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***miR-9a* regulates levels of both *rhomboid* mRNA and protein in the early *Drosophila melanogaster* embryo**

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1 Abstract

2 MicroRNAs have subtle and combinatorial effects on the expression levels of their
3 targets. Studying the consequences of a single microRNA knockout often proves difficult as
4 many such knockouts exhibit phenotypes only under stress conditions. This has led to the
5 hypothesis that microRNAs frequently act as buffers of noise in gene expression. Observing
6 and understanding buffering effects requires quantitative analysis of microRNA and target
7 expression in single cells. To this end, we have employed single molecule fluorescence *in situ*
8 hybridization, immunofluorescence, and high-resolution confocal microscopy to investigate
9 the effects of *miR-9a* loss on the expression of the serine-protease rhomboid in *Drosophila*
10 *melanogaster* early embryos. Our single-cell quantitative approach shows that *rhomboid*
11 mRNA exhibits the same spatial expression pattern in WT and *miR-9a* knockout embryos,
12 although the number of mRNA molecules per cell is higher when *miR-9a* is absent. However,
13 the level of rhomboid protein shows a much more dramatic increase in the *miR-9a* knockout.
14 Specifically, we see accumulation of rhomboid protein in *miR-9a* mutants by stage 5, much
15 earlier than in WT. The data therefore show that *miR-9a* functions in the regulation of
16 *rhomboid* activity by both inducing mRNA degradation and inhibiting translation in the
17 blastoderm embryo. Temporal regulation of neural proliferation and differentiation in
18 vertebrates by *miR-9* is well-established. We suggest that *miR-9* family microRNAs are
19 conserved regulators of timing in neurogenic processes. This work shows the power of single-
20 cell quantification as an experimental tool to study phenotypic consequences of microRNA
21 mis-regulation.

22 Introduction

23 The study of development in *Drosophila melanogaster* embryos, larvae, and adults has
24 provided an extremely important model for the study of microRNA (miRNA) biogenesis and
25 function (Matranga *et al.* 2005; Rand *et al.* 2005; Okamura *et al.* 2007). MicroRNAs are short
26 ~22 nucleotide long, single-stranded, endogenous RNAs found in animals and plants (Bartel
27 2004; Kozomara *et al.* 2019). MicroRNAs regulate gene expression post-transcriptionally by
28 recruiting the RNA-induced silencing complex (RISC) and then binding to specific sequences
29 on target mRNA molecules, usually in their 3'UTR. The binding of the miRNA-RISC triggers
30 repression of translation, deadenylation, and/or degradation of the target mRNA (Valencia-
31 Sanchez *et al.* 2006). It is estimated that the majority of animal mRNAs are targeted by
32 miRNAs (Friedman *et al.* 2009; Agarwal *et al.* 2015). An intriguing debate has arisen regarding
33 the phenotypic consequences of miRNA mis-regulation, with GOF (gain of function) and LOF
34 (loss of function) studies in different organisms finding that they act as either minor
35 modulators or key regulators of gene expression (Miska *et al.* 2007; Alvarez-Saavedra and
36 Horvitz 2010; Chen *et al.* 2014).

37 In many cases, individual effects of miRNAs on the expression of a target are relatively
38 small (Miska *et al.* 2007; Alvarez-Saavedra and Horvitz 2010; Chen *et al.* 2019a). In addition,
39 each miRNA may target hundreds of different transcripts, and many different miRNAs have
40 been found to act on the same targets (Peter 2010). It is therefore expected that a high degree
41 of quantitative precision is required to determine specific effects of miRNAs on gene
42 expression. Indeed, a complete understanding of miRNA function will only come from a
43 precise quantitative analysis of miRNA activity at the single cell level. Single cell studies of
44 miRNA effects on gene regulation may provide insight into mis-regulation phenotypes that

45 are not apparent at a tissue or organism level (Miska *et al.* 2007; Alvarez-Saavedra and Horvitz
46 2010). It has also been observed that the phenotypic effects of miRNA mutation or mis-
47 regulation are sometimes only revealed under particular conditions (e.g. dietary or
48 temperature stresses) (Li *et al.* 2009; Kennell *et al.* 2012). For example, flies lacking *miR-14*
49 are more sensible to salt stress compared to WT, while flies lacking *miR-7* present abnormal
50 expression of the proteins Yan and Ato only under temperature fluctuations (Xu *et al.* 2003;
51 Li *et al.* 2009). Such stress-dependent miRNA phenotypes have also been observed in other
52 organisms such as mouse and zebrafish (Van Rooij *et al.* 2007; Flynt *et al.* 2009). Thus, the
53 phenotypic consequences of miRNA mis-regulation may be subtle and cryptic.

54 The *mir-9* miRNA family is highly conserved in bilaterians and is a good example of a
55 miRNA that can exhibit both subtle and strong phenotypes (Coolen *et al.* 2013). Experiments
56 in a variety of vertebrate models show conservation of *mir-9* expression and function in
57 neurogenesis and neuronal progenitor proliferation. Over-expression of *mir-9* in zebrafish
58 embryos (Leucht *et al.* 2008), mouse embryonic cortex (Zhao *et al.* 2009) and chicken spinal
59 cord (Otaegi *et al.* 2011) leads to a reduction of the number of proliferating progenitors,
60 similarly to the observed effects in *Drosophila* (Li *et al.* 2006). Also common to these studies
61 is the observation that *mir-9* alteration (both loss and gain of function) results in a quite mild
62 phenotype (Shibata *et al.* 2011). This supports the idea that *mir-9* is not a biological switch
63 that allows the cell to adopt a certain fate, but a control factor to maintain a proper
64 development trajectory, possibly acting as a key component of a feedback control system.
65 *mir-9* dysfunction has been associated with a number of human pathologies, including various
66 kinds of cancer and neurodegenerative disorders (Coolen *et al.* 2013; He *et al.* 2017; Chen *et*
67 *al.* 2019b; Khafaei *et al.* 2019). In medulloblastomas (a paediatric brain cancer) tumour cells
68 appear to have a decreased expression of *mir-9*, while in a subclass of glioblastoma (an

69 aggressive adult brain cancer) tumour cells express *mir-9* at a higher level (Ferretti *et al.* 2009;
70 Kim *et al.* 2011). *mir-9* has been found to have a role also in cancers not directly related with
71 the nervous system, in which it may act as an oncogene or a tumour suppressor (Coolen *et al.*
72 2013). These dual roles and opposite effects, combined with observations of subtle and
73 cryptic phenotypes, has led to a model where miRNAs act to control or modulate the
74 dynamics of biological processes, and not as biological switches themselves.

75 Many studies have focused on *miR-9a* as a modulator of the specification and number
76 of *Drosophila* sensory organ precursor (SOP) cells, a key neuronal cell type that emerges
77 during embryonic stage 10 (Li *et al.* 2006; Cassidy *et al.* 2013). At embryonic stage 5, *miR-9a*
78 is expressed in the dorsal ectoderm and in the neuroectoderm: the germ layer in which the
79 future neuronal precursor cells will form (Fu *et al.* 2014; Gallicchio *et al.* 2021). It is possible
80 that *miR-9a* functions as a modulator of the genes required for proper ectoderm and
81 neuroectoderm specification. This early expression is reminiscent of *miR-1*, a miRNA involved
82 in mesoderm specification and muscle development, which is also expressed during early
83 embryogenesis exclusively in the presumptive mesoderm (Sokol and Ambros 2005).
84 Moreover, it has been suggested that both miRNAs might respond to the dorsal TF gradient
85 that activates and inhibits expression of genes involved in establishing germ layers (Biemar *et*
86 *al.* 2006). It is reported that *miR-9a* KO flies show defects on the wing margin (Li *et al.* 2006)
87 and an homozygous KO for *miR-1* causes lethality in second instar larvae, which die
88 immobilized and with abnormal musculature (Sokol and Ambros 2005). Nevertheless, no
89 differences in germ layer specification during embryogenesis have ever been observed in
90 either *miR-9a* or *miR-1* mutant (Fu *et al.* 2014). This is perhaps not surprising, as multiple
91 miRNAs often function redundantly, and it is rare that a specific biological process is strongly
92 affected when a single miRNA is knocked out (Liufu *et al.* 2017). However, when *miR-1* and

93 *miR-9a* are mutated together dramatic effects on embryonic development are observed (Fu
94 *et al.* 2014). The double knockout displayed an ectopic overexpression of *rhomboid* (*rho*), a
95 dorsal target gene expressed in the neuroectoderm, and a failure of gastrulation (Fu *et al.*
96 2014). *rho* possesses two *miR-9a* binding sites on its 3'UTR, indicating that *miR-9a* might
97 directly regulate *rho* mRNA degradation and/or translation.

98 We were therefore motivated to study *rho* expression in single cells and compare
99 quantification of mRNA number and protein levels between WT and *miR-9a* KO embryos in
100 the establishment of the embryonic domain of *rho* mRNA and protein. Using high resolution
101 confocal microscopy coupled with multiplex smFISH and IF we examined expression domains,
102 transcription dynamics and protein accumulation at the single cell level in whole mount
103 developing *D. melanogaster* embryos. In *miR-9a* KO mutants, we observed an increase in both
104 *rho* mRNA number per cell and Rho protein expression. We therefore conclude that *rho* is
105 directly targeted by *miR-9a*. Together, these results show that single-cell analysis and
106 quantification is a powerful approach to study miRNA function on target gene expression.

107 **Materials and Methods**

108 **Fly stocks, embryo collection, and fixing and larval dissection**

109 Flies were grown at 25 or 18°C. Embryos were collected after ~20 h and fixed in 1 V
110 heptane + 1 V 4% formaldehyde for 30 min shaking at 220 rpm. The embryos were then
111 washed and shaken vigorously for one minute in 100% methanol. Fixed embryos were stored
112 in methanol at -20°C. Larvae were dissected in 1× PBS, carcasses were fixed in 1 V 1× PBS +
113 1 V 10% formaldehyde for ~1 h, washed with methanol, and stored in methanol at -20°C.
114 Genotypes used for this study are: W [1118], (from Bloomington Drosophila Resource Centre)
115 and *mir-9a*^{E39} mutants (Li *et al.* 2006) generously gifted by the Fen-Biao Gao lab.

116

117 **Probe design, smFISH, and Immunofluorescence**

118 We applied an inexpensive version (Tsanov *et al.* 2016; Morales-Polanco *et al.* 2021)
119 of the conventional smFISH protocol in *Drosophila* (Trcek *et al.* 2017). Primary probes were
120 designed against the mature *rho* mRNA (*rhomboid_e*), the first *rho* intron (*rhomboid_i*) and a
121 genomic region flanking the *mir-9a* gene locus using the Biosearch Technologies Stellaris
122 probe Designer (version 4.2). All sequences were obtained from FlyBase. To the 5' end of each
123 probe was added the Flap sequence CCTCCTAAGTTTCGAGCTGGACTCAGTG. Multiple
124 secondary probes that are complementary to the Flap sequence were tagged with
125 fluorophores (CAL Fluor Orange 560, CAL Fluor Red 610, Quasar 670) to allow multiplexing.
126 Probes sequences are reported in Supplementary Data. For Immunofluorescence we used the
127 following antibodies: mouse anti-Dorsal (Developmental Studies Hybridoma Bank
128 #AB_528204) at 1:100, mouse anti-Spectrin (Developmental Studies Hybridoma Bank
129 #AB_520473) at 1:100, guinea-pig anti-Rho gently gifted from the Hayashi lab at 1:400 (Ogura

130 *et al.* 2018), goat anti-guinea pig IgG (H + L) Highly Cross-Adsorbed Secondary Antibody Alexa
131 Fluor 555 (Invitrogen #A21435) at 1:500, and goat anti-mouse IgG (H + L) Highly Cross-
132 Adsorbed Secondary Antibody Alexa Fluor 488 (Invitrogen #A32723) at 1:500. Further details
133 on reagents used are provided in the Reagents Table.

134

135 **Imaging and quantification**

136 Imaging was performed using a Leica SP8 Inverted Tandem Head confocal microscope
137 with LAS X v.3.5.1.18803 software (University of Manchester Bioimaging facility), using 40×,
138 and 100× magnifications. Deconvolution was performed using Huygens Pro v16.05 software.
139 Membrane segmentation was performed on Imaris (version 9.5.0), mRNA molecules and
140 Transcription sites were counted after membrane segmentation on Imaris 9.5.0 using the Cell
141 module. Protein fluorescence levels were measured using FIJI for Macintosh. From each
142 picture, five measurements of background mean intensity were taken. Each single
143 measurement was then adjusted using the formula: integrated density – (area × background
144 mean).

145

146 **Data availability statement**

147 Strains and plasmids are available upon request. The authors affirm that all data
148 necessary for confirming the conclusions of the article are present within the article, figures,
149 and tables.

150 Results

151 *rho* and *mir-9a* are co-expressed in the neurogenic ectoderm.

152 After the identification of Rhomboid (Rho) as an intramembrane serine protease in
153 *Drosophila*, Rho-like proteins have been identified in nearly every metazoan, suggesting a
154 conserved role for the family (Urban *et al.* 2001; Freeman 2014). Although the molecular and
155 cellular function of Rho-like proteins is well established, how their expression is post-
156 transcriptionally regulated has not been examined in detail. We therefore decided to
157 investigate if *miR-9a* and/or *miR-1* could directly regulate *rho* mRNA degradation and/or
158 translation. As *miR-1* is exclusively expressed in the mesoderm (Sokol and Ambros 2005; Fu
159 *et al.* 2014) and *miR-9a* in the dorsal and neurogenic ectoderm (Fu *et al.* 2014; Gallicchio *et*
160 *al.* 2021) largely overlapping *rho* (Ip *et al.* 1992a), we hypothesize that *miR-9a* might directly
161 target *rho*. We used TargetScan (Agarwal *et al.* 2018) and SeedVicious (Marco 2018) to
162 computationally verify the presence of two potential *miR-9a* binding sites in the *D.*
163 *melanogaster rho* 3'UTR. *rho* has 2 alternatively polyadenylated transcripts (based on the
164 most recent gene annotation in FlyBase), and the predicted *miR-9a* binding sites are both
165 located in the common 3'UTR region. In addition, we used SeedVicious to verify the presence
166 of *miR-9a* binding sites on Rho orthologs in beetle (*Tribolium castaneum*), worm
167 (*Caenorhabditis elegans*), zebrafish (*Danio rerio*), mouse (*Mus musculus*) and human, and the
168 non-model organisms mosquito (*Anopheles gambiae*), butterfly (*Heliconius melpomene*) and
169 mite (*Tetranychus urticae*) (Table 1).

170 We also employed nascent transcript smFISH to precisely establish the overlap in
171 expression domains of *rho* and the primary transcript of *miR-9a* (*pri-mir-9a*). To identify cells
172 that are actively transcribing *rho*, we designed probes against the first intron of *rho* to detect

173 active transcription sites (TS). As mature miRNAs are too short to be detected via smFISH, we
174 designed probes against ~1kb of sequence flanking the *mir-9a* hairpin to detect the larger
175 primary transcript. Using multiplex smiFISH, we were able to identify cells that are
176 transcribing both *rho* and *mir-9a* at the same time (Figure 1). As expected, *rho* expressing cells
177 are contained entirely within the *mir-9a* expression domain (Figure 1 A-B). Since it has been
178 widely observed that gene expression patterns are highly dynamic during stage 5 (Reeves *et*
179 *al.* 2012), we measured membrane introgression to distinguish between stage 5 sub-stages.
180 We find that both *rho* and *mir-9a* expression pattern become more defined at the ventral
181 edge of their expression domain as stage 5 proceeds (Figure 1 C-F). Interestingly, while *rho*
182 expressing cells are generally also expressing *mir-9a*, there are many cells at the ventral edge
183 that are expressing only *mir-9a* (Figure 1 C). As stage 5 progresses the two genes become co-
184 expressed in the same cells, which mark a clear boundary between neurogenic ectoderm and
185 presumptive mesoderm (Figure 1 D). It is therefore possible that the two genes respond
186 differently to the Dorsal gradient, specifically to the repressor *snail*, which has been shown to
187 repress both *mir-9a* and *rho* in the mesoderm (Hemavathy *et al.* 2004; Fu *et al.* 2014). Taken
188 together, the co-expression of *rho* and *mir-9a* and presence of conserved *miR-9a* target sites
189 suggest that *miR-9a* is a strong candidate to target *rho* mRNA during embryogenesis, and that
190 this role may be evolutionally conserved.

191

192 **Increased *rhomboid* mRNA copy number in *miR-9a*^{E39} mutants**

193 Combining high resolution confocal microscopy with smFISH, immunofluorescence
194 and segmentation allows us to count mRNA molecules in individual cells in *Drosophila* early
195 embryos. We quantified *rho* mRNA molecules per cell in WT and *mir-9a*^{E39} stage 5 embryos
196 (Figure 2 A-B). In order to tightly control the stage of embryonic development, we focused

197 only on stage 5 embryos that have a similar level of membrane introgression. As reported in
198 Fu et al. (2014) the *rho* expression pattern is not spatially or temporally different in *miR-9a*^{E39}
199 mutant embryos. We imaged and quantified six embryos per genotype and inspected many
200 more and we never saw an abnormal *rho* expression pattern. Nevertheless, when we
201 performed single cell segmentation and quantification, differences started to emerge (see
202 Figure 2 E and F). The data show that the 2 embryos have a spatially equivalent *rho* expression
203 pattern, but the mRNA number per cell is higher in *miR-9a*^{E39} mutant embryos. To corroborate
204 this observation, we performed two independent smFISH experiments using different
205 fluorophores (Figure 2, G-H), with 3 embryos per genotype. The number of cells that have low
206 or no detected *rho* expression varies from embryo to embryo, likely due to stochastic leaky
207 transcription or false positive detection and counting. After excluding cells with fewer than
208 10 counted *rho* mRNAs, we found that in both experiments, *miR-9a*^{E39} mutants possess a
209 higher number of *rho* mRNA per cell.

210 To further characterize the difference in *rho* mRNA number in single cells, we coupled
211 the intronic probes used in Figure 1 against *rho* introns with the probes used in Figure 2
212 against the mature *rho* transcripts, in order to simultaneously quantify *rho* TSs and mature
213 mRNA molecules (Figure 3). We used 100X images to separate and quantify *rho* TS number
214 per cell (maximum 2 per cell prior to replication and 4 per cell following). As the higher
215 magnification does not permit imaging of entire embryos, we focused on the central region
216 of the *rho* expressing stripe, again in stage 5 embryos with a similar membrane introgression
217 (Figure 3 A-B-C, A'-B'-C'). The comparison of *rho* mRNA distribution between WT and *miR-*
218 *9a*^{E39} embryos again shows that *miR-9a*^{E39} embryos have higher levels of *rho* mRNA number
219 per cell (Figure 3 E). The detection and quantification of *rho* TSs allowed us to distinguish
220 between cells that are differentially transcribing *rho*, and thus subgroup them in 3 classes:

221 cells with no TSs, cells with one TS and cells with two (or more) TSs. In Figure 3-F we reported
222 that cells with a higher number of TSs also show an increased number of *rho* mRNAs, and for
223 each group of cells *miR-9a*^{E39} have a generally higher number of transcripts with respect to
224 WT embryos. This becomes particularly evident for cells that are not transcribing *rho* at the
225 moment the embryo was fixed. It is important to note that very few cells have 3 or 4 TSs (<10
226 per image over ~700 segmented cells). These may represent cells following DNA replication,
227 or errors in the segmentation process. We are confident that these small numbers do not
228 significantly affect our analysis.

229

230 ***miR-9a* does not affect cell-to-cell variation in *rhomboid* mRNA number**

231 MicroRNAs are generally thought to have subtle effects on gene expression, mostly
232 acting as buffering factors against intrinsic and extrinsic noise. We therefore investigated
233 whether *miR-9a* might not only affect the number of *rho* transcripts per cell, but also cell-to-
234 cell variability in the number of mature mRNAs present. In order to quantify these effects, we
235 identified the immediate cell neighbours of each segmented cell, and then calculated how
236 variable the *rho* mRNA number per cell is amongst the identified neighbours. As variance
237 scales with mean, areas with high variance do not necessarily correspond to areas in which
238 the cell-to-cell variability is intrinsically higher. Other statistical parameters that have been
239 widely used in order to describe cell-to-cell variability are the coefficient of variation (CV) and
240 the Fano factor (FF) (Munsky *et al.* 2012; Foreman and Wollman 2020). FF is defined as
241 variance/mean while CV as standard deviation/mean. Thus, both measures are mean-
242 normalized. CV is a unitless parameter, and has been used to compare cell-to-cell variability
243 between mRNAs or protein levels resulting from the expression of different genes (Foreman
244 and Wollman 2020). On the other hand, FF has a dimension, and has been used to measure

245 how the observed data are dispersed from a Poisson distribution, which has FF equal to 1
246 (Thattai and Van Oudenaarden 2001; Hortsch and Kremling 2018). We therefore calculated
247 the FFs for the *rho* mRNA and TS counts reported in Figure 2 and Figure 3 (see Figure 4). We
248 observe that the FF is marginally higher in *miR-9a*^{E39} mutants. Closer inspection shows that
249 the FF is higher in *miR-9a*^{E39} mutants only in the group of cells with no transcription sites,
250 while groups of cells that have a single TS and 2 or more TSs have higher FF in the WT. We
251 speculate that the *miR-9a* buffering action on *rho* mRNA number per cell becomes more
252 evident and/or necessary in quiescent cells that are not actively transcribing *rho*.

253

254 **Rho is over-expressed in *miR-9a*^{E39} mutants during embryonic stage 5 and 6.**

255 As a change in mRNA levels does not necessarily linearly correlate with the change in
256 accumulation of the encoded protein (Koussounadis *et al.* 2015), we compared Rho protein
257 levels between WT and *miR-9a*^{E39} embryos. It has been reported that Rho protein expression
258 is detectable from the embryonic stages 10-11 in WT animals, despite *rho* mRNA being
259 transcribed much earlier during stage 5 (Llimargas and Casanova 1999). However, we find
260 that during stage 5, Rho protein was detectable in *miR-9a*^{E39} embryos. In Figure 5 we show
261 Rho staining in stage 5 and stage 6 WT and *miR-9a*^{E39} embryos with relative quantifications.
262 Anti-Dorsal antibody was used to provide a further control on the quality of the staining and
263 to orient the embryos. Fluorescence measurements were performed in FIJI by randomly
264 selecting 15 areas per embryo (5 in the anterior, 5 in the central and 5 in the posterior
265 regions). Quantifications shown in Figure 5 (panels C and F for stage 5 and 6 respectively)
266 clearly show that Rho levels are significantly higher (p-value < 0.0001 in both cases) in *miR-*
267 *9a*^{E39} mutants.

268 Discussion

269 *rho* has been one of the most studied Dorsal target genes. Its expression becomes
270 restricted to the neurogenic ectoderm in a precisely orchestrated manner: the low nuclear
271 levels of Dorsal in the dorsal ectoderm do not support *rho* activation, while *snail* represses its
272 transcription in the mesoderm (Ip *et al.* 1992b; Hong *et al.* 2008). *rho* has not been previously
273 studied as a direct target of miRNA regulation, but the combined effect of mutations in *miR-*
274 *1* and *miR-9a* on *rho* mRNA distribution motivated our investigation into *rho* regulation by
275 miRNAs (Fu *et al.* 2014). We found that the per cell copy number of *rho* mRNA is significantly
276 higher in *miR-9a*^{E39} mutant embryos (Figure 2 and Figure 3), suggesting *miR-9a* affects *rho*
277 mRNA stability or degradation. We could not find a clear effect of *miR-9a* on cell-to-cell
278 variability of the number of either *rho* mRNA transcription sites or mRNA molecules (Figure
279 4). Nevertheless, when we distinguish between cells that are and are not actively transcribing
280 *rho*, we find that the FF of cells with no transcription sites was significantly higher in *miR-9a*^{E39}
281 mutants. This leads us to suggest that, in WT animals, *rho* mRNA is “rapidly” degraded when
282 transcription stops, whereas this degradation is less efficient when *miR-9a* is removed, and
283 cell heterogeneity consequently increases. To our knowledge, this is the first study in which
284 mRNA copy number was compared in different genotypes using single cell quantitative
285 microscopy in order to uncover miRNA regulatory roles on target gene expression.

286 It has been shown that protein levels are usually more stable than mRNA levels (Perl
287 *et al.* 2017). The *miR-9a* regulatory effect on Rho protein accumulation might therefore be
288 more evident than the one we observed on the mRNA as it better reflects the integrated
289 activity over time. Rho is a transmembrane protease localized in the Golgi. While Fu *et al.*
290 reported *rho* mRNA patterns in double *miR-9a/miR-1* mutants (Fu *et al.* 2014), no information

291 on the protein pattern was previously available. We observed dramatic differences in timing
292 and level of Rho protein accumulation when comparing WT and *miR-9a*^{E39} embryos. In the
293 WT, Rho was only detectable from stage ~10, whereas in *miR-9a*^{E39} embryos it was clearly
294 present from stage 5, the same stage when we see *rho* transcription initiate. The early
295 accumulation of Rho protein appears to be inhibited by *miR-9a*. We suggest that translational
296 inhibition by *miR-9a* is released when a certain level of *rho* mRNA is reached, or in response
297 to an external signal later in development. We also note the possibility that early low levels
298 of Rho protein accumulation may be present but are undetectable with current technology.

299 Previous work on the *miR-9a/miR-1* double mutant shows that when *miR-1* is also
300 removed, greater developmental defects emerge leading to failure of gastrulation and ventral
301 midline enclosure (Fu *et al.* 2014). This phenotype suggests that these two miRNAs play an
302 important role in germ layer differentiation. Indeed, while *miR-9a* and *miR-1* involvement in
303 dorso-ventral (DV) axis patterning has not been definitively established, their expression
304 patterns indicate they are targets of DV specification (Biemar *et al.* 2006). Our current findings
305 provide convincing evidence for a role of *miR-9a* in the DV patterning process during early
306 *Drosophila* embryogenesis. We posit that *miR-9a* regulates *rho* mRNA accumulation and
307 translation, possibly affecting Epidermal Growth Factor Receptor (EGFR) signalling and
308 specification of the dorsal and neurogenic ectoderm (Golembo *et al.* 1996; Guichard *et al.*
309 1999). The role of *miR-1* is less clear as *miR-1* is not expressed in the same region as *rho*, and
310 therefore *miR-1* can affect *rho* expression only indirectly. *miR-1* is involved in muscle
311 development and is exclusively expressed in the mesoderm (Sokol and Ambros 2005). We
312 suggest that the combination of disrupted *miR-1* function in the mesoderm and *miR-9a*
313 function in the neurogenic ectoderm leads to disruption in establishment or maintenance of

314 an organized border between these two germ layers, as seen in the double mutants (Fu *et al.*
315 2014).

316 To conclude, we have shown in this work a new role of a the well conserved *miR-9a*
317 during early *Drosophila* embryogenesis. We have observed that *miR-9a* affects both *rho*
318 mRNA copy number per cell (possibly by degradation) and inhibits *rho* translation. Our
319 findings also show the importance of single-cell quantification when studying the effects of
320 miRNA regulation on target genes. As miRNAs act as weak modulators of gene expression,
321 single-cell quantitative approaches can reveal previously unknown effects on mRNA and
322 protein regulation by miRNAs. This work and the methods described can be easily applied to
323 many other miRNA-target gene networks to allow new insights into miRNA function during
324 development.

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334

335 **Competing interests**

336 The authors declare no competing interests.

337

338 **Author contributions**

339 LG, MR and SGJ conceived the project. Experiments were designed by LG and MR and
340 performed by LG. The manuscript was written by all the authors.

341

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485 **Figure legends**

486

487 **Figure 1. *rhomboid* and *miR-9a* are co-expressed in the neurogenic ectoderm.**

488 (A) Early and (B) middle stage 5 *D. melanogaster* embryos stained with probes against
489 *rhomboid* intron (yellow) and the primary transcript of *miR-9a* (magenta). (C-D) zooms of
490 highlighted areas in A and B respectively. In green is highlighted the presumptive ventral
491 midline, which separates mesoderm and ectoderm (pVM). (E-F) Brightfields of ventral borders
492 of the embryos in A and B showing membrane introgression (M.i.). Scalebars: 100 μm (A-B),
493 25 μm (C-D-E-F).

494

495 **Figure 2. *rhomboid* mRNA number per cell is higher in *miR-9a*^{E39} embryos**

496 (A) WT and (B) *miR-9a*^{E39} middle stage 5 embryos stained with a probe set against *Rhomboid*
497 transcripts. (C-D) Brightfields of a ventral region from embryos in A and B respectively
498 showing membrane introgression. (E-F) Computational reconstruction after segmentation of
499 the embryos in A and B. The colormap is based on mRNA number per cell with grey being low,
500 green intermediate and purple high. (G-H) Two independent quantifications of *rhomboid*
501 mRNA number in single cells in WT and *miR-9a*^{E39} mutant embryos. Each quantification was
502 performed using 3 embryos per genotype. Both p-values <0.0001. Scalebars: 100 μm (A-B),
503 25 μm (C-D).

504

505 **Figure 3. Detection and quantification of *rhomboid* transcription sites in single cells.**

506 Central region of (A) WT and (A') *miR-9a*^{E39} embryos respectively. Orientation is indicated by
507 the white arrow (Ant = Anterior embryonic region, Pos = Posterior embryonic region). (B-B')

508 Zoom from red area highlighted in A and A' respectively showing staining against *rhomboid*
509 intron (*rhomboid_i*, magenta), Spectrin to mark cellular membrane (yellow) and DAPI (grey).
510 (C-C') Zoom from red area highlighted in A and A' respectively showing staining against
511 *rhomboid* exon (*rhomboid_e*, green), Spectrin and DAPI. (D-D') Computational
512 reconstructions of the images in A and A' respectively. Each dot corresponds to a segmented
513 cell. The size of the dot corresponds to the number of *rhomboid* mRNAs detected with
514 *rhomboid_e*, while the colour corresponds to the number of detected transcription sites with
515 *rhomboid_i*. (E) Comparison between WT and *miR-9a^{E39}* *rhomboid* mRNA number per cell. p-
516 value = 0.0014. (F) Quantified cells are grouped depending on how many alleles are actively
517 transcribing the *rhomboid* locus: grey = 0 alleles active (p-value <0.0001), orange = 1 allele
518 active (p-value = 0.0021), red = 2 or more alleles active (p-value = 0.0259). Scalebars: 100 μ m
519 (A-A'), 25 μ m (B-C-B'-C').

520

521 **Figure 4. Fano factor quantification and comparison between WT and *miR-9a^{E39}* mutant**
522 **embryos.**

523 Computational reconstruction of Fano factor distribution calculated in neighbour clusters in
524 (A) WT and (B) *miR-9a^{E39}* stage 5 embryos. These two embryos are the same reported in figure
525 2 E-F respectively. (C-D) Comparison between Fano factor in WT and *miR-9a^{E39}* embryos in 2
526 independent experiments (n = 3 embryos each). P-value < 0.0001 in both graphs. (E-G)
527 Graphical reconstruction of Fano factor distribution calculated in neighbour cells clusters in a
528 WT and *miR-9a^{E39}* embryos, corresponding to Figure 3 A-A' respectively. (F) cells are sub-
529 grouped depending on their transcription sites number. p-values = 0.0147 (0 TS) and 0.0123
530 (1 TS), ns = non-significant.

531

532 **Figure 5. Rhomboid protein is over-expressed in *miR-9a*^{E39} embryos during stage 5 and 6.**
533 (A-B) Stage 5 WT and (A'-B') *miR-9a*^{E39} embryos respectively stained against Dorsal (red) and
534 Rhomboid (cyan). (C) Adjusted fluorescence levels from Rhomboid staining in stage 5 embryos
535 (n=3 per genotype). In each embryo 15 areas equally distributed along the Dorsal expression
536 border were quantified. Measurements are reported in Log10 scale. P-value < 0.0001. (D-E,
537 D'-E') Stage 6 WT and *miR-9a*^{E39} embryos respectively stained against Dorsal (red) and
538 Rhomboid (cyan). (F) Adjusted fluorescence levels from Rhomboid staining in stage 6 embryos
539 (n=3 per genotype). Quantified as in (C). P-value < 0.0001. Scalebars: 100 μ m in all panels.

540 Supplementary material

541 **Table S1.** Probes against *mir-9a*

Probe #	Probe Sequence (5'→ 3')
1	CCTCCTAAGTTTCGAGCTGGACTCAGTgttggtcaagtgactgtaac
2	CCTCCTAAGTTTCGAGCTGGACTCAGTccatcgattctcaatgttt
3	CCTCCTAAGTTTCGAGCTGGACTCAGTccattcttactctactctta
4	CCTCCTAAGTTTCGAGCTGGACTCAGTttatgcccacaaaacgaga
5	CCTCCTAAGTTTCGAGCTGGACTCAGTatatgcacgctacgacgatc
6	CCTCCTAAGTTTCGAGCTGGACTCAGTaagcgaggagctgcaatgat
7	CCTCCTAAGTTTCGAGCTGGACTCAGTgaaaactcgatgcatggga
8	CCTCCTAAGTTTCGAGCTGGACTCAGTgttcgtccagaaccagaaac
9	CCTCCTAAGTTTCGAGCTGGACTCAGTaggtaaagttggacgagcg
10	CCTCCTAAGTTTCGAGCTGGACTCAGTgcaacgagataatggtcgga
11	CCTCCTAAGTTTCGAGCTGGACTCAGTgtatgcattttgcatagact
12	CCTCCTAAGTTTCGAGCTGGACTCAGTgctgcgaatgcattgggaaa
13	CCTCCTAAGTTTCGAGCTGGACTCAGTtacaagtgtgttttgcgca
14	CCTCCTAAGTTTCGAGCTGGACTCAGTttggttttgctttttgtgt
15	CCTCCTAAGTTTCGAGCTGGACTCAGTtgtcgtcattgtttttgg
16	CCTCCTAAGTTTCGAGCTGGACTCAGTgcacacaatctaattccca
17	CCTCCTAAGTTTCGAGCTGGACTCAGTctcgtttcaccgactgtcg
18	CCTCCTAAGTTTCGAGCTGGACTCAGTcccatggacattcactcgat
19	CCTCCTAAGTTTCGAGCTGGACTCAGTatttatgttaagtctcgct
20	CCTCCTAAGTTTCGAGCTGGACTCAGTtttttttcgcttttggtgc
21	CCTCCTAAGTTTCGAGCTGGACTCAGTatgaacgcttatttcgctc
22	CCTCCTAAGTTTCGAGCTGGACTCAGTgctcctctttgttaaatt
23	CCTCCTAAGTTTCGAGCTGGACTCAGTctgcagatggttgaaggga
24	CCTCCTAAGTTTCGAGCTGGACTCAGTgtatatagagtcgattgtgt
25	CCTCCTAAGTTTCGAGCTGGACTCAGTaccaaagacaacatagcacc
26	CCTCCTAAGTTTCGAGCTGGACTCAGTttatcactcatacagctaga
27	CCTCCTAAGTTTCGAGCTGGACTCAGTtcggtaagctagctttatga
28	CCTCCTAAGTTTCGAGCTGGACTCAGTctgggcagacgctaata
29	CCTCCTAAGTTTCGAGCTGGACTCAGTaagtatacgcaatgtgggcc
30	CCTCCTAAGTTTCGAGCTGGACTCAGTgcttattttgatgtgttcc
31	CCTCCTAAGTTTCGAGCTGGACTCAGTatgcatggtgtacatatggg
32	CCTCCTAAGTTTCGAGCTGGACTCAGTttggccgtaaagccaaactg
33	CCTCCTAAGTTTCGAGCTGGACTCAGTggttttttgccttgccaaag
34	CCTCCTAAGTTTCGAGCTGGACTCAGTaacttactcgtttgtacgcg

35	CCTCCTAAGTTTCGAGCTGGACTCAGTccatgcaaaggtcgtatcta
36	CCTCCTAAGTTTCGAGCTGGACTCAGTgttcgcagggggtaaaacaa
37	CCTCCTAAGTTTCGAGCTGGACTCAGTtgtctttccacctcttttg
38	CCTCCTAAGTTTCGAGCTGGACTCAGTcaacgtcattgactgctgtt
39	CCTCCTAAGTTTCGAGCTGGACTCAGTaatcagtgttcatcaggtgc
40	CCTCCTAAGTTTCGAGCTGGACTCAGTgcctgcaaatgatctttcat
41	CCTCCTAAGTTTCGAGCTGGACTCAGTagcttgccgttattatcttg
42	CCTCCTAAGTTTCGAGCTGGACTCAGTgttggtgctatccgaaactt
43	CCTCCTAAGTTTCGAGCTGGACTCAGTtttcctctaaagttcctagc
44	CCTCCTAAGTTTCGAGCTGGACTCAGTgttcgacggcttaagagtg
45	CCTCCTAAGTTTCGAGCTGGACTCAGTgtttatggtgtttacaagtt
46	CCTCCTAAGTTTCGAGCTGGACTCAGTatggtatttgcttactttcc
47	CCTCCTAAGTTTCGAGCTGGACTCAGTataacttattgcacgctatt
48	CCTCCTAAGTTTCGAGCTGGACTCAGTgctttctattaagctgatca

542

543 **Table S2.** Probes against *rhomboid* exons (*rhomboid_e*)

Probe #	Probe Sequence (5'-> 3')
1	CCTCCTAAGTTTCGAGCTGGACTCAGTtgcacgcaactgactttcg
2	CCTCCTAAGTTTCGAGCTGGACTCAGTccgactttctcagttgatg
3	CCTCCTAAGTTTCGAGCTGGACTCAGTccacacacacgacaatttga
4	CCTCCTAAGTTTCGAGCTGGACTCAGTgatataatattctctgcttgc
5	CCTCCTAAGTTTCGAGCTGGACTCAGTatccaggagcttgattcag
6	CCTCCTAAGTTTCGAGCTGGACTCAGTaacttagtttgctgctcgt
7	CCTCCTAAGTTTCGAGCTGGACTCAGTtttttcggctcgacattg
8	CCTCCTAAGTTTCGAGCTGGACTCAGTtattcgacgttttcactc
9	CCTCCTAAGTTTCGAGCTGGACTCAGTccaatgtctttagtagct
10	CCTCCTAAGTTTCGAGCTGGACTCAGTaaatgcgtgggtttcttgta
11	CCTCCTAAGTTTCGAGCTGGACTCAGTgcctgtcgcaatgtttataa
12	CCTCCTAAGTTTCGAGCTGGACTCAGTgcgccgttgaagaaattctt
13	CCTCCTAAGTTTCGAGCTGGACTCAGTtctgcgttaagttctccatg
14	CCTCCTAAGTTTCGAGCTGGACTCAGTaaatccaccttggtttcgtt
15	CCTCCTAAGTTTCGAGCTGGACTCAGTatgtcaatgatggttctcctt
16	CCTCCTAAGTTTCGAGCTGGACTCAGTagttggaggaactggagcac
17	CCTCCTAAGTTTCGAGCTGGACTCAGTcaatcggtgtcgtacgacga
18	CCTCCTAAGTTTCGAGCTGGACTCAGTtgcggttgcagtagatgtg
19	CCTCCTAAGTTTCGAGCTGGACTCAGTtacttcagcaggccgatatc
20	CCTCCTAAGTTTCGAGCTGGACTCAGTatcactaggatgaaccaggg
21	CCTCCTAAGTTTCGAGCTGGACTCAGTggcgaagatggcaatctcaa

22	CCTCCTAAGTTTCGAGCTGGACTCAGTgaaattctgggcgggcattg
23	CCTCCTAAGTTTCGAGCTGGACTCAGTacggaatcggaacgggtagc
24	CCTCCTAAGTTTCGAGCTGGACTCAGTcgatagaccagcaccgaatc
25	CCTCCTAAGTTTCGAGCTGGACTCAGTctaaagaagcgccacacctg
26	CCTCCTAAGTTTCGAGCTGGACTCAGTagtggcgtgcaggaacatg
27	CCTCCTAAGTTTCGAGCTGGACTCAGTgatgacgatattgaagcca
28	CCTCCTAAGTTTCGAGCTGGACTCAGTctccaggggaatgccaaaaa
29	CCTCCTAAGTTTCGAGCTGGACTCAGTcagggatccggcaaaaacgc
30	CCTCCTAAGTTTCGAGCTGGACTCAGTaagacctccgagtcgacgac
31	CCTCCTAAGTTTCGAGCTGGACTCAGTtgaatgttgccagatgtg
32	CCTCCTAAGTTTCGAGCTGGACTCAGTtcttcatgtgcatagttc
33	CCTCCTAAGTTTCGAGCTGGACTCAGTtagatgacaacggatccgagt
34	CCTCCTAAGTTTCGAGCTGGACTCAGTtagagagcatagcccagatc
35	CCTCCTAAGTTTCGAGCTGGACTCAGTcgcttccatcgaagtattgg
36	CCTCCTAAGTTTCGAGCTGGACTCAGTaggtgggcaatgtacgacac
37	CCTCCTAAGTTTCGAGCTGGACTCAGTcagaaagccgatcgttagtc
38	CCTCCTAAGTTTCGAGCTGGACTCAGTggtgaccgaagtctttagc
39	CCTCCTAAGTTTCGAGCTGGACTCAGTcagatgagctgctctactc
40	CCTCCTAAGTTTCGAGCTGGACTCAGTcgaagacgggtaaggcacag
41	CCTCCTAAGTTTCGAGCTGGACTCAGTccgtggtgatcaggttgaaa
42	CCTCCTAAGTTTCGAGCTGGACTCAGTgcaacagatgctgggtaatc
43	CCTCCTAAGTTTCGAGCTGGACTCAGTcaaacttaggacactcccag
44	CCTCCTAAGTTTCGAGCTGGACTCAGTtagcatgctgacgactccgaa
45	CCTCCTAAGTTTCGAGCTGGACTCAGTaagctcaagcagattccgaa
46	CCTCCTAAGTTTCGAGCTGGACTCAGTaactctctgtctctgatct
47	CCTCCTAAGTTTCGAGCTGGACTCAGTgagtgaacttttctttcca
48	CCTCCTAAGTTTCGAGCTGGACTCAGTgtactatgtttcgaactga

544

545 **Table S3.** Probes against *rhomboid* intron (*rhomboid_i*)

Probe#	Probe sequence (5'-> 3')
1	CCTCCTAAGTTTCGAGCTGGACTCAGTttgtggctttagcttgtat
2	CCTCCTAAGTTTCGAGCTGGACTCAGTgcaatttgacatttcttgc
3	CCTCCTAAGTTTCGAGCTGGACTCAGTttttgccgctgtgacaattt
4	CCTCCTAAGTTTCGAGCTGGACTCAGTcgctctaaagttgaaatgct
5	CCTCCTAAGTTTCGAGCTGGACTCAGTgggcacacaggttgaacaaa
6	CCTCCTAAGTTTCGAGCTGGACTCAGTgagcgagagagatagagcga
7	CCTCCTAAGTTTCGAGCTGGACTCAGTtttttatgcttctgctgc
8	CCTCCTAAGTTTCGAGCTGGACTCAGTaacatagtttcacatggccc

9	CCTCCTAAGTTTCGAGCTGGACTCAGTtaattcattcggccttcttt
10	CCTCCTAAGTTTCGAGCTGGACTCAGTgcgggctttaagacataatg
11	CCTCCTAAGTTTCGAGCTGGACTCAGTgcatgaagaagagatgtcga
12	CCTCCTAAGTTTCGAGCTGGACTCAGTagactgcaccgaatgtccat
13	CCTCCTAAGTTTCGAGCTGGACTCAGTaggcacgaaatcgcagtcgg
14	CCTCCTAAGTTTCGAGCTGGACTCAGTcggttgcttagcaatttcaa
15	CCTCCTAAGTTTCGAGCTGGACTCAGTgctcttttcatgttcttcat
16	CCTCCTAAGTTTCGAGCTGGACTCAGTgaaaaagtgagtgggtgccg
17	CCTCCTAAGTTTCGAGCTGGACTCAGTcgttgttgcttttgtgtctg
18	CCTCCTAAGTTTCGAGCTGGACTCAGTtcaagtcgattgcacacac
19	CCTCCTAAGTTTCGAGCTGGACTCAGTcactcacactcattgtgttc
20	CCTCCTAAGTTTCGAGCTGGACTCAGTtaacaaattcattgccttgc
21	CCTCCTAAGTTTCGAGCTGGACTCAGTctgttcgtcgcaacaaggaa
22	CCTCCTAAGTTTCGAGCTGGACTCAGTgtcacagcacaatcttctt
23	CCTCCTAAGTTTCGAGCTGGACTCAGTtgtaaatcgattcgattca
24	CCTCCTAAGTTTCGAGCTGGACTCAGTccagagacatttctcacia
25	CCTCCTAAGTTTCGAGCTGGACTCAGTgccaggcattattgtaattc
26	CCTCCTAAGTTTCGAGCTGGACTCAGTatggccaactaatcagctaa
27	CCTCCTAAGTTTCGAGCTGGACTCAGTcagccctgaaatcatcttcg
28	CCTCCTAAGTTTCGAGCTGGACTCAGTtagcttgtgtagcatctacg
29	CCTCCTAAGTTTCGAGCTGGACTCAGTcccgaaattagctggacaaa
30	CCTCCTAAGTTTCGAGCTGGACTCAGTatttgatgggccaagtttgc
31	CCTCCTAAGTTTCGAGCTGGACTCAGTgtcagcttgtgtgagctaac
32	CCTCCTAAGTTTCGAGCTGGACTCAGTttttccccgaagggaact
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34	CCTCCTAAGTTTCGAGCTGGACTCAGTaagcgaaggaaaagcctgct
35	CCTCCTAAGTTTCGAGCTGGACTCAGTtgtggggaaatgcagcagaa
36	CCTCCTAAGTTTCGAGCTGGACTCAGTcgagcacaacaaagagca
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38	CCTCCTAAGTTTCGAGCTGGACTCAGTgcgccgttgaagaaattctg

546

Table 1





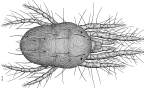




	Organism	Transcript	microRNA	Position on 3'UTR	Site type
	<i>Drosophila melanogaster</i>	rho-RA/RB	dme-miR-9a/b/c-5p	340	8mer
				1075	7_A1
	<i>Tribolium castaneum</i>	TC034044	tca-miR-9b-5p	416	7_m8
			tca-miR-9a/e/c-5p	188	8mer
				417	8mer
	<i>Anopheles gambiae</i>	AGAP005058 RA/RB	aga-miR-9a/b/c	405	7_A1
				904	8mer
				3197	8mer
	<i>Heliconius melpomene</i>	HMEL008701-RA	hme-miR-9b	710	8mer
			hme-miR-9a	1561	8mer
	<i>Tetranychus urticae</i>	tetur14g02680.1	tur-miR-9-5p	138	7_A1
	<i>Caenorhabditis elegans</i>	rho-1	cel-miR-79-3p	54	7_m8
	<i>Danio rerio</i>	Rhbdl3-203	dre-miR-9-5p	464	7_m8
	<i>Mus musculus</i>	Rhbdl3-201	mmu-miR-9-5p	1046	7_m8
	<i>Homo sapiens</i>	RHBDL3-201/203	hsa-miR-9-3p	2988	7_A1

Figure 1

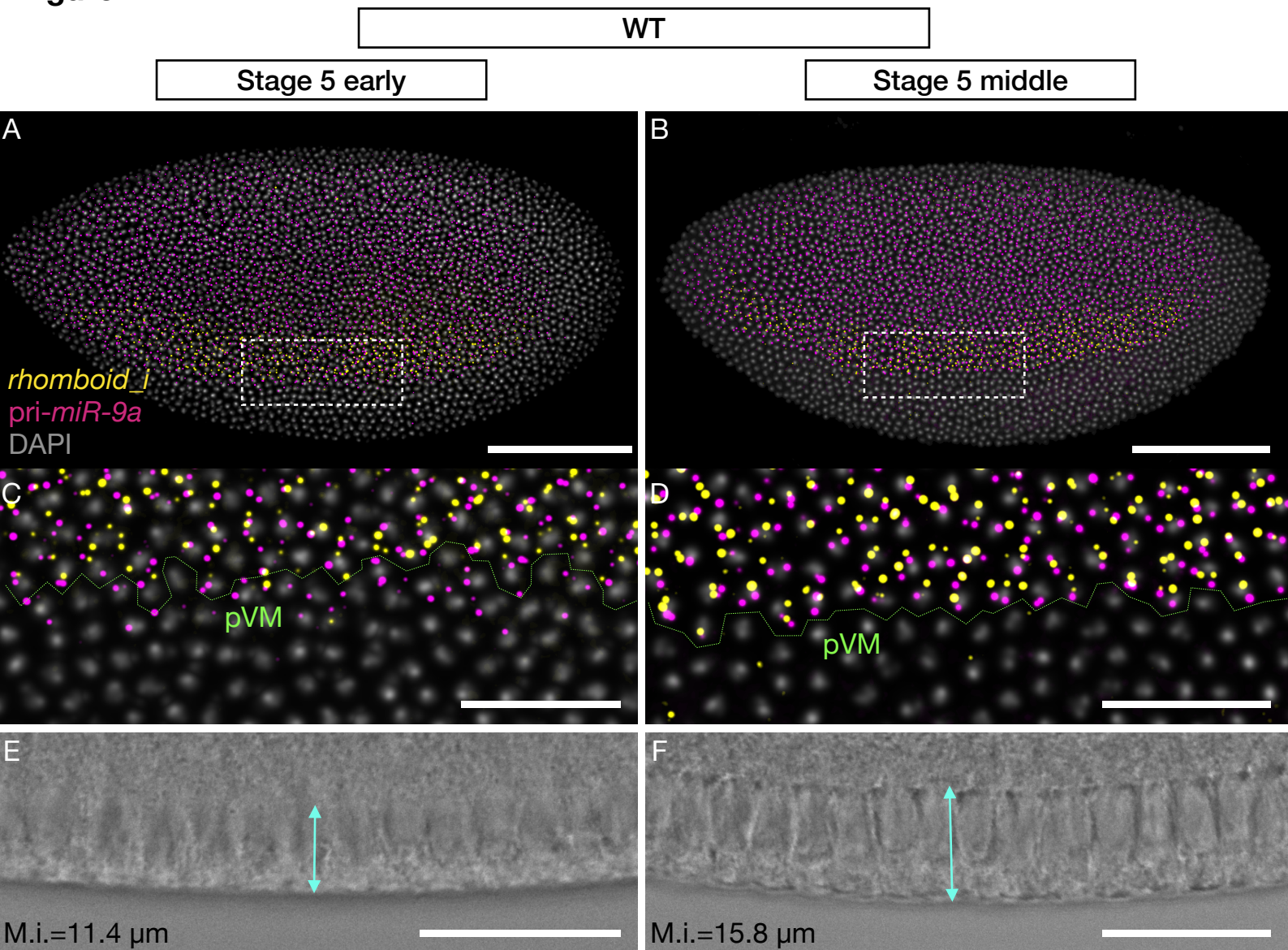


Figure 2

WT

miR-9a^{E39}

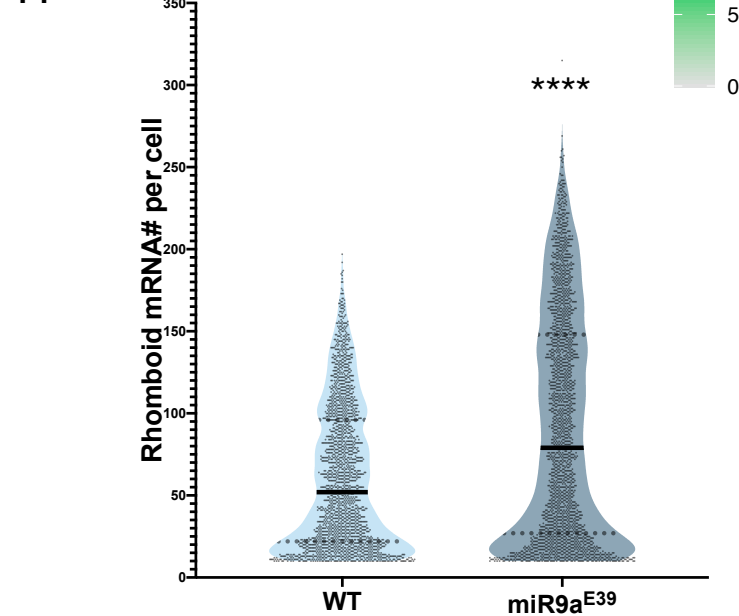
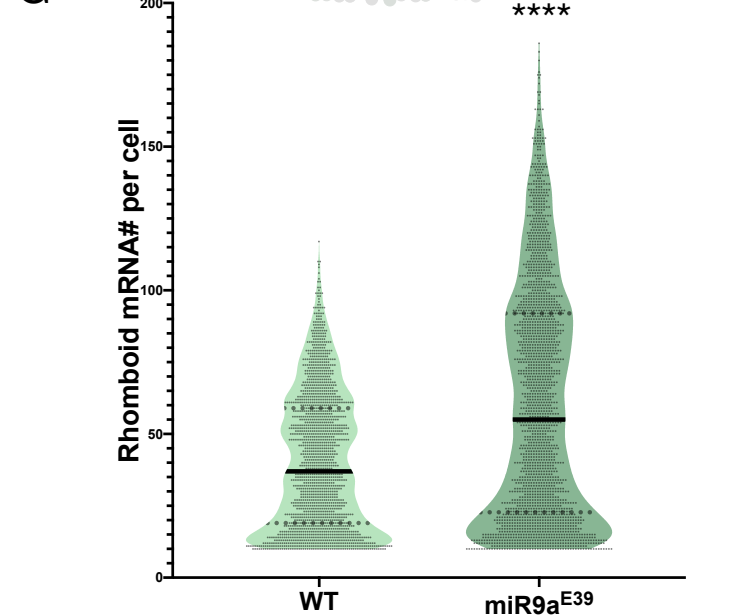
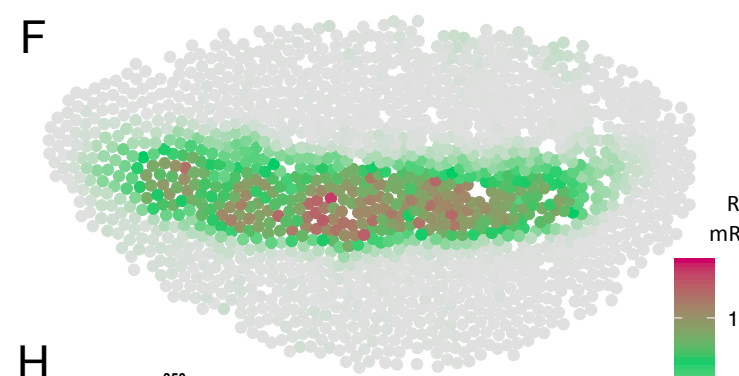
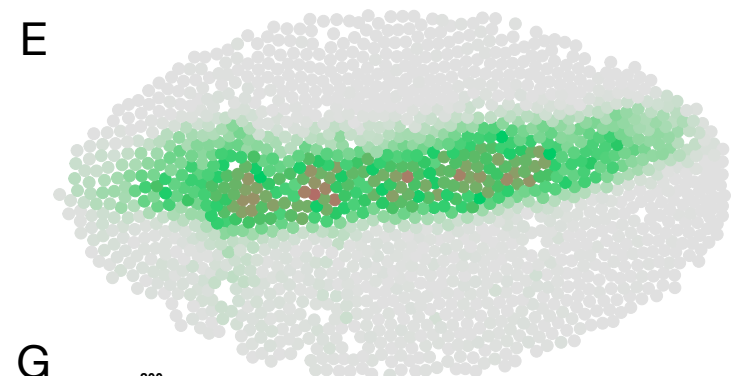
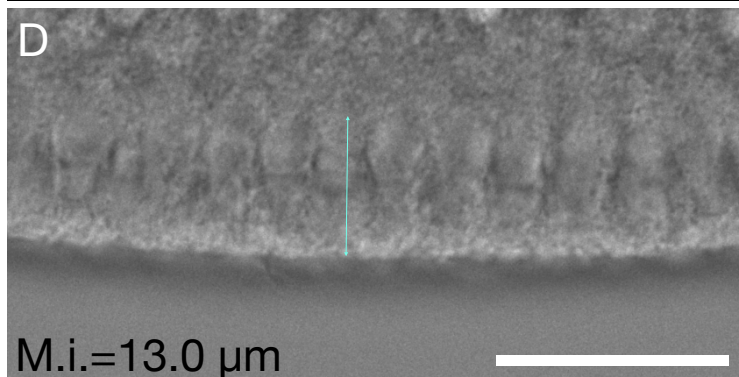
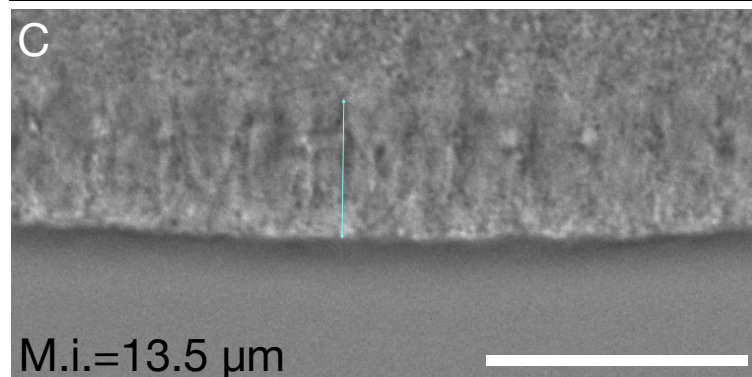
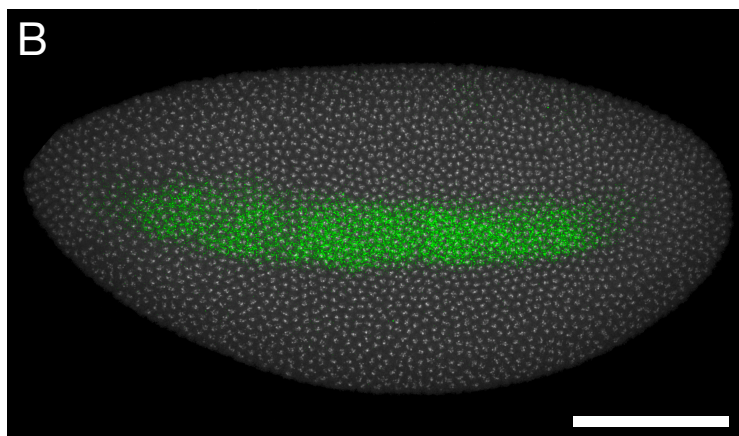
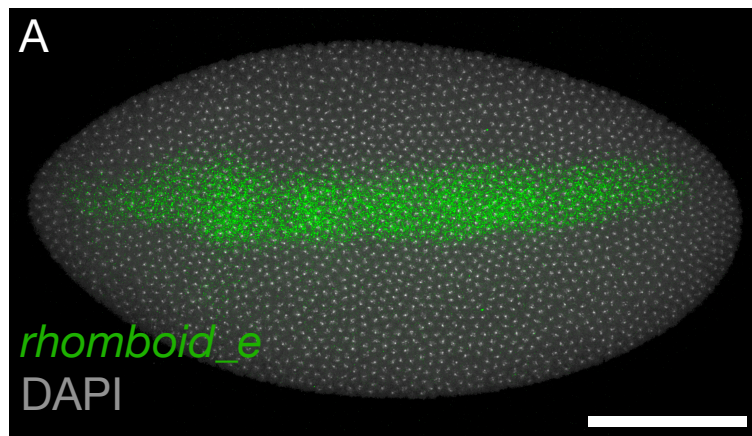


Figure 3

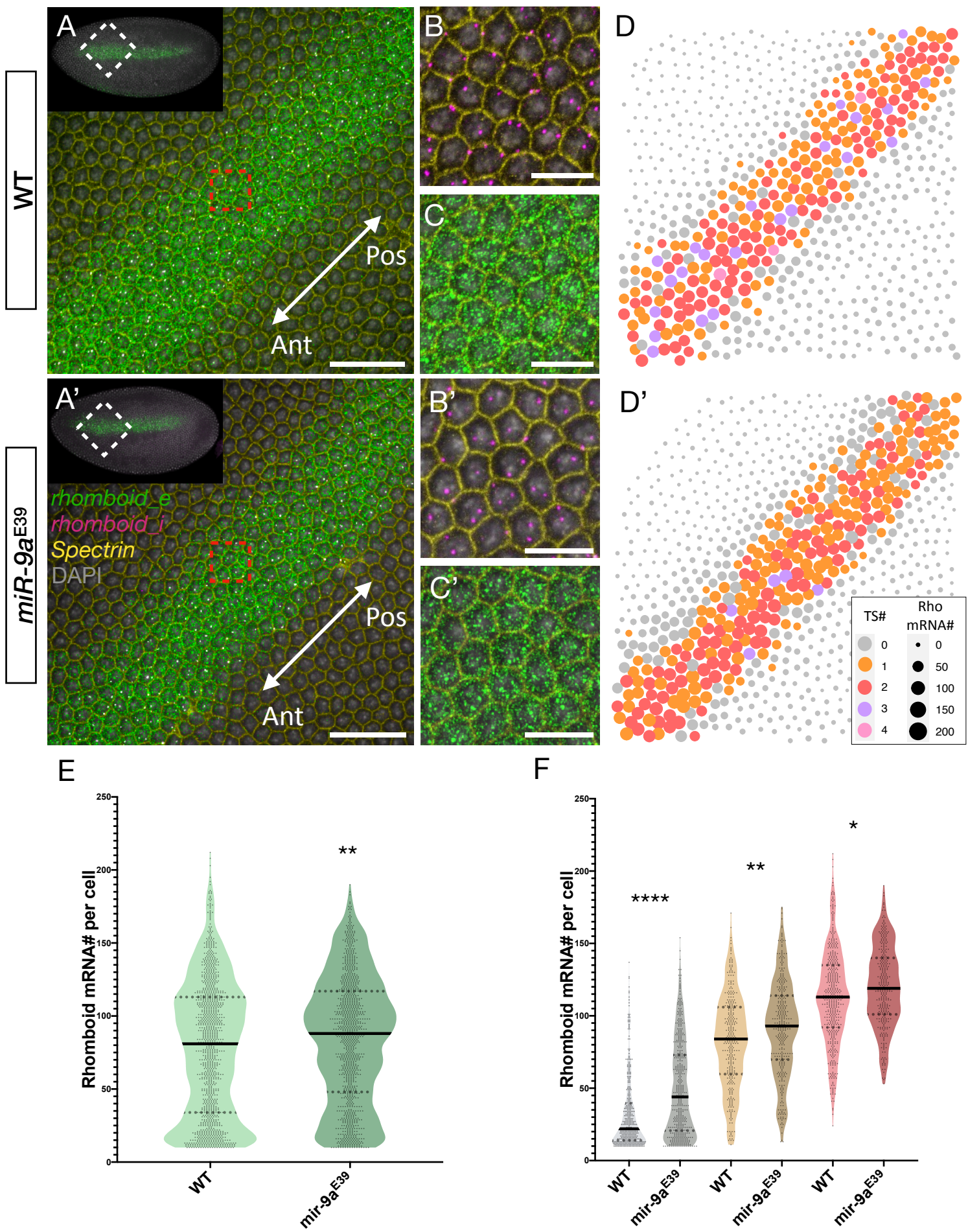


Figure 4

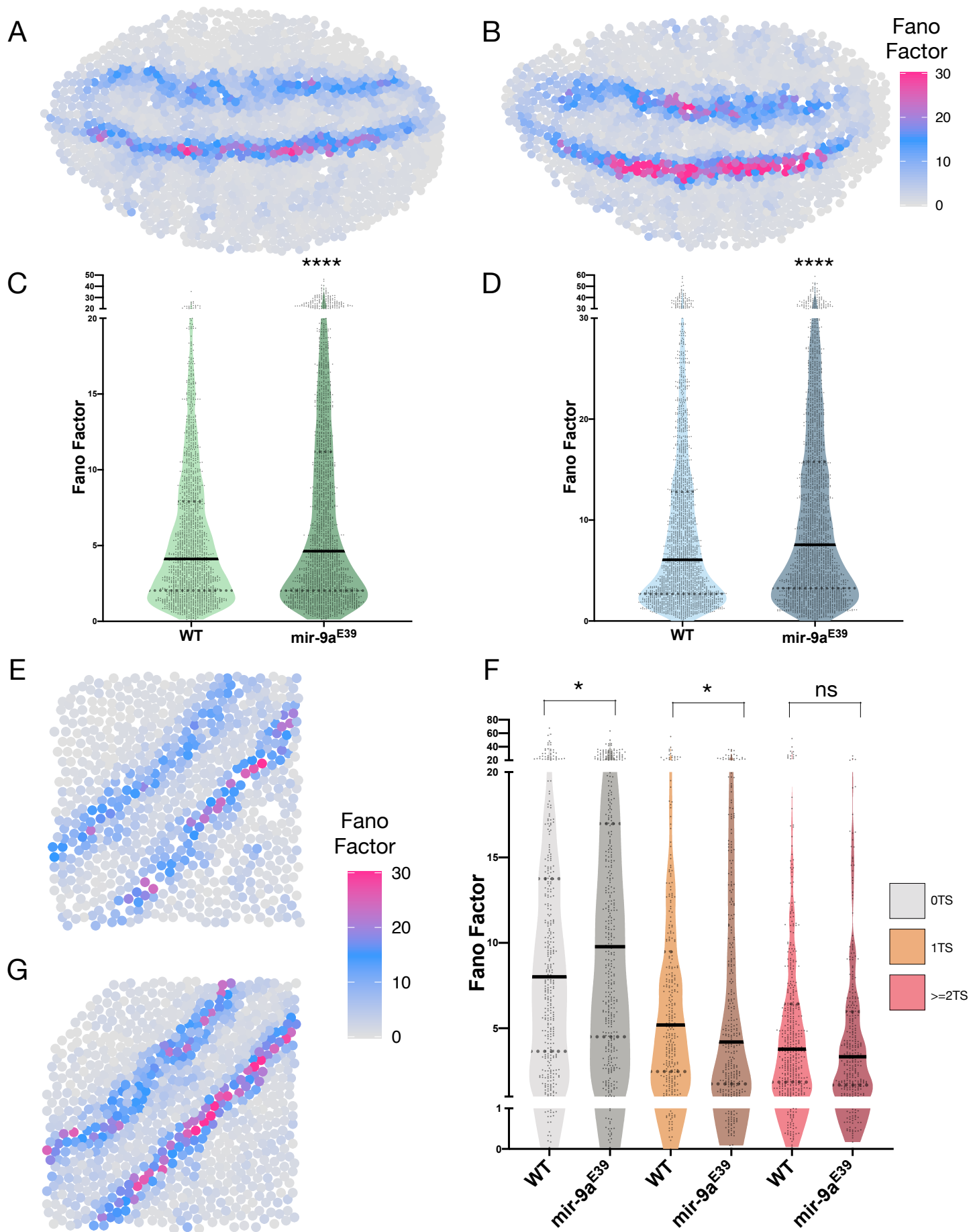
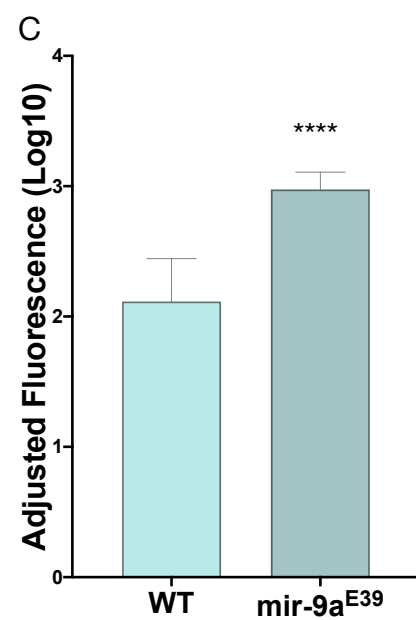
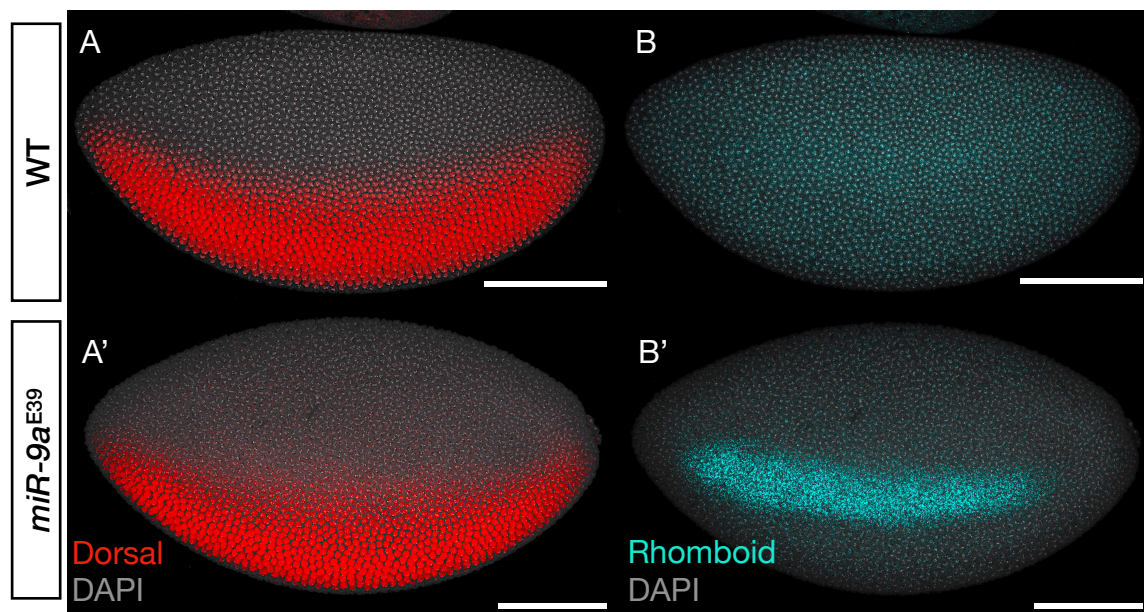


Figure 5

Stage 5



Stage 6

