997 Green fluorescent
613 protein/β-galactosidase double reporters for visualizing Drosophila gene expression
614 patterns. Dev. Genet. 20: 338–347.
615 Trcek, T., T. Lionnet, H. Shroff, and R. Lehmann, 2017 mRNA quantification using single-
616 molecule FISH in Drosophila embryos. Nat. Protoc. 12: 1326–1347.
617 Tsanov, N., A. Samacoits, R. Chouaib, A. M. Traboulsi, T. Gostan *et al.*, 2016 SmiFISH and
618 FISH-quant - A flexible single RNA detection approach with super-resolution capability.
619 Nucleic Acids Res. 44:.
620 Wheeler, B. M., A. M. Heimberg, V. N. Moy, E. A. Sperling, T. W. Holstein *et al.*, 2009 The
621 deep evolution of metazoan microRNAs. Evol. Dev. 11: 50–68.
622 Yoo, A. S., A. X. Sun, L. Li, A. Shcheglovitov, T. Portmann *et al.*, 2011 MicroRNA-mediated
623 conversion of human fibroblasts to neurons. Nature 476: 228–231.
624 Zhao, C., G. Sun, S. Li, and Y. Shi, 2009 A feedback regulatory loop involving microRNA-9 and
625 nuclear receptor TLX in neural stem cell fate determination. Nat. Struct. Mol. Biol. 16:
626 365–371.
627



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630 **Supplementary Figure S1** Single Channels from Fig.4

631 (A) sfGFP probes 40X. (B) *Senseless* probes 40X. (C) pre-*miR-9a* probes 40X. (D) sfGFP

632 antibody 40X. (E) Marge 40X. (F) Merge 100X. (G) sfGFP probes 100X. (H) *Senseless*

633 probes 100X. (I) pre-*miR-9a* probes 100X. (L) sfGFP antibody 100X.