The microRNA miR-7 regulates Tramtrack69 in a developmental switch in Drosophila follicle cells

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SUMMARY
Development in multicellular organisms includes both small incremental changes and major switches of cell differentiation and proliferation status. During Drosophila oogenesis, the follicular epithelial cells undergo two major developmental switches that cause global changes in the cell-cycle program. One, the switch from the endoreplication cycle to a gene-amplification phase, during which special genomic regions undergo repeated site-specific replication, is attributed to Notch downregulation, ecdysone signaling activation and upregulation of the zinc-finger protein Tramtrack69 (Ttk69). Here, we report that the microRNA miR-7 exerts an additional layer of regulation in this developmental switch by regulating Ttk69 transcripts. miR-7 recognizes the 3' UTR of ttk69 transcripts and regulates Ttk69 expression in a dose-dependent manner. Overexpression of miR-7 effectively blocks the switch from the endocycle to gene amplification through its regulation of ttk69. miR-7 and Ttk69 also coordinate other cell differentiation events, such as vitelline membrane protein expression, that lead to the formation of the mature egg. Our studies reveal the important role miR-7 plays in developmental decision-making in association with signal-transduction pathways.

KEY WORDS: MicroRNA, Follicular epithelium, Notch signaling, EcR signaling, Ttk69 (Ttk), VM32E, Gene amplification

INTRODUCTION
Precise control of DNA replication is crucial for genome stability, cell division and growth control. Most somatic cells replicate DNA once per cell cycle, to maintain the standard numbers of chromosomes and genes. In some cancerous and differentiated cells, however, this rule is broken and DNA is copied without cell division. The Drosophila egg chamber, the developmental unit of oogenesis, is an excellent model system in which to study how developmental signals control different DNA-replication and cell-cycle patterns (Dobens and Raftery, 2000; Klusza and Deng, 2011). During oogenesis, the somatically derived follicle cells undergo mitosis until stage 6 followed by three rounds of endocycling, during which they replicate their genomic contents without division. Finally, at stage 10B they switch into a phase known as gene amplification, during which specific genomic regions are selectively amplified (e.g. the chorion gene region); this stage is therefore referred to as the chorion gene-amplification or simply the amplification stage (Calvi et al., 1998; Cayirlioglu et al., 2001). This endocycle-to-amplification (E/A) switch, which is marked by a change from the oscillating genome-wide DNA replication pattern to a focused site-specific pattern, allows maximum production of materials required for eggshell synthesis during egg production and has served as a model system in which to determine how DNA replication origins are selected and fired (Calvi and Spradling, 1999; Tower, 2004; Claycomb and Orr-Weaver, 2005). Similar gene amplification has also been found in certain types of cancer cells; the genomic regions that contain the Myc gene and neighboring DNA sequence are selectively amplified (Collins and Groudine, 1982; Lee et al., 1984).

The E/A switch in main body follicle cells is triggered by two signaling events: downregulation of Notch signaling, which is normally active during endocycle stages; and activation of the hormonal ecdysone receptor (EcR) pathway. Both occur at oogenesis stage 10B and are necessary for upregulated expression of a zinc-finger protein Tramtrack69 kDa isoform (Ttk69; Ttk – FlyBase), which also has a role in the E/A switch (Jordan et al., 2006; Sun et al., 2008; Boyle and Berg, 2009). Other factors important for DNA replication and cell-cycle regulation are required for gene amplification, such as E2F, RBF, components of the origin recognition complex, Doubleparked and epigenetic regulators (Royzman et al., 1999; Cayirlioglu et al., 2001; Cayirlioglu et al., 2003).

Changes in the cell-cycle pattern in follicle cells are strictly temporally regulated. In addition to developmental signaling pathways, a class of small regulatory RNAs, the endogenously encoded microRNA (miRNA), has also been shown to be involved in temporal regulation of cell differentiation in development. The very first mutant-miRNA phenotype observed (lin-4 and let-7) was the heterochronic phenotype in the roundworm Caenorhabditis elegans (Reinhart et al., 2000; Grishok et al., 2001). The larval cells of these heterochronic mutants remain in the first-instar larval stage and do not progress to the second instar. Similarly, their homologs in Drosophila (miR-125 and let-7) are involved in temporal regulation of metamorphic processes (Caygill and Johnston, 2008; Sokol et al., 2008), suggesting a conserved role for these miRNAs in temporal regulation of development. Our recent study revealed the miRNA biogenesis pathway functions in the transition from the mitotic cycle to the endocycle in the follicle cells of the fly egg chamber (Poulton et al., 2011). Here, we report that miR-7 modulates the E/A switch by acting directly on Ttk69 transcripts.

MATERIALS AND METHODS
Fly stocks and genetic clones
The following alleles were used in this study: FRT42D miR-7\( ^{\Delta} \) (a gift from R. W. Carthew, Northwestern University, Evanston, IL, USA), a null allele generated by excision of the miR-7 sequence, last two exons of \( b^{l} \) and two exons of hillarin (Li and Carthew 2005), UAS-miR-7.140 (a gift from S. M. Cohen, Institute of Molecular and Cellular Biology, Singapore) (Stark et al., 2003), ttk69-RNAi (Vienna Drosophila RNAi Center), UAS-Ttk69

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RESULTS
Overexpression of miR-7 leads to a failure to switch to the gene amplification stage

Previously, we have reported that the miRNA biogenesis pathway, including Dicer-1 and Pasha, is involved in regulation of the temporal pattern of Notch signaling and the cell-cycle switches in the follicular epithelium (Poulton et al., 2011). To identify the miRNA(s) involved in this regulation, we looked at miR-7, which has been reported to regulate Notch and EGFR pathways in Drosophila imaginal discs and sensory organ precursors (Li et al., 2009). To determine whether miR-7 plays a role in regulating follicle cell differentiation, we overexpressed miR-7 (Stark et al., 2003) using the flip-out-GAL4 system (Pignoni and Zipsursky, 1997). The normal cell-fate changes that occur in follicle cells can be revealed by staining for two transcription factors, Cut and Hindsight (Hnt), which have complementary expression patterns in follicle cells during oogenesis (Sun and Deng, 2005; Sun and Deng, 2007). Normally, Cut is expressed in early (stages 1-6) and late oogenesis (stage 10B to later), whereas Hnt is expressed only in midoogenesis (stages 7-10A), when follicle cells are undergoing endoreplication (Fig. 1A,B). When antibody markers for Cut and Hnt expression were used, no defect was detected in miR-7-overexpressing cells during the switch from the mitotic cycle to the endocycle (stages 6/7 of oogenesis). Surprisingly, miR-7 overexpression caused defects in the expression patterns of both Cut and Hnt after stage 10A, i.e. prolonged Hnt expression beyond stage 10A (98%, n=257 clones), whereas wild-type cells had already lost Hnt expression (Fig. 1C) and failed to express Cut protein (87%, n=186) (Fig. 1D), which is normally upregulated in main-body follicle cells at stage 10B. Within these miR-7 overexpression clones, we noticed that Cut expression was not at the same level from cell to cell. This variegation of Cut expression was probably caused by the variegation of the bipartite GAL4/UAS system in the follicle cell lineage (Skora and Spradling, 2010). In cells with higher levels of Gal4-induced RFP and miR-7 expression, Cut expression generally appeared lower (Fig. 1D*). Together, these results indicate that follicle-cell differentiation is disrupted when miR-7 is overexpressed during late oogenesis.

During stage 10B, the changes in cell signaling activity and gene expression culminate in the E/A switch, during which specific genomic loci are replicated repeatedly. To determine whether miR-7 overexpression resulted in a disruption of this DNA-replication-pattern change, we used bromodeoxyuridine (BrdU), a synthetic thymine analog, labeling to determine whether DNA replication was affected. In stage 10A or younger follicle cells, BrdU labeling is detected in the entire nucleus in an oscillating pattern, demonstrating that the cells are still going through periods of genome-wide replication, during either mitosis or endoreplication. After the E/A switch, the BrdU pattern takes the form of distinct foci that label only specific chromosomal loci in the wild type. In miR-7 overexpressing cells, BrdU labeling the entire nucleus in an oscillating pattern (91%, n=178; Fig. 1E), whereas neighboring wild-type follicle cells presented the typical pattern of foci. Taken together, these results indicate that overexpressed miR-7 can cause defects in cell fate and the E/A switch in follicle cells.

miR-7 overexpression does not affect Notch activation or EcR signaling during the E/A switch
Notch signaling downregulation is necessary for follicle cells to leave the endocycle and begin synchronous gene amplification. Forced continued activation of Notch signaling in these cells results in a failure to undergo the E/A switch, in Hnt downregulation and in Cut upregulation (Sun et al., 2008). To determine how miR-7 regulates the E/A switch in follicle cells, we first asked whether the failure to undergo the E/A switch in miR-7-overexpressing follicle cells was caused by a disturbance in Notch signaling, i.e. whether miR-7 overexpression extended Notch activity. To this end, we examined the expression of the Notch activity reporter E(spl)-CD2, which contains the regulatory sequence of the E(spl) gene, a direct target of Notch intracellular domain (NICD) and its nuclear partner Suppressor of hairless [Su(H)], fused to the rat CD2 gene (de Celis et al., 1998). Normally, Notch is activated in stage 7-10A follicle cells, as...
indicated by increased CD2 staining. If overexpression of miR-7 disrupts the E/A switch by inhibiting the downregulation of Notch signaling at stage 10B, then we would predict that CD2 levels should remain high in those cells, but that result was not observed (91% showed normal levels of CD2 expression, n=301; Fig. 2A). In addition, the expression of two other Notch reporters, Gbe-lacZ (Furriols and Bray, 2001) and m7-lacZ (Assa-Kunik et al., 2007), remained unchanged in follicle cells with miR-7 overexpression (data not shown), indicating that miR-7 does not regulate Notch activity [NICD/Su(H) mediated activation] in follicle cells.

In addition to Notch, our previous findings have demonstrated that the EcR pathway also regulates the E/A switch (Sun et al., 2008). Downregulation of Notch signaling allows activation of the EcR pathway at stage 10B for the E/A switch (Sun et al., 2008). An accurate reporter of EcR activity in the follicle cells at this stage is hs-Gal4-EcR.LBD, UAS-nlacZ (Kozlova and Thummel, 2002), which has a Gal4 DNA-binding domain fused with the EcR ligand-binding domain. The presence of ecysone in the cell triggers the fused protein to activate UAS-lacZ expression. This reporter reveals uniform upregulation of lacZ expression at stage 10B in main-body follicle cells (Sun et al., 2008). Because this reporter also uses the Gal4/UAS system to monitor EcR activity, we generated a heat-shock-inducible construct, hs-miR-7, and made a transgenic fly line. Heat-shock-induced expression of miR-7 caused decreased levels of Cut in follicle cells (Fig. 2B), suggesting this transgene behaves in a similar way to the aforementioned UAS-miR-7 during the E/A switch. However, no significant difference in β-Galactosidase antibody staining was detected (Fig. 2B′), indicating normal EcR activity in these cells. To confirm this result, we also used an EcRE-lacZ reporter (BL 4516), which contains the ecdysone-responsive element upstream of the lacZ reporter gene. Its expression is normally detected in all follicle cells after stage 9. miR-7 overexpression did not change the pattern of this reporter either (data not shown). These results indicate that miR-7 does not appear able to regulate ecysone activity when overexpressed; the phenotypes associated with miR-7 overexpression in follicle cells are therefore probably not a result of altered EcR signaling.

miR-7 regulates Ttk69 expression dose dependently during the E/A switch

Because neither Notch nor EcR pathways appear to be the target of miR-7 in follicle cells during the E/A switch, we turned our attention...
Development 140 (4)

Fig. 2. miR-7 does not regulate Notch or ecdysone signaling during the E/A switch. (A–A″) Notch activation is marked with E(Spl)-CD2 expression. No increased CD2 staining (white in A and green in A″) was observed in stage 10B follicle cells with overexpressed miR-7 (red in A′ and A″). (A″) Intensity analysis representing the CD2 expression level of the blue-boxed area in A (clone cells marked with asterisks). (Plot was generated using ImageJ v1.46j interactive 3D surface plot.) miR-7 overexpression was induced by flip-out Gal4 driven UAS-miR-7, and marked with β-Galactosidase expression (red) (B–B″). Follicle cells with heat shock-induced overexpression of miR-7 (hs-miR-7) were revealed by Cut downregulation (white in B, outlined). In this stage 10B egg chamber, no clear difference was detected between miR-7-overexpressing cells and neighboring wild-type cells in ecdysone signaling activity, as revealed by β-Galactosidase staining (red in B′ and B″) of the hs-Gal4-ECD; UAS-LZ reporter. (C–C″) In a control experiment without hs-miR-7, the patched β-Galactosidase staining (red in C′ and C″) did not have any effect on the Cut expression (C). DAPI (blue) was used to mark cell nuclei. Posterior is towards the right. Scale bars: 10 µm.

to Ttk69, a zinc-finger protein isoform whose upregulation is required for the E/A switch (Sun et al., 2008). In normal developing egg chambers, Ttk69 is expressed in follicle cells at low levels, from the germarium until stage 10A (Fig. 3A); at stage 10B, in response to high levels of ecdysone signaling. Ttk69 levels sharply increase (Fig. 3A) (Sun et al., 2008). Manipulation of Ttk69 expression levels during the E/A switch also affects the expression of Cut and Hnt (Sun et al., 2008). To test the possibility that miR-7 affects the E/A switch by regulating Ttk69 levels, we measured Ttk69 expression in follicle cells overexpressing miR-7. In stage 10B cells overexpressing miR-7, Ttk69 expression was lower than in wild-type neighboring cells (87%, n=306; Fig. 3B), suggesting miR-7 can regulate Ttk69 expression levels during the E/A switch.

To determine whether miR-7 endogenously regulates Ttk69 expression in follicle cells, we performed a loss-of-function analysis using the FLP-FRT technique and a UAS-Ttk69 sensor line containing GFP expressed under the tubulin promoter followed by the 3′ UTR sequence of ttk69. In the wild-type background, the expression of this GFP sensor was hardly detectable. If the ttk69 3′UTR contains the miR-7 target site, then loss of miR-7 should lead to derepression of the ttk69 3′UTR sensor, and we should observe increased GFP signal (supplementary material Fig. S1). We therefore introduced this ttk69 3′UTR sensor line into the miR-7 loss-of-function background. Consistent with the hypothesis that ttk69 is a target of miR-7, we observed strong GFP signal in miR-7−/− follicle-cell clones, whereas wild-type neighbor cells lacked GFP expression (Fig. 4A). To verify that ttk69 is a direct target of miR-7, we also generated a mutated ttk69 3′UTR sensor with the miR-7 target sequence TCTTCCA mutated to TCTagCA (supplementary material Fig. S1). As expected, expressing this mutated ttk69 3′UTR sensor in the miR-7 loss-of-function background produced no difference in GFP expression in clone cells and wild-type neighbor cells (Fig. 4B). These data strongly suggest that miR-7 directly regulates Ttk69 expression through the single miR-7 target site at the ttk69 3′UTR.

To determine whether the regulation of Ttk69 levels is important to miR-7-induced phenotypes in follicle cells, we co-expressed miR-7 and Ttk69 in follicle cells and examined Cut and Hnt expression levels. To avoid repression of misexpressed Ttk69 by miR-7, we used a UAS-Ttk69 transgene constructed from ttk69 cDNA without the 3′UTR and thus lacking the miR-7 binding site. If miR-7 regulates the E/A switch by controlling ttk69 transcripts,
with extra Ttk69 present in miR-7-overexpressing follicle cells, we should not observe downregulated Cut and prolonged Hnt phenotypes. Indeed, we found that the Cut downregulation phenotype was alleviated (96%, n = 104; Fig. 4C), and in some cases we were able to find slightly increased Cut (Fig. 4C, arrows).

Similarly, the increased levels of Hnt we observed at stage 10B in response to miR-7 overexpression alone were no longer present when we co-expressed Ttk69 (95%, n = 228; Fig. 4D). In addition, the BrdU labeling showed the typical focal pattern in clone cells with co-expression of Ttk69 and miR-7 (89%, n = 107; Fig. 4E), indicating that these cells enter the gene amplification cycle similar to their wild-type neighbors.

Loss of miR-7 is not sufficient to activate premature E/A switch

Overexpression of miR-7 can disrupt the E/A switch, and expression of markers such as Hnt and Cut suggests that miR-7 plays a role in regulating these processes. Furthermore, we have demonstrated that miR-7 does repress Ttk69 (Fig. 3B), and increased Ttk69 levels are important in promoting the E/A transition (Sun et al., 2008). We therefore asked whether loss of miR-7 and Ttk69 are involved in these developmental changes in follicle cells that lead to egg maturation, and whether they act in ways similar to those used in their control of the E/A switch. To this end, we studied the expression of a vitelline membrane protein, VM32E (Bernardi et al., 2009), in egg chambers with genetic manipulations of miR-7 and/or ttk69. An antibody against VM32E revealed no expression in early oogenesis (Fig. 6A; supplementary material Fig. S2); starting at stage 9, low levels of VM32E was detected, mostly at the apical side of the follicle cells (Fig. 6A; supplementary material Fig. S2). At stages 10A and 10B, VM32E protein was readily detected in follicle cells and in the extracellular space between follicle cells and the oocyte (Fig. 6A; supplementary material Fig. S2). The distribution patterns on the apical and basal surfaces differ; on the apical side, VM32E shows a more...
continuous pattern, but on the basal side and the cytoplasm, it shows punctate staining (supplementary material Fig. S2). We did not observe VM32E expression in the anterior columnar follicle cells, the centripetal cells, or the posterior follicle cells (Fig. 6A).

To determine the relationship between miR-7 and VM32E expression, we examined the levels of VM32E protein in response to overexpression or deletion of miR-7. Overexpression of miR-7 resulted in loss of VM32E expression after stage 10 [Fig. 6B; supplementary material Fig. S3; 83% (n=97) during stages 9/10A and 91% (n=171) at stage 10B], whereas in mutant miR-7 clones, we found increased VM32E [Fig. 6C; supplementary material Fig. S4; 60% (n=143) during stages 9/10A and 36% (n=178) at stage 10B]. At the apical side, VM32E protein appeared to be able to diffuse to cover the adjacent wild-type cell (supplementary material Fig. S4, arrowhead). To determine whether miR-7 affected VM32E expression at the transcriptional or post-transcriptional level, we expressed a reporter of VM32E gene expression, -253/-39 lacZ (Bernardi et al., 2009), in egg chambers overexpressing miR-7. This transgenic line bears the -253/-39 region of the VM32E promoter and is fused to a lacZ reporter gene. The -253/-39 lacZ shows a patchy expression pattern in follicular epithelium. In cells overexpressing miR-7, we observed downregulation of β-Gal (data not shown). These results, together with the fact that no miR-7 target site was predicted at the 3′UTR of VM32E mRNA, indicate that miR-7 indirectly regulates VM32E expression in follicle cells.

Because the above results suggest that miR-7 affects both Ttk69 and VM32E expression, we asked whether Ttk69 has an effect on VM32E expression. Indeed, in follicle cells overexpressing Ttk69, we observed weak upregulation of VM32E (67.3%, n=447; Fig. 6D). Conversely, we found decreased VM32E both in clones

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**Fig. 4. Direct regulation of Ttk69 by miR-7.** (A-B″) miR-7<sup>Δ</sup> clone cells (absence of red, outlined). (A-A″) Strong GFP signal from the ttk69 3′UTR sensor line in clone cells; GFP was absent in wild-type neighbor cells. (B-B″) GFP from the mutated 3′UTR sensor line was expressed in both the miR-7<sup>Δ</sup> clone (outlined) and wild-type follicle cells. (C-C″) Overexpression of Ttk69 rescued the decreased Cut caused by overexpression of miR-7 (compare with Fig. 1D-D″), we occasionally observed higher Cut levels in these cells (arrows). (D-D″) Lack of prolonged Hnt staining in the co-expression of Ttk69 and miR-7 cells. (E-E″) Punctate BrdU labeling was restored when Ttk69 was co-expressed with miR-7 (outlined). (F) Quantification of extra Ttk69 expression needed to rescue the Hnt and Cut phenotypes in stage 10B cells with overexpressed miR-7. DAPI (blue) was used to mark cell nuclei. Posterior is towards the right. Scale bars: 10 µm.

**Fig. 5. Loss of miR-7 is not sufficient to activate premature E/A switch.** (A-A″) Oscillating genomic BrdU incorporation (arrows) can be observed in miR-7<sup>Δ</sup> follicle-cell clones (absence of red) and in wild-type cells. DAPI (blue) was used to mark cell nuclei. Posterior is towards the right. Scale bars: 10 µm.
in which Ttk69 was knocked down by RNAi (data not shown) and in mutant ttk69 clones (Fig. 6E; supplementary material Fig. S5). These results indicate that Ttk69 is necessary and sufficient to promote VM32E expression.

Because genetic manipulations of miR-7 or ttk69 resulted in altered levels of VM32E expression, we asked whether VM32E regulation by miR-7 is mediated by Ttk69. To examine this possibility, we used the mosaic analysis with a repressible cell marker (MARCM) technique to misexpress ttk-RNAi in miR-7Δ1 follicle cell clones (Lee and Luo, 2001). If miR-7 regulates VM32E levels by repressing Ttk69, then downregulation of Ttk69 in miR-7 mutant cells should lead to decreased VM32E, as we observed under ttk69 knockdown alone. Indeed, in miR-7 and ttk69 double loss-of-function cells generated by the MARCM technique, VM32E expression was suppressed (82.3%, n=138; Fig. 7A,B). These findings suggest that Ttk69 acts downstream of miR-7 to promote VM32E expression.

**DISCUSSION**

The follicular epithelium, with two clear global changes in cell-cycle programs and differentiation status, has served as an excellent model in the study of developmental switches (reviewed by Klusza and Deng, 2011). The rise and fall (activation and downregulation) of Notch signaling in the follicle cells have direct effects on the two developmental switches. miRNAs, however, play important modulatory roles in controlling the timing of these two switches (Poulton et al., 2011; this study), but clear differences exist. During the mitotic-to-endocycle switch, the miRNAs directly regulate the
timing of Notch activation by acting on cis-expressed Delta (Poulton et al., 2011). During the E/A switch, the miRNA miR-7, does not seem to have a direct role in regulating Notch activity; instead, it acts directly on the transcription factor Ttk69, which is also a target of Notch and EcR signaling in this process.

The phenotypes of loss- and gain-of-function of miR-7 during the E/A switch can be explained by its direct regulation of Ttk69. 

\[ \text{tkt69 loss-of-function (Sun et al., 2008) and miR-7 overexpression follicle cell clones both exhibit the following phenotypes: failure to switch from endocycling to gene amplification; continued Hnt expression; and lack of Cut upregulation. By contrast, loss-of \text{miR-7} \text{ (which induces Ttk69 upregulation) or forced overexpression of Ttk69 (Sun et al., 2008) could not shift the E/A switch earlier. Similarly, in the regulation of vitelline membrane synthesis, genetic epistatic analysis of miR-7 and the let-7 and miR-125 heterochronic microRNAs process in Drosophila by the let-7 and miR-125 heterochronic microRNAs.} \]

Intriguingly, the level of miR-7 expression in the ovary is low when compared with other miRNAs (supplementary material Fig. S6) suggesting a dose-dependent regulation by miR-7. We considered the possible role of Yan in follicle cells, but cloning analysis of Yan mutations did not generate any defect during the E/A switch (data not shown), suggesting Yan is not a relevant target in follicle cells. Therefore, the miR-7 function depends strongly on the cellular context in which it is expressed. Intriguingly, the level of miR-7 expression in the ovary is low when compared with other miRNAs (supplementary material Fig. S6) (Berezikov et al., 2011). When a miR-7 reporter was used to examine its expression in follicle cells (Stark et al., 2003), no obvious signal was detected (data not shown). As such a low level of miR-7 can modulate important developmental processes in follicle cells, the efficiency of miRNA use must be very high.

miRNAs have been found to be involved in important aspects of animal and plant development (Bushati and Cohen, 2007; Carlsbecker et al., 2010), as well as in a variety of human diseases, including cancer and cardiovascular diseases (Stefani, 2007; Liu and Olson, 2010). Many of their targets are transcription factors, which themselves have a broad range of target genes; this type of regulation allows miRNAs to have a more global effect in influencing cell-fate changes and other cellular events. Our studies of the involvement of miRNAs in temporal regulation in follicle cell development reveals the collaborative effect of miRNAs, signaling pathways and transcription factors in developmental decision making. In this system, transcription factor Ttk69 emerges as a common target and plays a crucial role in coordinating the effects of the miRNA and signaling pathways.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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