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

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Abstract

MicroRNAs can have subtle and combinatorial effects on the levels of the targets and pathways they act on. Studying the consequences of a single microRNA knockout often proves difficult as many such knockouts exhibit phenotypes only under stress conditions. This has often led to the hypothesis that microRNAs buffer the effects of intrinsic and environmental stochasticity on gene expression. Observing and understanding this buffering effect entails quantitative analysis of microRNA and target expression in single cells. To this end, we have employed single molecule fluorescence in situ hybridization, immunofluorescence, and high-resolution confocal microscopy to investigate the effects of miR-9a loss on the expression of the serine-protease Rhomboid in Drosophila melanogaster early embryos. Our single-cell quantitative approach shows that spatially, the rhomboid mRNA pattern is identical in WT and miR-9a knockout embryos. However, we find that the number of mRNA molecules per cell is higher when miR-9a is absent, and the level and temporal accumulation of rhomboid protein shows a more dramatic increase in the miR-9a knockout. Specifically, we see accumulation of rhomboid protein in miR-9a mutants by stage 5, much earlier than in WT. The data therefore show that miR-9a functions in the regulation of rhomboid mRNA and protein levels. While further work is required to establish whether rhomboid is a direct target of miR-9 in Drosophila, our results further establish the miR-9 family microRNAs as conserved regulators of timing in neurogenic processes. This work shows the power of single-cell quantification as an experimental tool to study phenotypic consequences of microRNA mis-regulation.
**Introduction**

*Drosophila melanogaster* embryonic, larval, and adult development has provided an extremely important model for the study of microRNA (miRNA) biogenesis and function (Matranga *et al.* 2005; Rand *et al.* 2005; Okamura *et al.* 2007). MicroRNAs are short ~22 nucleotide, single-stranded, endogenous RNAs found in animals and plants (Bartel 2004; Kozomara *et al.* 2019). MicroRNAs regulate gene expression post-transcriptionally by recruiting the RNA-induced silencing complex (RISC) and then binding to specific sequences on target mRNA molecules, usually in their 3’UTR. The binding of the miRISC triggers repression of translation, deadenylation, and/or degradation of the target mRNA (Valencia-Sanchez *et al.* 2006). It is estimated that the majority of animal mRNAs are targeted by miRNAs (Friedman *et al.* 2009; Agarwal *et al.* 2015). An intriguing dichotomy regarding the phenotypic consequences of miRNA mis-regulation has arisen, with GOF (gain of function) and LOF (loss of function) studies in different organisms suggesting different functional modes: LOF studies find that miRNAs are minor modulators, whereas GOF studies reveal them to be key regulators of gene expression (Reinhart *et al.* 2000; Miska *et al.* 2007; Alvarez-Saavedra and Horvitz 2010; Chen *et al.* 2014).

In many cases, individual effects of miRNAs on the expression of a target are relatively small (Miska *et al.* 2007; Alvarez-Saavedra and Horvitz 2010; Chen *et al.* 2019a). In addition, each miRNA may target hundreds of different transcripts, and many different miRNAs have been found to act on the same targets (Peter 2010). It is therefore expected that a high degree of quantitative precision is required to determine specific effects of miRNAs on gene expression. Indeed, a complete understanding of miRNA function will only come from a precise quantitative analysis of miRNA activity at the single cell level. Single cell
studies of miRNA effects on gene regulation may provide insight into cellular phenotypes that are not apparent at a tissue or organism level (Miska et al. 2007; Alvarez-Saavedra and Horvitz 2010). It has also been observed that the phenotypic effects of miRNA mutation or mis-regulation are sometimes only revealed under specific, often stressful, conditions (e.g. dietary restriction, temperature stress) (Li et al. 2009; Kennell et al. 2012). For example, flies lacking miR-14 are more sensible to salt stress compared to WT, while flies lacking miR-7 present abnormal expression of the proteins Yan and Ato only under temperature fluctuations (Xu et al. 2003; Li et al. 2009). Such stress-dependent miRNA phenotypes have also been observed in other organisms such as mouse and zebrafish (Van Rooij et al. 2007; Flynt et al. 2009). Thus, the phenotypic consequences of miRNA mis-regulation may be subtle and cryptic until particular environmental conditions expose the cellular level dysfunction.

The mir-9 miRNA family is highly conserved in bilaterians and is a good example of a miRNA that can exhibit both subtle and strong phenotypes (Coolen et al. 2013). Experiments in a variety of vertebrate models show conservation of mir-9 expression and function in neurogenesis and neuronal progenitor proliferation. Over-expression of mir-9 in zebrafish embryos (Leucht et al. 2008), mouse embryonic cortex (Zhao et al. 2009) and chicken spinal cord (Otaegi et al. 2011) leads to a reduction of the number of proliferating progenitors, similarly to the observed effects in Drosophila (Li et al. 2006). However, when miR-9a was knocked out in Drosophila, the phenotype was quite mild, leading to a modest increase of sensory organ progenitors (SOPs) as well as some subtle wing-defects that were dependent on the genetic background (Li et al. 2006; Bejarano et al. 2010; Coolen et al. 2013). The overall complexity of miRNA-target genes networks and the observation that miRNAs do not generally have large effects on the levels of individual target genes lead to a model
suggesting that many miRNA function not as biological switches but rather as modulators or buffers of gene expression by fine-tuning the response to intrinsic and extrinsic noise (Liu et al. 2017).

*miR-9* dysfunction has been associated with a number of human pathologies, including various kinds of cancer and neurodegenerative disorders (Coolen et al. 2013; He et al. 2017; Chen et al. 2019b; Khafaei et al. 2019). In medulloblastomas (a paediatric brain cancer) tumour cells appear to have decreased expression of *miR-9*, while in a subclass of glioblastoma (an aggressive adult brain cancer) tumour cells express *miR-9* at a higher level (Ferretti et al. 2009; Kim et al. 2011). *miR-9* has been found to have a role also in cancers not directly related with the nervous system, in which it may act as an oncogene or a tumour suppressor (Coolen et al. 2013).

The conserved role *miR-9* plays in regulation of Enhancer of split-HLH/HES family gene function in vertebrates and invertebrates strongly suggests an important ancestral function of *miR-9* (Bonev et al. 2011, 2012; Coolen et al. 2012; Soto et al. 2020). Work across a range of model organisms, including a number of studies in *Drosophila*, have focused on *miR-9α* as a modulator of the specification of *Drosophila* SOPs, a key neuronal cell type that emerges around embryonic stage 10 (Li et al. 2006; Cassidy et al. 2013). At embryonic stage 5, *miR-9α* is expressed in the dorsal ectoderm and neurogenic ectoderm: the germ layer where the future neuronal precursor cells will form (Fu et al. 2014; Gallicchio et al. 2021). This early expression throughout the neuroectoderm is reminiscent of early *miR-1* expression throughout the mesoderm, where *miR-1* functions in muscle development (Sokol and Ambros 2005). It has been suggested that both miRNAs likely respond to the Dorsal transcription factor (TF) gradient that activates and inhibits expression of genes involved in establishing the germ layers (Biemar et al. 2006). It is reported that *miR-9α*...
knock out (KO) flies show defects on the wing margin (Li et al. 2006) and an homozygous KO for miR-1 causes lethality in second instar larvae, which die immobilized and with abnormal musculature (Sokol and Ambros 2005). When miR-1 and miR-9a are mutated together, dramatic effects on embryonic development are observed (Fu et al. 2014). The double KO exhibits a disrupted pattern of rhomboid (rho) expression and a failure of gastrulation (Fu et al. 2014). Drosophila rho encodes a transmembrane serine protease (Rho), localized in the Golgi apparatus, that processes the epidermal growth factor (EGF) ligand Spitz, and is therefore necessary for proper EGF signalling by (Bang and Kintner 2000; Urban et al. 2001). The pattern of rho expression is determined by dl activation and snail repression inputs (Bier et al. 1990). rho also has two predicted miR-9a binding sites in its 3’UTR. Together these observations suggest a role of miR-9a as a direct or indirect regulator of rho mRNA expression and/or translation.

We were therefore motivated to study rho expression and cellular phenotype in miR-9a mutant embryos at the single-cell level. In particular, single-cell quantitative approaches may reveal phenotypic consequences of relatively mild effects of miRNA mutations on gene expression levels, which might be lost when a population of cells is considered (Linsen et al. 2008). Using high resolution confocal microscopy coupled with multiplex smiFISH and IF we examined expression domains, transcription dynamics and protein accumulation at the single cell level in whole mount developing D. melanogaster embryos. In miR-9a KO mutants, we observed an increase in both rho mRNA number per cell and Rho protein expression, concluding that miR-9a deletion affects rhomboid mRNA expression and protein accumulation. Together, these results show that single-cell analysis and quantification is a powerful approach to study miRNA function on target gene expression.
Materials and Methods

Fly stocks, embryo collection, and fixing and larval dissection

Flies were grown at 25 or 18°C. Embryos were collected after ∼20 h and fixed in 1 V heptane + 1 V 4% formaldehyde for 30 min 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 1× PBS, carcasses were fixed in 1 V 1× PBS + 1 V 10% formaldehyde for ∼1 h, washed with methanol, and stored in methanol at −20°C. Genotypes used for this study are: W [1118], (from Bloomington Drosophila Resource Centre) and miR-9a^E39 mutants (Li et al. 2006) generously gifted by the Fen-Biao Gao lab.

Probe design, smFISH, and Immunofluorescence

We applied an inexpensive version (Tsanov et al. 2016; Morales-Polanco et al. 2021) of the conventional smFISH protocol in Drosophila (Trcek et al. 2017). Primary probes were designed against the mature rho mRNA (rhomboid_e), the first rho intron (rhomboid_i) and a genomic region flanking the mir-9a gene locus using the Biosearch Technologies Stellaris probe Designer (version 4.2). All sequences were obtained from FlyBase. 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. Probes sequences are reported in the Tables S1-S3. For Immunofluorescence we used the following antibodies: mouse anti-Dorsal (Developmental Studies Hybridoma Bank #AB_528204) at 1:100, mouse anti-Spectrin (Developmental Studies Hybridoma Bank
#AB_520473) at 1:100, guinea-pig anti-Rho gently gifted from the Hayashi lab at 1:400 (Ogura et al. 2018), goat anti-guinea pig IgG (H + L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor 555 (Invitrogen #A21435) at 1:500, and goat anti-mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor 488 (Invitrogen #A32723) at 1:500.

Further details on reagents used are provided in the Reagents Table.

**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 40×, and 100× magnifications. Deconvolution was performed using Huygens Pro v16.05 software. Membrane segmentation was performed on Imaris (version 9.5.0), mRNA molecules and Transcription sites were counted after membrane segmentation on Imaris 9.5.0 using the Cell module. Protein fluorescence levels were measured using FIJI for Macintosh. From each picture, five measurements of background mean intensity were taken. Each single measurement was then adjusted using the formula: integrated density – (area × background mean).
**Results**

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

Following the discovery of Rhomboid (Rho) as an intramembrane serine protease in *Drosophila*, Rho-like proteins have subsequently been identified in nearly every metazoan, (Urban et al. 2001; Freeman 2014). Although the molecular and cellular function of Rho-like proteins is well established, there are still a number of questions about their expression and possible post-transcriptional regulation. Given the strong rho phenotype exhibited in the *miR-1-miR-9a* double mutant we decided to investigate if *miR-9a* and/or *miR-1* could directly regulate rho mRNA degradation and/or translation. As *miR-1* is exclusively expressed in the mesoderm (Sokol and Ambros 2005; Fu et al. 2014) and *miR-9a* in the dorsal and neurogenic ectoderm (Fu et al. 2014; Gallicchio et al. 2021) largely overlapping *rho* (Ip et al. 1992a), we hypothesize that *miR-9a* might directly target rho. We used TargetScan (Agarwal et al. 2018) and SeedVicious (Marco 2018) to computationally identify the presence of two potential *miR-9a* binding sites in the *D. melanogaster rho* 3'UTR (Figure 1). *rho* has 2 alternatively polyadenylated transcripts (based on the most recent gene annotation in FlyBase), and the predicted *miR-9a* binding sites are both located in the common 3'UTR region. In addition, we used SeedVicious (Marco 2018) to search for *miR-9a* binding sites in Rho orthologs in beetle (*Tribolium castaneum*), worm (*Caenorhabditis elegans*), zebrafish (*Danio rerio*), mouse (*Mus musculus*) and human, and the non-model organisms mosquito (*Anopheles gambie*), butterfly (*Heliconius melpomene*) and mite (*Tetranychus urticae*) (Table 1). This analysis shows that *miR-9* family members have predicted binding sites on several rho orthologs. Evidence of conserved miRNA target sites
in homologous genes is often an indicator of functional significance (Grün et al. 2005; Friedman et al. 2009).

We then employed nascent transcript smFISH to precisely establish the overlap in expression domains of rho and the primary transcript of miR-9a (pri-mir-9a). To identify cells that are actively transcribing rho, we designed probes against the first intron of rho to detect active transcription sites (TS). As mature miRNAs are too short to be detected via smFISH, we designed probes against ~1kb of sequence flanking the mir-9a hairpin to detect the larger primary transcript. Using multiplex smFISH, we were able to identify cells that are transcribing both rho and mir-9a at the same time (Figure 2). As rho transcription is activated by Dorsal during embryonic stage 5 (pre-gastrulating embryo) we have focused our imaging on this specific embryonic stage. As expected, rho-expressing cells are contained entirely within the mir-9a expression domain (Figure 2 A-B). Since it has been widely observed that gene expression patterns are highly dynamic during stage 5 (Reeves et al. 2012), we measured membrane introgression to distinguish between stage 5 sub-stages. We find that both rho and mir-9a expression pattern become more refined at the ventral edge of their expression domain as stage 5 proceeds (Figure 2 C-F). Interestingly, while rho-expressing cells are generally also expressing mir-9a, there are many cells precisely at the ventral edge of the neuroectoderm that are expressing only mir-9a (Figure 2 C). As stage 5 progresses, the expression patterns of the two genes become more defines and the ventral expression border of both rho and mir-9a marks a clear boundary between neurogenic ectoderm and presumptive mesoderm (Figure 2 D). It is therefore likely that the two genes respond differently to the Dorsal gradient and to the mesodermal repressor snail, which has been shown to repress both mir-9a and rho in the mesoderm (Hemavathy et al. 2004; Fu et al. 2014). Taken together, the co-expression of rho and mir-9a and presence of conserved
miR-9a target sites suggest that miR-9a is a strong candidate to target rho mRNA during embryogenesis, and that this role may be evolutionally conserved.

**Increased rhomboid mRNA copy number in miR-9a<sup>E39</sup> mutants**

Combining high resolution confocal microscopy with smFISH, immunofluorescence and segmentation allows us to count mRNA molecules in individual cells in *Drosophila* early embryos. We quantified rho mRNAs per cell in WT and miR-9a<sup>E39</sup> (described in Li et al. 2006) stage 5 embryos (Figure 3 A-B). In order to precisely determine the stage of embryonic development, we focused only on stage 5 embryos that have a similar level of membrane introgression. As reported in Fu et al. (2014) the rho expression pattern is not spatially or temporally different in miR-9a<sup>E39</sup> mutant embryos. We imaged and quantified expression in six embryos per genotype. We also inspected many more and never saw abnormal rho expression patterns. Nevertheless, when we performed single cell segmentation and quantification, differences started to emerge (see Figure 3 E and F). The data show that the 2 embryos have a spatially equivalent rho expression pattern, but the number of mRNAs per cell is higher in miR-9a<sup>E39</sup> mutant embryos. To further support this observation, we performed two independent smFISH experiments using different fluorophores (Figure 3, G-H), with 3 embryos per genotype. The number of cells that have low or no detected rho expression varies from embryo to embryo, likely due to stochastic leaky transcription or false positive detection and counting. After excluding cells with fewer than 10 counted rho mRNAs, we found that in both experiments, miR-9a<sup>E39</sup> mutants possess a higher number of rho mRNA per cell.

To further characterize the difference in rho mRNA number in single cells, we simultaneously quantified rho transcript sites (TSs) and mature mRNA molecules (Figure 4),
using the intronic probes used in Figure 2 with rho intronic probes used in Figure 3 targeting
the mature rho transcripts. We segment and quantify rho TS number per cell (maximum 2
per cell prior to replication and 4 per cell following). As the higher magnification does not
permit imaging of entire embryos, we focused on the central region of the rho expressing
stripe, again in stage 5 embryos with a similar membrane introgression (Figure 4 A-B-C, A’-
B’-C’). The comparison of rho mRNA distribution between WT and miR-9aE39 embryos again
shows that miR-9aE39 embryos have higher numbers of rho mRNAs per cell (Figure 4-E). The
detection and quantification of rho TSs allowed us to distinguish between cells that are
differentially transcribing rho, and thus subgroup them in 3 classes: cells with no TSs, cells
with one TS and cells with two (or more) TSs. In Figure 4-F we reported that cells with a
higher number of TSs also show an increased number of rho mRNAs, and for each group of
cells, miR-9aE39 embryos have a generally higher number of transcripts with respect to WT
embryos. This becomes particularly evident for cells that are not transcribing rho at the
moment the embryo was fixed. It is important to note that very few cells have 3 or 4 TSs
(<10 per image over ~700 segmented cells). These may represent cells following DNA
replication, or errors in the segmentation process. We are confident that these small
numbers do not significantly affect our analysis and we did not observe a change in the
number of cells with no TSs, 1 TS or 2 (or more) TSs between the two genotypes (Figure 4-
G).

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

MicroRNAs are frequently found to have subtle effects on gene expression, acting as
buffering factors against intrinsic and extrinsic noise. We therefore investigated whether
miR-9a might not only affect the number of rho transcripts per cell, but also cell-to-cell
variability in the number of mature mRNAs present. In order to quantify these effects, we identified the immediate cell neighbours of each segmented cell, and then calculated how variable the *rho* mRNA number per cell is amongst the identified neighbours. As variance scales with mean, areas with high variance do not necessarily correspond to areas in which the cell-to-cell variability is intrinsically higher. Other statistical parameters that have been widely used in order to describe cell-to-cell variability are the coefficient of variation (CV) and the Fano factor (FF) (Munsky *et al.* 2012; Foreman and Wollman 2020). FF is defined as variance/mean while CV as standard deviation/mean. Thus, both measures are mean-normalized. CV is a unitless parameter, and has been used to compare cell-to-cell variability between mRNAs or protein levels resulting from the expression of different genes (Foreman and Wollman 2020). On the other hand, FF has a dimension, and has been used to measure how the observed data are dispersed from a Poisson distribution (Thattai and Van Oudenaarden 2001; Hortsch and Kremling 2018). As we are comparing measurements relative to the same gene between two genotypes, we calculated the FFs for the *rho* mRNA and TS counts reported in Figure 3 and Figure 4 (see Figure 5). We observe that the FF is marginally higher in *miR-9a*E39 mutants compared to WT, and we posit that it is significantly different because of the very high number of observations, while the effect size is indeed small. Closer inspection shows that the FF is higher in *miR-9a*E39 mutants only in the group of cells with no transcription sites, as might be expected, while groups of cells that have a single TS and 2 or more TSs have higher FF in the WT. We speculate that the *miR-9a* buffering action on *rho* mRNA number per cell becomes more evident and/or necessary in quiescent cells that are not actively transcribing *rho*.

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

rho has been one of the most studied Dorsal target genes. Its expression becomes restricted to the neurogenic ectoderm in a precisely orchestrated manner: the low nuclear levels of Dorsal in the dorsal ectoderm do not support rho activation, while snail represses its transcription in the mesoderm (Ip et al. 1992b; Hong et al. 2008). rho has not been previously studied as a direct target of miRNA regulation, but the combined effect of mutations in miR-1 and miR-9a on rho mRNA distribution motivated our investigation into rho regulation by miRNAs (Fu et al. 2014). We found that the per cell copy number of rho mRNA is significantly higher in miR-9a E39 mutant embryos (Figure 3 and Figure 4), suggesting miR-9a affects rho mRNA stability or degradation. Further work is required to determine if this is a direct or indirect effect. We could not find a clear role for miR-9a in stabilizing cell-to-cell variability of either the number of rho mRNA transcription sites or mRNA molecules (Figure 5). Nevertheless, when we distinguish between cells that are and are not actively transcribing rho, we find that the FF of cells with no transcription sites was significantly higher in miR-9aE39 mutants. This leads us to suggest that, in WT animals, rho mRNA is rapidly degraded when transcription stops, whereas this degradation is less efficient when miR-9a is removed, and cell heterogeneity consequently increases. To our knowledge, this is the first study in which mRNA copy number was compared in different genotypes using single cell quantitative microscopy in order to uncover miRNA regulatory roles on target gene expression.

It has been shown that protein levels are usually more stable than mRNA levels (Perl et al. 2017). The miR-9a regulatory effect on Rho protein accumulation might therefore be more evident than the one we observed on the mRNA as it better reflects the integrated
activity over time. Rho is a transmembrane protease localized in the Golgi. While Fu et al. reported rho mRNA patterns in double miR-9a/miR-1 mutants (Fu et al. 2014), no information on the protein pattern was previously available. We observed dramatic differences in timing and level of Rho protein accumulation when comparing WT and miR-9a embryos. In the WT, Rho was only detectable from stage ~10, whereas in miR-9a embryos it was clearly present from stage 5, the same stage when we see the initiation of rho transcription. The early accumulation of Rho protein appears to be inhibited by miR-9a. We suggest that the most parsimonious explanation would be direct translational inhibition by miR-9a which is diminished as a certain level of rho mRNA is reached, or in response to an external signal later in development. A clear demonstration that the predicted miR-9a target sites in the rho UTR are functional is needed to further support this hypothesis. We also note the possibility that early low levels of Rho protein may be present but are undetectable with current technology.

Previous work on the miR-9a/miR-1 double mutant shows that when miR-1 is also removed, strong phenotypic defects emerge leading to failure of gastrulation and ventral midline enclosure (Fu et al. 2014). This phenotype suggests that these two miRNAs play an important role in germ layer differentiation. Indeed, while a role for miR-9a and miR-1 involvement in dorso-ventral (DV) axis patterning has not been definitively established, their expression patterns indicate they are early targets of DV specification (Sokol and Ambros 2005; Biemar et al. 2006). Our current findings provide convincing evidence for a role of miR-9a in the DV patterning process during early Drosophila embryogenesis. We posit that miR-9a regulates rho mRNA accumulation and translation, possibly affecting epidermal growth factor receptor (EGFR) signalling and specification of the dorsal and neurogenic ectoderm (Golembo et al. 1996; Guichard et al. 1999). The role of miR-1 is less clear as miR-
1 is not expressed in the same region as rho, and therefore miR-1 can affect rho expression only indirectly. miR-1 is involved in muscle development and is exclusively expressed in the mesoderm (Sokol and Ambros 2005). We suggest that the combination of disrupted miR-1 function in the mesoderm and miR-9a function in the neurogenic ectoderm leads to disruption in establishment or maintenance of an organized border between these two germ layers, as seen in the double mutants (Fu et al. 2014).

To conclude, we have demonstrated a new function for miR-9a during early Drosophila embryogenesis. We have observed that miR-9a affects both rho mRNA copy number per cell (possibly by degradation) and rho protein levels. Our findings also show the importance of single-cell quantification when studying the effects of miRNA regulation on target genes. As miRNAs act as weak modulators of gene expression, single-cell quantitative approaches can reveal previously unknown effects on mRNA and protein regulation by miRNAs. This work and the methods described can be easily applied to many other miRNA-target gene networks to allow new insights into miRNA function during development.
Data availability statement

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

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Competing interests

The authors declare no competing interests.

Author contributions

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

Figure 1. Binding sites of mir-9a on rhomboid 3’UTR. Schematic representation of the Drosophila melanogaster miR-9a binding sites on rhomboid 3’UTR. ORF = open reading frame. Numbers above 3’UTR region indicates nucleotide position from the 3’UTR start (indicated with number 0). Only the canonical microRNA binding site types have been considered.

Figure 2. rhomboid and miR-9a are co-expressed in the neurogenic ectoderm.
(A) Early and (B) middle stage 5 D. melanogaster embryos stained with probes against rhomboid intron (yellow) and the primary transcript of miR-9a (magenta). (C-D) Closer sections of highlighted areas in A and B respectively. In green is highlighted the presumptive ventral midline, which separates mesoderm and ectoderm (pVM). (E-F) Brightfields of ventral borders of the embryos in A and B showing membrane introgression (M.i.). Scalebars: 100 μm (A-B), 25 μm (C-D-E-F).

Figure 3. rhomboid mRNA number per cell is higher in miR-9aE39 embryos
(A) WT and (B) miR-9aE39 middle stage 5 embryos stained with a probe set against Rhomboid transcripts. (C-D) Brightfields of a ventral region from embryos in A and B respectively showing membrane introgression. (E-F) Computational reconstruction after segmentation of the embryos in A and B. The colormap is based on mRNA number per cell with grey being low, green intermediate and purple high. (G-H) Two independent quantifications of rhomboid mRNA number in single cells in WT and miR-9aE39 mutant embryos. Each
quantification was performed using 3 embryos per genotype. Both p-values <0.0001.

Scalebars: 100 μm (A-B), 25 μm (C-D).

Figure 4. Detection and quantification of rhomboid transcription sites in single cells.

Central region of (A) WT and (A’) miR-9aE39 embryos respectively. Orientation is indicated by the white arrow (Ant = Anterior embryonic region, Pos = Posterior embryonic region). (B-B’) Zoom from red area highlighted in A and A’ respectively showing staining against rhomboid intron (rhomboid_i, magenta), Spectrin to mark cellular membrane (yellow) and DAPI (grey).

(C-C’) Zoom from red area highlighted in A and A’ respectively showing staining against rhomboid exon (rhomboid_e, green), Spectrin and DAPI. (D-D’) Computational reconstructions of the images in A and A’ respectively. Each dot corresponds to a segmented cell. The size of the dot corresponds to the number of rhomboid mRNAs detected with rhomboid_e, while the colour corresponds to the number of detected transcription sites with rhomboid_i. (E) Comparison between WT and miR-9aE39 rhomboid mRNA number per cell. p-value = 0.0014. (F) Quantified cells are grouped depending on how many alleles are actively transcribing the rhomboid locus: grey = 0 alleles active (p-value <0.0001), orange = 1 allele active (p-value = 0.0021), red = 2 or more alleles active (p-value = 0.0259). (G) Bar plot reporting the number of segmented cells belonging to each transcription site group. Colours as in (F). Scalebars: 100 μm (A-A’), 25 μm (B-C-B’-C’).

Figure 5. Fano factor quantification and comparison between WT and miR-9aE39 mutant embryos.

Computational reconstruction of Fano factor distribution calculated in neighbour clusters in (A) WT and (B) miR-9aE39 stage 5 embryos. These two embryos are the same reported in figure 3 E-F respectively. (C-D) Comparison between Fano factor in WT and miR-9aE39
embryos in 2 independent experiments (n = 3 embryos each). P-value < 0.0001 in both graphs. (E-G) Graphical reconstruction of Fano factor distribution calculated in neighbour cells clusters in a WT and \textit{miR-9a}^{E39} embryos, corresponding to Figure 4 A-A’ respectively. (F) cells are sub-grouped depending on their transcription sites number. p-values = 0.0147 (0 TS) and 0.0123 (1 TS), ns = non-significant.

Figure 6. Rhomboid protein is over-expressed in \textit{miR-9a}^{E39} embryos during stage 5 and 6.

(A-B) Stage 5 WT and (A’-B’) \textit{miR-9a}^{E39} embryos respectively stained against Dorsal (red) and Rhomboid (cyan). (C) Adjusted fluorescence levels from Rhomboid staining in stage 5 embryos (n=3 per genotype). In each embryo 15 areas equally distributed along the Dorsal expression border were quantified. Measurements are reported in Log10 scale. P-value < 0.0001. (D-E, D’-E’) Stage 6 WT and \textit{miR-9a}^{E39} embryos respectively stained against Dorsal (red) and Rhomboid (cyan). (F) Adjusted fluorescence levels from Rhomboid staining in stage 6 embryos (n=3 per genotype). Quantified as in (C). P-value < 0.0001. Scalebars: 100 μm in all panels.
| Organism                  | Transcript                  | microRNA          | Position on 3'UTR | Site type |
|--------------------------|-----------------------------|-------------------|-------------------|-----------|
| *Drosophila melanogaster* | rho-RA/RB                   | dme-miR-9a/b/c-5p | 340               | 8mer      |
|                          |                             |                   | 1075              |           |
| *Tribolium castaneum*    | TC034044                    | tca-miR-9b-5p     | 416               | 7_m8      |
|                          |                             | tca-miR-9a/e-5p   | 188               | 8mer      |
|                          |                             |                   | 417               |           |
| *Anopheles gambiae*      | AGAP005058 RA/RB            | aga-miR-9a/b/c    | 405               | 7_A1      |
|                          |                             |                   | 904               | 8mer      |
|                          |                             |                   | 3197              |           |
| *Heliconius melpomene*   | HMEL008701-RA               | hme-miR-9b        | 710               | 8mer      |
|                          |                             | hme-miR-9a        | 1561              | 8mer      |
| *Tetranychus urticae*    | tetur14g02680.1             | tur-miR-9-5p      | 138               | 7_A1      |
| *Caenorhabditis elegans* | rho-1                       | cel-miR-79-3p     | 54                | 7_m8      |
| *Danio rerio*            | Rhbdl3-203                  | dre-miR-9-5p      | 464               | 7_m8      |
| *Mus musculus*           | Rhbdl3-201                  | mmu-miR-9-5p      | 1046              | 7_m8      |
| *Homo sapiens*           | RHBDL3-201/203              | hsa-miR-9-3p      | 2988              | 7_A1      |
Figure 2

A. Stage 5 early

B. Stage 5 middle

C. pVM

D. pVM

E. M.i.=11.4 μm

F. M.i.=15.8 μm

rhomboid_i
pri-miR-9a
DAPI
Figure 3

WT

miR-9a\textsuperscript{E39}

rhomboid

DAPI

M.i.=13.5 \mu m

M.i.=13.0 \mu m

WT

miR-9a\textsuperscript{E39}

G

H

Rhomboid mRNA\# per cell

0

50

100

150

200

250

300

350

****

****

Rhomboid mRNA\# per cell

0

50

100

150

200

250

300

350

****

****
Figure 4

A, B, C: Image panels showing the distribution of rhomboid mRNA per cell in WT and miR-9a E39 conditions.

A': Image showing the same distribution as A, but with a different staining protocol.

B': Image showing a different angle or section of the tissue.

C': Image showing a different section or staining protocol.

D: Graph showing the number of cells with rhomboid mRNA expression over different time points (TS).

E: Violin plot showing the distribution of rhomboid mRNA per cell in WT and miR-9a E39 conditions.

F: Violin plot showing the distribution of rhomboid mRNA per cell over different TS.

G: Bar graph showing the number of cells with rhomboid mRNA expression over different TS conditions.

Legend:
- **: P < 0.001
- *: P < 0.05
- ns: Not significant

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Figure 5

A

B

C

D

E

F

G
Figure 6

Stage 5

WT

miR-9a<sup>E39</sup>

Dorsal

DAPI

Rhomboid

DAPI

Stage 6

WT

miR-9a<sup>E39</sup>

Dorsal

DAPI

Rhomboid

DAPI

Adjusted Fluorescence (Log10)