Regulation of pattern formation and gene amplification during *Drosophila* oogenesis by the miR-318 microRNA

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Abstract

Pattern formation during epithelial development requires the coordination of multiple signaling pathways. Here, we investigate the functions of an ovary-enriched miRNA, miR-318, in epithelial development during Drosophila oogenesis. miR-318 maternal loss-of-function mutants were female sterile and laid eggs with abnormal morphology. Removal of miR-318 disrupted the dorsal-anterior follicle cell patterning, resulting in abnormal dorsal appendages. miR-318 mutant females also produced thin and fragile eggshells, due to impaired chorion gene amplification. We provide evidence that the ecdysone signaling pathway activates expression of miR-318 and that miR-318 cooperates with Tramtrack69 (Ttk69) to control the switch from endocycling to chorion gene amplification during differentiation of the follicular epithelium. The multiple functions of miR-318 in oogenesis illustrate the importance of miRNAs in maintaining cell fate and promoting the developmental transition in the female follicular epithelium.

Introduction

microRNAs (miRNAs) are small non-coding RNAs that act to silence target mRNAs by promoting mRNA degradation and/or protein translation inhibition. The importance of miRNAs in animal development and human disease has been well established through genetic and genomic studies (Bushati and Cohen 2007; Ebert and Sharp 2012; Flynt and Lai 2008; Kloosterman and Plasterk 2006;
Mendell and Olson 2012; Sun and Lai 2013; van Rooij and Olson 2012). However, understanding the cellular functions of individual miRNAs in different biological settings remains a major challenge as many miRNAs have been identified in different species.

miRNAs have been implicated in a variety of important roles during Drosophila oogenesis (Dai et al. 2012). Oogenesis takes place within the ovarioles, each of which contains an assembly-line of developing egg chambers. Each egg chamber consists of 16 interconnected germ-line cells, including 15 nurse cells and one oocyte, surrounded by a monolayer of approximately 1000 somatically derived follicle cells (Sprading, 1993). A complex exchange of signals between the germ-line cells and the surrounding follicle cells is required for oocyte development and eggshell patterning (Berg 2005; Dobens and Raftery 2000).

Previous studies reported that mutations for components of miRNA biogenesis pathway, including Dicer-1, Loquacious, Pasha, Drosha and Ago-1, resulted in defects in germ-line stem cell division and in follicle cell development (Azzam et al. 2012; Hatfield et al. 2005; Jin and Xie 2007; Park et al. 2007). Ninety-three miRNAs are expressed in the Drosophila ovary (Czech et al. 2008), but functions have been assigned to only a few of them. miR-184 controls germ-line stem cell differentiation and dorsoventral patterning by regulating Saxophone and K10 (Iovino et al. 2009). miR-7 and miR-278 target Dacapo (Dap) to regulate cell cycle progression in germ-line stem cells (Yu et al. 2009). miR-7 also regulates Ttk69 to control a developmental switch in the follicle cells (Huang et al. 2013). miR-279
represses STAT expression in both the follicle cells and migratory border cells to control cell fate (Yoon et al. 2011). miR-989 regulates border cell migration through multiple target genes (Kugler et al. 2013). Thus, it appears that individual miRNAs act in a variety of ways to control different aspects of oogenesis.

Here, we report on the role of ovary-enriched miRNA, miR-318. Drosophila miR-318 shares the same “seed” sequence with two other miRNAs, miR-3 and miR-309, comprising the miR-3 seed family (Figure S1). Though all three miRNAs can in principle target the same mRNAs, their spatial and temporal expression differs. miR-3 and miR-309 are part of a polycistronic miRNA complex expressed strongly in the early embryo, with roles in regulation of the maternal-zygotic transition (Bushati et al. 2008). miR-3 and miR-309 are expressed at very low levels, if at all, in the ovary (Czech et al., 2008; Ruby et al., 2007). In contrast, miR-318 is the 7th most abundant miRNA in the ovary, comprising over 6% of total miRNA sequence reads (Czech et al. 2008). We present evidence that miR-318 acts in the somatic follicle cells to regulate eggshell patterning and biogenesis during Drosophila oogenesis.
Results

miR-318 mutant females produce defective eggs

miR-318 is located in close proximity to miR-994 in the interval between two protein coding genes (Figure 1A). To study miR-318 function, we made use of a targeted knock-out allele in which 126 bp genomic fragment including the miR-318 hairpin was replaced with green fluorescence protein (GFP) and mini-white reporter genes (Figure 1A; (Chen et al. 2014)). A second genetically independent allele, miR-318^Δ2, was produced by imprecise excision of a P-element insertion line P[w[+mC]=GSV6]GS15934, resulting in a deletion of 1.8Kb. miR-318^Δ2 removed miR-318 and miR-994, as well as part of the coding region of Ire-1B (Figure 1A). miRNA quantitative PCR confirmed that the mature miR-318 miRNA was absent in RNA samples prepared from the miR-318^Δ1a/miR-318^Δ1b (Δ1a and Δ1b are equivalent alleles from two independent homologous recombination events) and miR-318^Δ1/miR-318^Δ2 mutants (Figure 1B). In addition, we noted that the expression of miR-994 was reduced to 11% in the miR-318^Δ1a/miR-318^Δ1b mutant and to 7% in the miR-318^Δ1/miR-318^Δ2 mutant, compared to the control, suggesting that miR-318^Δ1 is a hypomorphic allele for miR-994 (Figure 1B). The trans-heterozygous combination miR-318^Δ1/miR-318^Δ2 was used for phenotypic assays. We also made use of a miR-318 transgene, consisting of the interval between the flanking protein genes, but lacking miR-994 sequences (Figure 1A). The transgene was recombined onto the miR-318^Δ1 chromosome, and restored miR-318 expression to ~40% of wild type control levels, without restoring
miR-994 expression (Δ1, 318R, Figure 1B).

miR-318 mutant flies were fully viable and did not show obvious morphological defects. However, females lacking miR-318 laid eggs with a range of defects in eggshell formation and patterning (Figure 1C). More than 90% of eggs from miR-318 mutant mothers also showed defects in dorsal pattern: the dorsal appendages were shifted toward posterior and were closer together and shorter than in wild type embryos (Figure 1C). The operculum was also slightly enlarged. These defects were fully rescued by introducing the miR-318 rescue transgene in the mutant background (Δ1, 318R/Δ2, Figure 1C).

In addition, 76% of eggs from miR-318 mutant mothers failed to hatch into first instar larvae (Figure 1D). Most of the eggs appeared not to be fertilized, and the micropyle was reduced or absent (visualized by scanning electron microscopy, Figure S2A and B). In addition, eggs had a transparent and thin eggshell and were fragile (Figure 1C). 12% of eggs had collapsed by 48 hours after laying (Figure 1E). All these defects were fully rescued by introducing the miR-318 rescue transgene into the mutant background. Suppression of these phenotypes by expression of the rescue transgene suggests that the defects are attributable to the loss of miR-318. miR-994 appears to be dispensable in this context. Thus miR-318 is required for egg maturation, and for eggshell patterning.

miR-318 is expressed in the follicle cells

Consistent with previous deep sequencing data, quantitative miRNA PCR
showed that miR-318 was undetectable in adult males, but was expressed in adult females and enriched in ovaries (Figure S3A and B). To visualize miR-318 expression in vivo, we made use of the miR-318\(^{\Delta1}\) allele, in which GFP was integrated into the miR-318 locus in place of the miRNA hairpin (Figure 1A). This placed GFP expression under control of the endogenous miR-318 regulatory elements. Ovaries were dissected from miR-318\(^{\Delta1/+}\) heterozygous adult females and stained with antibody to GFP (Figure 2A-C'). GFP was not detected in early stage egg chambers (Figure 2A and A'). Beginning at stage 9, GFP was observed in most follicle cells surrounding the oocyte (Figure 2B and B'). From stage 10 onward, GFP persisted in the follicle cells, including the border cells whose function is required to make the micropyle (Figure 2C and C', data not shown for later stages). We noted that miR-318 expression was undetectable in the polar cells within the border cell cluster and the posterior polar cells (labeled with a polar cell specific marker FasIII, Figure S4A-C''').

As an independent means to visualize miR-318 activity, we used a sensor transgene that expresses GFP under the control of a ubiquitously expressed tubulin promoter and has two perfect miR-318 target sites in its 3'UTR. miRNA activity represses GFP expression from this type of sensor transgene. GFP expression from a control sensor transgene (lacking the miRNA target sites) was uniform in egg chambers of various stages (Figure 2D and D'). However, GFP expression was suppressed in the follicle cells in stage 10 egg chambers of flies carrying the miR-318 sensor (Figure 2E-F'). The reduced GFP expression in the
follicle cells was not observed when the miR-318 sensor was introduced into the miR-318 mutant (Figure 1G and G’), confirming that the difference in expression in the follicle cells vs the germ line was due to the activity of the miRNA in the follicle cells. Expression of miR-318 in the follicle cells beginning in stage 9 is consistent with the phenotypic evidence for a role in eggshell formation and patterning.

**Eggshell patterning**

Patterning the eggshell along the dorsal-ventral and anterior-posterior axis requires the EGFR and Dpp signaling pathways (BERG 2005; DOBENS and RAFTERY 2000). Broad (Br), a zinc-finger transcription factor, functions downstream of these two signaling pathways to specify the dorsal appendages (DENG and BOWNES 1997). Beginning at stage 6, Broad is expressed in all oocyte-associated follicle cells and subsequently repressed in the anterior midline region in early stage 10B egg chambers. Later, Broad expression increases in the two groups of dorsal-anterior follicle cells that play an essential function in the specification of the dorsal appendages (DENG and BOWNES 1997). In miR-318 mutant ovaries, Broad expression was not repatterned to clear the dorsal anterior region in 8 of the 15 stage 10B egg chambers examined (Figure 3A-B”). Fewer cells showed reduced Broad expression in the anterior midline region and the domain was irregular in shape. In addition, only very few of the laterally-positioned follicle cells adjacent to this domain displayed the normal increase in Broad expression in the dorsal appendage field (14/15 mutant samples, arrow in Figure 3B). Introducing the
miR-318 transgene rescued these defects (Figure 3C-C’). Although miR-318 activity is required for the normal pattern of changes in Br expression that depend on EGFR and DPP activity, miR-318 expression itself does not appear to be regulated by either of these pathways (Figure S5A-D’). This suggests that miR-318 activity may be required for the correct interpretation of these patterning cues, or for the ability of cells to respond normally to them.

**Chorion gene amplification**

During late oogenesis, follicle cells produce the chorion proteins that make the eggshell. Molecular and genetic studies have shown that developmentally regulated amplification of the chorion genes is required to support eggshell biogenesis (CAVXI et al. 1998; CAVXI and SPRADLING 1999; TOWER 2004). The fragile eggshell phenotype led us to explore the possibility that miR-318 might be involved in chorion gene amplification. To test this, we used incorporation of a thymidine analog, 5-ethynyl-2’-deoxyuridine (EdU), to monitor DNA replication. In wild-type controls, the sites of chorion gene amplification are seen as 4-6 subnuclear foci of EdU incorporation in each of the follicle cell nuclei (Figure 4A-A’’). Higher magnification views are shown in Figure S6A-A”). miR-318 mutant egg chambers showed a range of abnormalities in EdU incorporation (Figure 4B-B’’ and Figure S6B-B”). In each egg chamber, a few cells showed EdU incorporation uniformly throughout the nucleus (e.g. arrowhead in Figure 4B and Figure S6B), suggesting that all of the DNA was replicating. Cells in the dorsal
anterior region (where the Broad expression domain was affected) showed little or no incorporation of EdU (arrow in Figure 4B; arrow and dotted arrow in Figure S6B). This suggested a reduction in chorion gene amplification in this region. In addition, a generally reduced EdU incorporation was also observed in 15/20 mutant samples examined. These defects were rescued by restoring miRNA expression with the rescue transgene (Figure 4C-C″ and Figure S6C-C″).

To more precisely examine the effects of removing miR-318 we used the FLP-FRT system (XU and RUBIN 1993) to create clones of miR-318 mutant in the follicle cells. Use of genetic mosaics allows direct comparison of mutant cells with their age-matched neighbors in the same egg chamber. In this experiment the miR-318 mutant cells were marked by 2 copies of GFP (brighter green), compared to the heterozygous GFP/+ cells which carried one mutant copy of miR-318 and the +/+ cells with 2 wild-type copies of miR-318. As in the intact egg chamber, we observed a proportion of cells in the clones with EdU incorporation throughout the nucleus (Figure 4D-D″, arrowhead). 28% of nuclei in the mutant clones showed this phenotype (Figure 4F). 54% of the nuclei in the mutant clones showed reduced EdU incorporation (Fig 4D-D″, dotted arrow; Figure 4F), while 4% showed no Edu incorporation (Figure 4E-E″, arrow; Figure 4F). We also noted that mutant nuclei with reduced Edu signals in chorion amplification foci appeared to show a weaker diffuse Edu incorporation in the rest of nucleus (Figure 4D-D″ and E-E″, star).
**Delayed exit from follicle cell endocyling**

The observation of uniform incorporation of EdU in some *miR-318* mutant cells at stage 10B, suggested that *miR-318* might be required for the transition of follicle cells from whole genome replication to selective chorion gene amplification. Follicle cell ploidy increases by endoreplication of the DNA without subsequent cell division. Endoreplication stops during stage 10A/B. Cut expression is normally very low during endocycles but increases to higher levels in most follicle cells by stage 10B ([Sun and Deng 2005](#)). Conversely, expression of another transcription factor, Hindsight (Hnt), shows the opposite pattern, being higher during the endocycles and decreasing at stage 10B ([Sun and Deng 2007](#)). Increased Cut expression and decreased Hnt expression provide markers for the transition from endocycles to gene amplification ([Huang et al. 2013](#)). We compared the expression patterns of Cut and Hnt in mutant and wild type clones in stage 10B egg chambers. As shown in Figure 5A-A”, Hnt expression was higher in miR-318 mutant clones compared to control clones. Consistently, Cut expression appeared to be lower in *miR-318* mutant clones compared to control clones (Figure 5B-B”). These results suggest that loss of *miR-318* compromises the transition from endocycles to chorion gene amplification in the follicular epithelium.

Given that Loss of *miR-318* delayed exit from follicle cell endocyling, we next asked whether *miR-318* overexpression would be able to advance this process and lead to premature chorion gene amplification. To this end, we induced ectopic expression of *miR-318* using the flip-out GAL4 system in the follicle cells. In stage 9
egg chambers, miR-318 overexpression cells showed an oscillating genomic EdU incorporation pattern similar to that observed in adjacent wild type cells (Figure 6A-A’). Consistent with this, the expression of Hnt and Cut were unchanged in miR-318 overexpressing cells (Figure 6B-C’). Thus, overexpression of miR-318 was not sufficient to activate premature gene amplification.

**EcR signaling is required for miR-318 expression**

Activation of the ecdysone signaling pathway promotes the transition from endocycling to gene amplification in the follicle cells (Sun et al. 2008). We therefore asked whether the steroid hormone 20-hydroxyecdysone (20E) and its nuclear receptor were involved in regulating miR-318 expression. Expression of a dominant-negative form of the ecdysone receptor (EcR) in the clones of follicle cells led to loss of a miR-318–GFP reporter transgene (Figure 7A-B’). To further test whether this regulation is directly through the regulatory elements of miR-318, we prepared a reporter construct containing 852 bp fragment of genomic DNA upstream of miR-318. This fragment, contains a putative ecdysone response element (EcRE, Figure 7C), similar to the consensus EcRE sequence through which the EcR/Usp heterodimeric complex binds to DNA to mediate ecdysone regulated gene expression (Cherbas et al. 1991; Yao et al. 1992). 20E treatment increased miR-318 luciferase reported activity by 1.5 fold in S2 cells (Figure 7D). A mutated luciferase reporter that lacked the EcRE showed a blunted response to 20E treatment (Figure 7D), indicating that the EcRE was functionally
important in mediating the response to ecdysone in S2 cells.

**miR-318 interacts with ttk69**

Previous studies have shown that regulation of Ttk69, a zinc-finger protein, plays an important role during late oogenesis (FRENCH et al. 2003). At stage 10B, Ttk69 functions to promote dorsal appendage tube elongation and chorion gene amplification (BOYLE and BERG 2009; PETERS et al. 2013; SUN et al. 2008). A recent report has found that, *miR-7* miRNA regulates Ttk69 during the transition from endocycles to gene amplification. *miR-7* overexpression was able to repress Ttk69 expression and delay the switch from endocycles to amplification, but loss of miR-7 was not sufficient to advance this process (HUANG et al. 2013).

In light of the gene amplification defect we observed in *miR-318* mutants, we asked whether *miR-318* interacts with Ttk69. To test this, we examined the effects of introducing one copy of ttk69 mutant allele *ttk*\(^{1e11}\) into the *miR-318* mutant background. Heterozygous *ttk*\(^{1e11}/+\) females laid normal eggs. However, removing one copy of ttk69 increased the severity of the *miR-318* mutant egg defect, including short dorsal appendages (Figure 8A). 79% of eggs from *miR-318*\(^{A1}\) *ttk*\(^{1e11}/ miR-318*\(^{A2}\) mothers collapsed, compared to 13% in the *miR-318*\(^{A1}/miR-318*\(^{A2}\) mutants (Figure 8B). Whereas 24% of eggs from *miR-318* mutant females were able to develop into first instar larvae, none of eggs from *miR-318*\(^{A1}/ttk*^{1e11}/ miR-318*^{A2}\) females hatched into first instar larvae (Figure 8C).
Similar results were obtained when using a deficiency chromosome that covered the ttk69 locus (Figure 8B and C).

To determine whether these more severe defects in eggshell morphology were associated with the impairment of chorion gene amplification, we analyzed the pattern of gene amplification using EdU incorporation. Egg chambers from miR-318\textsuperscript{\textdagger} ttk\textsuperscript{\textdagger\textdagger}1e11/ miR-318\textsuperscript{\textdagger\textdagger}2 females showed a more severe reduction of EdU incorporation in the chorion gene amplification pattern, and a higher frequency of nuclei showing overall EdU labeling, compared to the miR-318\textsuperscript{\textdagger}/miR-318\textsuperscript{\textdagger\textdagger} mutants (Figure 8D-G'; quantified in Figure 8H). This analysis revealed that 63\% of stage 10B egg chambers from miR-318\textsuperscript{\textdagger} ttk\textsuperscript{\textdagger\textdagger}1e11/ miR-318\textsuperscript{\textdagger\textdagger}2 females had severe defective EdU incorporation and 31\% had moderate defective EdU incorporation, compared to 18\% of stage 10B egg chambers from miR-318\textsuperscript{\textdagger}/miR-318\textsuperscript{\textdagger\textdagger} females had severe defects and 59\% had moderate defects (Figure 8H). This suggests that removal of one copy of ttk69 enhanced the gene amplification defects in miR-318 mutant females.

The genetic interaction between miR-318 and ttk69 prompted us to test whether miR-318 was able to activate Ttk69 expression in the follicle cells. To do this, we performed immunofluorescence staining with an antibody against Ttk69. However, Ttk69 expression was not affected in miR-318 mutant clones compared to control clones in the follicle cells in stage 10B egg chambers (Figure 8I-I''). These results suggest that miR-318 acts cooperatively with Ttk69 to control gene amplification.
Discussion

In this study, we have shown that miR-318, one member of the miR-3 seed family, was highly expressed in ovaries and acted as a novel regulator in pattern formation and chorion gene amplification during *Drosophila* oogenesis. In contrast to this, miR-3 and miR-309, two other members of this family, function exclusively during embryogenesis (Bushati *et al.* 2008). As miRNAs within the same miRNA family shares the same “seed” sequence, multiple members of the same miRNA family could function redundantly by acting on the same set of target genes. For instance, miR-11 and miR-6, two members of the miR-2 seed family miRNAs, function to control the level of apoptosis by repressing expression of the proapoptotic genes including rpr, hid and skl (Ge *et al.* 2012). Different roles between miR-318 and miR-3/309 provided one example that members of the same miRNA family could have distinct functions during development, potentially by acting on different sets of target genes. As a large number of miRNA knock out mutants have been generated in *Drosophila*, the phenotypical analysis of individual miRNA mutants will help us to understand the function redundancy and diversity of miRNAs within the same miRNA family in different developmental processes (Chen *et al.* 2014).

Our analysis of miR-318 in *Drosophila* oogenesis revealed several features of its expression and function. (1) The spatial and temporal expression pattern of miR-318 is tightly controlled during oogenesis. miR-318 is uniformly expressed in
somatically derived follicle cells during late oogenesis. It has been shown that spatial gene expression patterns in the follicle cells follow a simple combinatorial code based on six basic shapes, including the uniform pattern (Cheung et al. 2011; Yakoby et al. 2008). The uniform expression pattern of miR-318 in the follicle cells may reflect its function as a uniform repressor. Although EGFR and Dpp pathways control the expression of multiple genes in the follicle cells, they appear not to be involved in regulating miR-318 expression. In contrast, miR-318 expression is activated by the ecdysone signaling pathway through the EcRE site at miR-318 locus. The direct regulation of miR-318 by EcR highlights the importance of miRNAs in mediating downstream effects of EcR signaling. (2) Multiple functions of miR-318 during late oogenesis. The morphological defects of miR-318 mutant eggs suggest connections between miR-318 and EGFR and Dpp signaling pathways. The enlarged operculum in miR-318 mutant resembles the phenotype observed when Dpp is overexpressed (Twombly et al. 1996). Given that reduced Dpp activity did not compromise miR-318 expression, it is possible that miR-318 might function to limit Dpp activity. In the main body of follicle cells, mutation of miR-318 resulted in decreased Br expression in two dorsal-anterior domains and the reduced size of the midline region. These defects would be consistent with reduced EGFR activity (Roth 1998; Schupbach 1987). Except for its roles in patterning follicular epithelium, miR-318 also functions in eggshell biogenesis. Reduced EdU incorporation at chorion gene loci in miR-318 mutant follicle cells suggests that miR-318 is important for efficient chorion gene amplification in the follicle cells.
The chorion gene amplification programs are also coupled with the endocycles-to-amplification transition in the development of follicle cells (McConnell et al. 2012). Delayed exit from follicle cell endocycling in miR-318 mutants indicates that miR-318 is essential for this developmental switch. (3)

Cooperation of miR-318 and the transcriptional repressor Ttk69 in controlling the endocycles-to-amplification switch. It has been reported that the cell cycle transition from endocycling to gene amplification requires downregulation of the Notch pathway, activation of the EcR pathway and upregulation of Ttk69 (Sun et al. 2008). The enhancement of gene amplification defects in miR-318 mutants with reduced Ttk69 activities demonstrates that these two negative regulators of gene expression function together to control the switch from endocycling to gene amplification. EcR pathway promotes the activity of Ttk69, and in turn to trigger gene amplification (Sun et al. 2008). The fact that EcR signaling controls miR-318 expression further supports the notion that miR-318 acts cooperatively with Ttk69 (Figure 8J). (4) Spatial differences in miR-318 effects on gene amplification. In stage 10B, gene amplification occurs synchronously in all follicle cells over the oocyte (Calvi et al. 1998). We have shown that some cells had more reduced EdU incorporation than others in the miR-318 mutant follicular epithelium. Especially, EdU incorporation was severely affected in dorsal-anterior follicle cells. These results raise the possibility that the roles of miR-318 vary in different domains of the follicular epithelium. How does the uniformly expressed miR-318 affect gene amplification with a spatial difference? It is conceivable that miR-318 might
interact with its potential targets in specific regions of the follicular epithelium.

As miRNAs normally downregulate target gene expression, the mRNA levels of miR-318 targets whose expression leads to pattern formation and chorion gene amplification defects in miR-318 mutants would be expected to be increased in the mutant ovaries. We performed RNA deep sequencing analysis from RNA samples prepared from both wild type and miR-318 mutant ovaries and identified a number of genes which were upregulated in the mutant ovaries. However, the overlapping hits between our identified genes and predicted miR-318 targets from TargetScanFly and miRNA.org were not apparently involved in regulating pattern formation and chorion gene amplification. Thus, we did not verify this further. As miR-318 is expressed only in a sub-population of cells, assaying target RNA or protein level through RNA in situ hybridization or antibody staining in egg chambers with miR-318 mutant clones would be a better way to verify the predicted targets. Although we have been unable to identify the specific targets for miR-318, our results indeed demonstrate that miR-318 is one important regulator in oogenesis and future investigations on its targets could potentially provide more mechanistic insights in pattern formation and developmental gene amplification.

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**Conflict of Interest**

The authors declare no conflict of interest.

**References**


PETERS, N. C., N. H. THAYER, S. A. KERR, M. TOMPA and C. A. BERG, 2013 Following the 'tracks': Tramtrack69


Materials and methods

Drosophila stocks and genetics

Fly strains used: Df(3R)BSC479 (Bloomington stock center BL24983 removes miR-318), ttk\textsuperscript{le11} (BL4164), Df(3R)ED6361 (BL24143), FRT82B (BL2035), FRT82B ttk\textsuperscript{le11} (WM. Deng), P[w[+mC]=GSV6]GS15934 (Drosophila Genetic Resource Center, DGRC206424), c323-GAL4 (Brian Calvi), miR-318 control sensor (lab stock), UAS-miR-318 (BL41161), UAS-EcR\textsuperscript{DN} (BL9449), UAS-EGFR\textsuperscript{RNAi} (BL25781), UAS-dpp\textsuperscript{RNAi} (BL25782), UAS-EGFR\textsuperscript{DN} (JC. Pastor-Pareja), HsFLP; Act5C>y+>Gal4 UAS-GFP56T/(CyO) (JC. Pastor-Pareja), HsFlp; Act5C>y+>GAL4 UAS-myr.RFP/CyO (JC. Pastor-Pareja) and hsFlp; FRT82B arm-LacZ/TM3 Ser (P. Rørth).

The miR-318 genomic rescue construct was created by PCR amplification of genomic fragments containing the miR-318 region. The DNA was amplified in two fragments so that the miR-994 sequences could be left out. The fragments were then cloned into the site-specific integration vector pAttB. Primers for fragment 1 were 5'-CGTCTAGAAAAATCTATGTTGGTTCGATAC-3' with 5'-CGGCGGCCGCTAAATTCAGGACGCGATCGAAG-3', and for fragment 2 (with miR-318) were 5'-CGGCGGCCGCAAACAGCCTAGATCCGAATGCG-3' and 5'-CGGCGGCCGCTTGGGTGTACTTTGTTTTTCTG-3'.

To generate the miR-318 sensor construct, oligonucleotides containing two copies of the sequence complementary to miR-318 were annealed and cloned downstream of the EGFP coding region of Tub-EGFP in pCaSpeR4 (BRENNECKE et al.)
**Mutant generation**

A modified targeting vector was used to make a GFP knock-in allele by homologous recombination. An EGFP fragment cut from pEGFP-N1 was subcloned into pW25 to generate the targeting vector pW25-EGFP. Approximately 4 Kb fragments of genomic DNA flanking miR-318 were amplified and cloned into pW25-EGFP using the following primers:

5'-GCGGCCGCGAGAACAGATTCCAATTGACAT-3' and
5'-GCGGCCGCCACGCAAGGCACTCGGATACTC-3' for upstream flanking sequence,
and 5'-GGCGCGCCGGAAACCTTAAATCATACCAAT-3' and
5'-GGCGCGCCGTCAGGCAATGTCAAGTAGAAG-3' for downstream flanking sequence.

Targeting was performed as described in (Weng et al. 2009).

The miR-318\(^{\Delta 2}\) mutant was made by mobilization of P\{w[+mC]=GSV6\}GS15934, using standard procedures. The deletion removed a 1.8 kb genomic DNA (from Ch3R:6233008 to Ch3R:6234810), determined by sequencing the DNA fragment spanning the breakpoints.

**Clonal analysis**

We used FLP/FRT recombination technique to generate loss-of-function mutant clones in the follicle cells. miR-318\(^{\Delta 1}\) was first recombined onto a FRT82B chromosome. To induce miR-318 mutant clones, adult female flies with genotype
HsFLP/+; FRT82B miR-318Δ1/FRT82B arm-lacZ were heat shocked at 37 °C for 1 hour and then incubated at 25 °C for 3-4 days before ovary dissection. As miR-318Δ1 is a GFP knock-in allele, null mutant clones were marked by the presence of two copies of GFP and wild type twin clones were labeled by the loss of a GFP marker. We used the flip-out system to generate overexpression clones in the follicle cells. To induce ectopic expression of miR-318 or EcRDN, adult female flies with genotype HsFLP/++; Act5C>y+>Gal4 UAS-GFP.S56T/+; UAS-miR-318/+ or HsFLP/++; Act5C>y+>Gal4 UAS-myr.RFP /UAS-EcRDN were heat shocked at 37 °C for 1 hour and then incubated at 25 °C for 3-4 days before ovary dissection. Clones were marked by the presence of GFP or RFP.

**Immunocytochemistry, EdU labeling and microscopy**

For immunostaining, ovaries of 3-5 days old adult female flies were dissected in ice-cold PBS and fixed for 15 min in PBS containing 4% paraformaldehyde. Fixed ovaries were washed 3 times with PBS, 0.1 % Triton X100 (PBT) and blocked in PBT with 5% NGS for 1 hour at room temperature. Samples were incubated with primary antibodies overnight at 4 °C, then washed 3 times and incubated with secondary antibodies for 2 hours and DAPI for 20 mins at room temperature. Samples were washed 3 times and mounted in 1:1 PBS-Glycerol or Vectashield (Vector labs). Primary antibodies: chicken anti-GFP (1:2000, Abcam), rabbit anti-RFP (1:500, Abcam,ab62341), mouse anti-β-galactosidase (1:2000, Promega), mouse anti-Broad-core (25E9.D7; 1:100, Developmental Studies Hybridoma Bank,
DSHB, Iowa City, IA, USA), mouse anti-Cut (2B10; 1:15, DSHB), mouse anti-hnt (1G9; 1:15, DSHB), mouse anti-FasIII (7G10; 1:100, DSHB). anti-Ttk69 was raised in rabbit against a GST-Ttk69 fusion protein containing the C-terminal amino acids 475 to 641 of ttk69. Secondary antibodies were Alexa Fluor 488, 555 or 633-conjugated (1:250 or 1:500, Molecular Probes). DAPI (1 μg/ml, Sigma, St. Louis, MO, USA) was used to stain for nuclei. EdU staining was performed using the Click-iT EdU Alexa Fluor 555 Imaging Kit (C10338, Life technologies). Dissected ovaries were incubated for 1 hour at 25°C in Grace’s medium (Life technologies) containing 10 μm EdU and then fixed and immunostained as described above. Samples were washed for 20 mins in PBT and then incubated with EdU reaction cocktail for 30 min. Ovaries were then washed for 2 hours before adding the mounting medium. Optical sections were acquired using a Zeiss LSM510 confocal microscope at 1 μm intervals and converted into a single image using maximum intensity projection. For scanning electron microscope analysis, eggs were mounted directly and imaged with a JEOL JSM-6360LV scanning electron microscope.

Cell culture, transfection and luciferase assays

Transfections were performed with X-tremeGENE reagent according to the manufacturer's protocol (Roche). S2 cells were cultured in Schneider's medium supplemented with 10% FBS. 20-hydroxyecdysone (20E) (Enzo Life Sciences, ALX-370-012-M005) was added to the medium at a final concentration of 10×10⁻⁶
M at 6 hours after transfection. The luciferase reporter vector containing a wild type miR-318 regulatory sequence was prepared as the following. A 852-bp sequence upstream of the miR-994/318 stem loops was amplified and cloned into pGL3 using the following primers: CCGGTACAAAAATCTATGTTGGTTCGATACT and CGCTCGAGTAAATTCAGGACGCGATCGAAG. A mutated version that lacked the putative EcRE was cloned into the same vector by a two-step PCR method. Two additional primers used were

TGACCCACAAATACGCCCTGAATAGTCACGTTGTGGGATTC and CAGGGCGTATTTGTGGGTCA. Transfections were done in 24-well plates with 500 ng of firefly luciferase reporter plasmid and 50 ng of renilla luciferase plasmid as a control. Transfections were done with three technical replicates in four independent experiments. Firefly and Renilla luciferase were measured using the Dual luciferase assays kit protocol 48 hours after transfection.

**Quantitative real-time PCR for mature miRNAs**

Total RNA was isolated from 10 pairs of ovaries with TRIZol reagent (Invitrogen). Reverse transcription reactions were done on 10 ng of total RNA with miRNA-specific stem-loop RT primers. Real-time PCR was performed using the TaqMan miRNA assay kit protocol on an ABI7500 fast real-time PCR machine. miRNA levels were normalized to miR-184.
Figure legends

Figure 1: Generation and characterization of miR-318 mutant.

(A) Organization of the miR-318 locus. miR-318 is located on chromosome 3R at 86B4 on the cytogenetic map. Filled pentagons indicate the positions of miR-318 and miR-994. Open pentagons indicate the positions of adjacent predicted genes. Deletions of miR-318Δ1 and miR-318Δ2 mutant alleles are indicated by the bracketed area. miR-318Δ1 was generated by ends-out gene targeting. One hundred twenty six base pairs including miR-318 stem loop was replaced by the GFP and miniwhite genes. The integrated DNA is indicated below. miR-318Δ2 was produced by imprecise excision of P{w[+mC]=GSV6}GS15934 and the deletion spans 1.8 kb. The miR-318 genomic rescue transgene is shown at the bottom. The bracketed area indicates the removed DNA including miR-994.

(B) Mature miRNA for miR-318 is absent in miR-318Δ1 and miR-318Δ2 mutants. Quantitative RT-PCR analysis of mature miR-318 and miR-994 miRNA levels in RNAs extracted from ovaries of wild type control and two combinations of miR-318 mutant alleles and the miR-318 rescue transgenic flies. Δ1 denotes the miR-318Δ1 mutant allele. Δ1a and Δ1b are from two independent homologous recombination events. Δ2 denotes miR-318Δ2 mutant allele. 318R denotes the miR-318 genomic rescue transgene. miR-8 served as a control miRNA that is expressed in ovaries. miRNA expression levels were normalized to miR-184. Error bars represent standard deviation from three independent experiments.

(C) miR-318 maternal loss-of-function mutants produce morphologically
abnormal eggs. Shown are phase contrast images of dorsal views of eggs from wild type control (a), miR-318 mutant (b) and miR-318 rescue (c) female flies. All the genotypes are indicated.

(D) The hatching rate of eggs laid by miR-318 mutant females is reduced. Shown is the percentage of hatched eggs from female flies of indicated genotypes. Error bars represent standard deviation from three independent experiments. n>200 for each experiment. p<0.05.

(E) Eggs laid by miR-318 mutant females are fragile. Shown is the percentage of collapsed eggs from female flies of indicated genotypes. Error bars represent standard deviation from three independent experiments. n>200 for each experiment.

Figure 2: Expression of miR-318 during oogenesis.

(A-C'') miR-318 is expressed in the follicle cells in stage 9 (B and B') and stage 10 (C and C') egg chambers, but not in early stage egg chambers (A and A'). Shown are confocal images of egg chambers from miR-318<sup>Δ1</sup>/+ heterozygous females, stained with anti-GFP (Green) and DAPI (Blue). As GFP is integrated into the miR-318 locus in the miR-318<sup>Δ1</sup> allele, the pattern of GFP expression reflects miR-318 expression. Arrow in A indicates egg chambers at early stages.

(D-E') Mature miR-318 is expressed in the follicle cells in a stage 10 egg chamber. Shown are confocal images of egg chambers from wild type females expressing control sensor or miR-318 sensor transgenes, stained with anti-GFP (Green) and
DAPI (Blue). Note miR-318 sensor GFP expression got repressed in the follicle cells in a stage 10 egg chamber.

(F-G’) Repression of the miR-318 sensor GFP in the follicle cells is due to the presence of miR-318. Shown are confocal images of stage 10 egg chambers from wild type and miR-318\(^{-2}/Df\) mutant females expressing the miR-318 sensor transgene, stained with anti-GFP (Green) and DAPI (Blue). Df denotes the deficiency chromosome Df(3R)BSC479. Note the difference of GFP expression level in the follicle cells. Scale bars: 100 um.

**Figure 3: Loss of miR-318 causes defects in follicle cell patterning.**

(A-C'') The expression pattern of BR is abnormal in stage 10B egg chambers from miR-318 mutants. Shown are maximum intensity projections of Z-stack confocal images of stage 10B egg chambers from female flies of indicated genotype. Samples were stained with anti-BR (Red) and DAPI (Blue) to visualize the nuclei. Arrow indicates the dorsal anterior region. Merged images are shown in the right panels. Scale bar: 100 um.

**Figure 4: Removal of miR-318 impairs chorion gene amplification.**

(A-C'') The EdU incorporation pattern is abnormal in intact stage 10B egg chambers from miR-318 mutants. Shown are maximum intensity projections of confocal Z-stacks of stage 10B egg chambers from females of indicated genotypes. Samples were labeled with EdU (Red), anti-BR (Purple) and DAPI (Blue) to
visualize the nuclei. Gene amplification was assayed by incorporation of EdU, which labels sites of active DNA replication as subnuclear foci. In wild type stage 10B egg chambers, four to six EdU foci with different intensity and size were observed in each follicle cell nucleus. Synchronous EdU incorporation was seen. In contrast to this, miR-318 mutant egg chambers showed apparent defects in EdU incorporation. e.g. EdU incorporation throughout the nucleus (Arrowhead in B) and absent EdU incorporation (Arrow in B). Merged images are shown in the right panels. See Figure S6 for higher magnification. Scale Bar: 50 um.

(D-E’’) The EdU incorporation pattern is abnormal in miR-318 mutant clones of stage 10B egg chambers. Shown are maximum intensity projections of Z-stacks of confocal images of stage 10B egg chambers with miR-318 mutant clones in the follicle cells. miR-318 mutant clones were marked by two copies of GFP, and the lack of GFP indicated wild type clones. Dash lines marks the mutant clone areas. Samples were labeled with EdU (Red), anti-GFP (Green) and DAPI (Blue). Four mutant clones from two egg chambers are shown. Different classes of DNA replication patterns were seen according to the intensity and size of EdU foci: EdU incorporation throughout the nucleus (Arrowhead in D), reduced EdU incorporation (Dotted arrow in D) and absent EdU incorporation (Arrow in E). Star indicates a weaker diffuse Edu incorporation in the rest of nucleus. Scale Bar: 10 um.

(F) Quantitative analysis of EdU incorporation patterns. The level of EdU fluorescence intensity in each nucleus was measured with ImageJ. Comparison
was made for the follicle cells within the same egg chamber. Reduced EdU incorporation was defined as the EdU intensity in the mutant nucleus is less than 90% of the average level of wild type nuclei. n=167 nuclei from 10 separate egg chambers.

**Figure 5: miR-318 mutation impairs the developmental switch from endocycles to amplification.**

(A-B'') Hnt is upregulated and Cut is down-regulated in miR-318 mutant follicle cell clones in stage 10B egg chambers. Shown are maximum intensity projections of Z-stacks of confocal images of stage 10B egg chambers with miR-318 mutant clones in the follicle cells. Samples were stained with anti-Hnt (Grey in A; Purple in A''), anti-Cut (Grey in B; Purple in B''), anti-GFP (Green) and DAPI (Blue). Quantitative analysis is shown in the right panels (A''' and B''''). n=5 (in A'') and 8 (in B'''). p<0.05 for each comparison. Scale bars: 10 um.

**Figure 6: Overexpression of miR-318 does not cause premature gene amplification.**

(A-C'') Overexpression of miR-318 does not affect EdU incorporation, Hnt and Cut expression in the follicle cells in stage 9 egg chambers. Shown are maximum intensity projections of Z-stacks of confocal images of stage 9 egg chambers with miR-318 overexpression clones in the follicle cells. miR-318 overexpression clones were generated using Flip-out system and marked by the presence of GFP.
Samples were labeled with EdU (Red in A), anti-Hnt (Red in B), anti-Cut (Red in C), anti-GFP (Green) and DAPI (Blue). Scale bar: 50 um.

**Figure 7: EcR signaling is required for miR-318 expression.**

(A-B’’) miR-318 expression is absent in the follicle cells overexpressing EcR_DN. Shown are confocal images of stage 10B egg chambers from miR-318^1/+ heterozygous female flies with control or EcR_DN overexpression clones in the follicle cells. GFP expression reflects miR-318 expression in the miR-318^1 allele as described previously. Clones were marked by the presence of RFP. Samples were stained with anti-GFP (Green), anti-RFP (Red) and DAPI (Blue). Scale bar: 50 um.

(C) Predicted EcRE sequence in the miR-318 regulatory region is shown relative to EcRE consensus sequence. Mismatch is underlined. The position is shown relative to miR-318 stem loop region.

(D) Luciferase activity assays of wild type and mutated miR-318 regulatory region luciferase reporters upon 20E treatment in S2 cells. Fold changes were measured as luciferase activity of 20E-treated versus untreated samples. Control samples were co-transfected with the empty vector. Error bars represent standard deviation from four independent experiments. P<0.05.

**Figure 8: miR-318 interacts with ttk69 to promote gene amplification.**

(A) Removal of one copy of ttk69 enhances the morphologically abnormal egg phenotype in the miR-318 mutant background. Shown are phase contrast images
of dorsal views of eggs from ttk^{le11} heterozygous control (a), miR-318 mutant (b) and miR-318 mutant with one copy of ttk^{le11} (c) female flies.

(B) Removal of one copy of ttk69 increases the percentage of collapsed eggs in the miR-318 mutant background. Shown is the percentage of collapsed eggs from female flies of indicated genotypes. Error bars represent standard deviation from three independent experiments. n>200 for each experiment. p<0.05.

(C) Removal of one copy of ttk69 causes severely reduced hatching rate of eggs in the miR-318 mutant background. Shown is the percentage of hatched eggs from female flies of indicated genotypes. Error bars represent standard deviation from three independent experiments. n>200 for each experiment.

(D-G’) Removal of one copy of ttk69 enhances gene amplification defects in the miR-318 mutant background. Shown are maximum intensity projections of confocal Z-stacks of stage 10B egg chambers from females of indicated genotypes. Samples were labeled with EdU (Red) and DAPI (Blue). Scale bar: 50 um.

(H) Quantitative analysis of EdU intensity from D to G’. Three classes of egg chambers were observed according to the intensity of EdU foci: Class I (Normal), Class II (Moderate) and Class III (Severe). n=50 for w^{1118}, 53 for ttk^{le11}/+, 34 for Δ1/Δ2, 38 for Δ1 ttk^{le11}/Δ2.

(I-I’’) Ttk69 expression is not affected in miR-318 mutant follicle cells. Shown are maximum intensity projections of Z-stacks of confocal images of stage 10B egg chambers with miR-318 mutant clones in the follicle cells. Samples were stained with anti-Ttk69 (Purple), anti-GFP (Green) and DAPI (Blue). Quantitative analysis
is shown in the right panel (I''). n=6. Scale bar: 20 um.

(J) A diagram of Ttk69 and miR-318 regulation by the ecdysone signaling pathway during the Endocycle/Gene amplification switch.
Figure 1

A miR-318 locus

B

miR-318\^1

CG6325 miR-318

miR-318\^2

miR-318 Rescue (not including miR-994)

miR-318

miR-318 \land miR-994 \land miR-8

w1118 \Delta1/\Delta2 \Delta1,318R/\Delta2

D Hatching rate

E Percentage of eggs collapsed

w1118 \Delta1/\Delta2 \Delta1,318R/\Delta2
Figure 2

GFP

Before stage 9

Stage 9

Stage 10

miR-318 sensor

Control sensor

miR-318 sensor

+/

Δ2/Df

DAPI

miR-318-GFP

Control sensor

miR-318 sensor
Figure 3

BR | DAPI | Merge
---|------|------
A  | A'   | A''  |
B  | B'   | B''  |
C  | C'   | C''  |

w1118
Δ1/Δ2
Δ1,318R/Δ2
Figure 4

EdU | DAPI | BR | Merge
---|------|----|-----
A | A' | A'' | A'''
B | B' | B'' | B'''
C | C' | C'' | C'''

D | D' | D''
E | E' | E''

F

- Normal EdU incorporation
- Reduced EdU incorporation
- No EdU incorporation
- EdU incorporation throughout the nucleus
Figure 7

C

EcRE consensus sequence

AGGTCANTGACCC

G T A T

G T A T

-651/-639 TGTTCAATTGACCC

D

Fold changes

Control miR-318 wt miR-318 ΔEcRE

**
Figure 8

A

EdU

Merge

B

Percentage of eggs collapsed

C

Hatching rate

D

EdU

Merge

E

EdU

Merge

F

EdU

Merge

G

EdU

Merge

H

Class 1 (Normal)

Class 2 (Moderate)

Class 3 (Severe)

J

miR-318

Gene amplification

Ecdysone signaling pathway

Endocycle

Ctrl

Δ1/Δ1