

Redundant and incoherent regulations of multiple phenotypes suggest microRNAs' role in stability control

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Each microRNA (miRNA) represses a web of target genes and, through them, controls multiple phenotypes. The difficulties inherent in such controls cast doubt on how effective miRNAs are in driving phenotypic changes. A “simple regulation” model posits “one target–one phenotype” control under which most targeting is nonfunctional. In an alternative “coordinate regulation” model, multiple targets are assumed to control the same phenotypes coherently, and most targeting is functional. Both models have some empirical support but pose different conceptual challenges. Here, we concurrently analyze multiple targets and phenotypes associated with the miRNA-310 family (miR310s) of *Drosophila*. Phenotypic rescue in the *mir310s* knockout background is achieved by promoter-directed RNA interference that restores wild-type expression. For one phenotype (eggshell morphology), we observed redundant regulation, hence rejecting “simple regulation” in favor of the “coordinate regulation” model. For other phenotypes (egg-hatching and male fertility), however, one gene shows full rescue, but three other rescues aggravate the phenotype. Overall, phenotypic controls by miR310s do not support either model. Like a thermostat that controls both heating and cooling elements to regulate temperature, redundancy and incoherence in regulation generally suggest some capacity in stability control. Our results therefore support the published view that miRNAs play a role in the canalization of transcriptome and, hence, phenotypes.

[Supplemental material is available for this article.]

MicroRNAs (miRNAs) are a class of small RNAs, 22 nt long, that broadly and weakly repress gene expression in animals (Lai 2003; Bartel 2004; Bartel 2009). They are very abundant, are often highly conserved, and, unlike other regulatory genes such as transcription factors (TFs), are exclusive down-regulators of their direct targets (Chen and Rajewsky 2007; Hobert 2008; Martinez and Walhout 2009). The repression by miRNAs is puzzling as each miRNA targets a large number of genes, and naturally, the mean repression is weak. Even highly expressed miRNAs often repress many of their targets by <50% (Baek et al. 2008; Selbach et al. 2008; Eichhorn et al. 2014). The puzzle is further compounded by the existence of many miRNAs expressed at low levels.

The puzzle of broad and weak target repression suggests to many investigators that miRNA targeting may often be biologically insignificant. An early model invokes “simple regulation,” which posits “one target gene–one phenotype” control. Despite subsequent challenges (e.g., Hunter et al. 2013), this simple model continues to receive recent support (Flynt and Lai 2008; Ecsedi et al. 2015; Pinzon et al. 2017). As illustrated in Figure 1A, a miRNA (miR-X) down-regulates many target genes (Y_1, Y_2, \dots, Y_n), and the down-regulation leads to changes in a few phenotypes (Z_1, Z_2 , etc). In this view, a major gene Y_1 is principally responsible for the phenotype Z_1 (Grosshans et al. 2005; Iovino et al. 2009; Bejarano et al. 2010; Smibert and Lai 2010; Ecsedi et al. 2015), and another target gene Y_2 may be responsible for a phenotype Z_2 . Overall, for any miRNA, only a few targets are biologically rel-

evant, and most others are noises (Seitz 2009; Pinzon et al. 2017). The view is tantamount to suggesting that >90% of the total repression capacity of the entire miRNA repertoire is noise (see Discussion).

In an alternative “coordinated regulation” hypothesis, multiple targets may be deployed to control the same phenotype in a coordinated manner (Subramanyam et al. 2011; Lai 2015; Cicek et al. 2016; Mahmoudi and Cairns 2017). In Figure 1B, targeting via both Y_1 and Y_2 enhances phenotype Z_1 , while Y_2, Y_3 , and Y_n targeting reduces phenotype Z_2 . These actions may be additive but could also be redundant if each target by itself can fully rescue the phenotype. Redundant control has been suggested to be central to miRNA function (Tan et al. 2012; Lee and Hyun 2014) and could account for the large number of target genes. Such a control circuit, however, could be difficult to assemble since all target genes are required to influence the phenotypes in the same direction.

While many studies have supported the “simple regulation” hypothesis by finding a single gene capable of rescuing the phenotype of interest (Karres et al. 2007; Hyun et al. 2009), few have ruled out redundancy, by which other target genes may also regulate the same phenotype to a similar extent. Curiously, in studies that deal with the same miRNA (Let-7) and the same phenotype (vulva rupture), opposite conclusions were reached (Hunter et al. 2013; Ecsedi et al. 2015). To rigorously test the models, it will be

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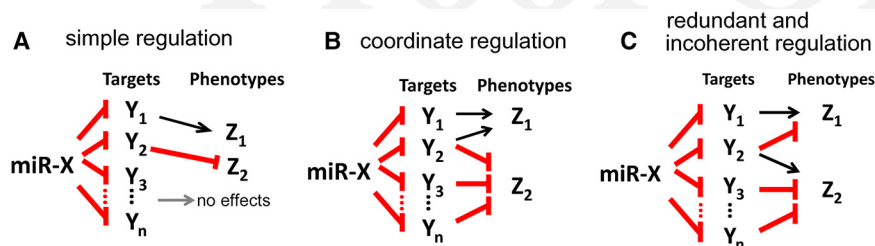


Figure 1. Models of phenotypic regulation by miRNA. (A) “Simple regulation”: A miRNA represses one target gene to control a phenotype. Different targets are responsible for different phenotypes. Red (blunt-headed) arrow indicates repression. (B) “Coordinated regulation”: A miRNA represses multiple targets, which in turn govern the same phenotype in the same direction. Pleiotropy, where a single target (e.g., Y₂) influences more than one phenotype, is common. (C) In the “redundant and incoherent regulation” model, a miRNA affects each phenotype through multiple targets redundantly and often incoherently. Pleiotropy is also common.

necessary to analyze each target gene individually and restore each expression to the wild-type level. Several phenotypes should also be examined simultaneously. Here, we study the miRNA-310 cluster (miR310s) of *Drosophila*. Because the four members of F2 miR310s share the same seed (see Fig. 2A; Supplemental Fig. S1; Supplemental Text S4) and are clustered, they are often studied as a single functional unit (Tang et al. 2010; Tsurudome et al. 2010; Pancratov et al. 2013; Cicek et al. 2016). Cross-species rescues using *Drosophila pseudoobscura* miR310s are also carried out to probe functional conservation.

Results

The criteria for identifying miRNA targets responsible for a phenotype are as follows (see Fig. 1A,B): First, the phenotype Z is associated with the deletion of miRNA-X. Second, a set of target genes, including gene Y, is shown to be up-regulated in the deletion background. Third, restoring the expression of Y to its wild-type pattern results in the rescue of phenotype Z in the miR-X deletion background. If the phenotypic rescue is complete, gene Y is often declared the major gene for the control of Z. Strictly speaking, it is necessary to rule out other genes having a comparable phenotypic effect, but this is rarely done (see Discussion).

Defects in egg morphology, hatchability, and male fertility

The *mir310s* deletions were generated in *Drosophila melanogaster* in our earlier study (Tang et al. 2010). Quantitative real-time PCR (qRT-PCR) measurements show that the knockout lines (*mir310s*⁻) lack all *mir310s* expression (Fig. 2B; Supplemental Fig. S3). Since miR310s are most highly expressed in the ovaries and testes (Bejarano et al. 2012; Marco 2014), eggs and sperm are the tissues we focused on in this study.

First, we note that the dorsal appendages attached to the egg-shell for respiration (Suzanne et al. 2001; Berg 2008) are defective in the *mir310s* deletion background. The total length of dorsal appendages is reduced by ~10% in the eggs of *mir310s*⁻ flies (Fig. 3A, B). The fraction of the total length occupied by the paddle region is 43.2 ± 0.4% in the control and 38.8 ± 0.5% in the *mir310s*⁻ eggs (Fig. 3C). Second, we measured egg hatchability, which is ~32% in *mir310s*⁻ eggs but 81.6% in the control (Fig. 3D). The deformity in the dorsal appendage appears to reflect functional differences inside the eggs.

Third, for male reproduction, since there is no visible morphological defect, male fertility was measured at two levels of

sperm exhaustion by mating males to two batches of females in succession. Since sperm is stored in seminal vesicles, males with a reduced capacity in sperm making would have a lower fertility count only under sperm-depletion conditions (Wu 1983). In *mir310s*⁻ males, the progeny count was reduced by half in the first round (78 progeny per male vs. 36) and by 87% in the second (30 vs. four) (Fig. 3E).

We then reintroduced *mir310s* into the *mir310s*⁻ background to rescue the phenotypes. The construct (Dm310s, for *mir310s* of *D. melanogaster*) has been described earlier (Tang et al. 2010). The transgenic line expresses all members of

miR310s at a level close to the wild type (Fig. 2B; Supplemental Fig. S3) and exhibits full phenotypic rescue (Fig. 3C–E). These observations confirm that the observed phenotypes are due to the *mir310s* knockout.

Target identification and the selection of five genes: *DI*, *E2f2*, *Ecr*, *Mad*, and *Mef2*

We next aimed to identify target genes through which miR310s exert their phenotypic effects. We predicted miR310s targets using TargetScan, PicTar, and PITA (Grun et al. 2005; Lewis et al. 2005; Kertesz et al. 2007; Kheradpour et al. 2007; Ruby et al. 2007) and selected 47 potential targets for qRT-PCR validation (for details, see Methods and Supplemental Fig. S4). These genes were chosen in consideration of their possible roles in dorsal appendage formation, follicle cell development, and male fertility. In comparison with the control flies, most of the 47 predicted targets are up-regulated during early and late oogenesis in *mir310s*⁻ females (see Supplemental Fig. S5). We further selected candidate target genes for in vitro validation (for details, see Supplement Text S1). Five genes—*DI*, *E2f2*, *Ecr*, *Mad*, and *Mef2*—were chosen for their possible functional involvement (see Methods; Supplemental Text S1), but they merely represent a small sample of the total target pool.

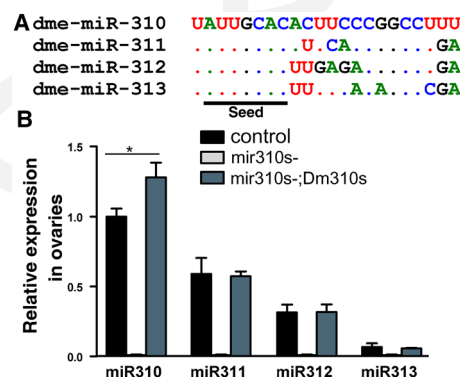


Figure 2. Sequence and expression of miR310s. (A) Mature sequences of the miR310 cluster in *Drosophila melanogaster* (Dm310s). The four members share a conserved seed (underlined). (B) Relative expression of miR310s in ovaries. *mir310s*⁻ denotes the knockout line in which no expression is detectable. The miR310s cluster is reintroduced in the *mir310s*⁻;UAS-Dm310s line in which miR310s is driven by the UAS element. Assays were taken by qRT-PCR in triplicate using 2S RNA as the endogenous control. Error bars, SEs by Student's *t*-test. (*) *P* < 0.05.

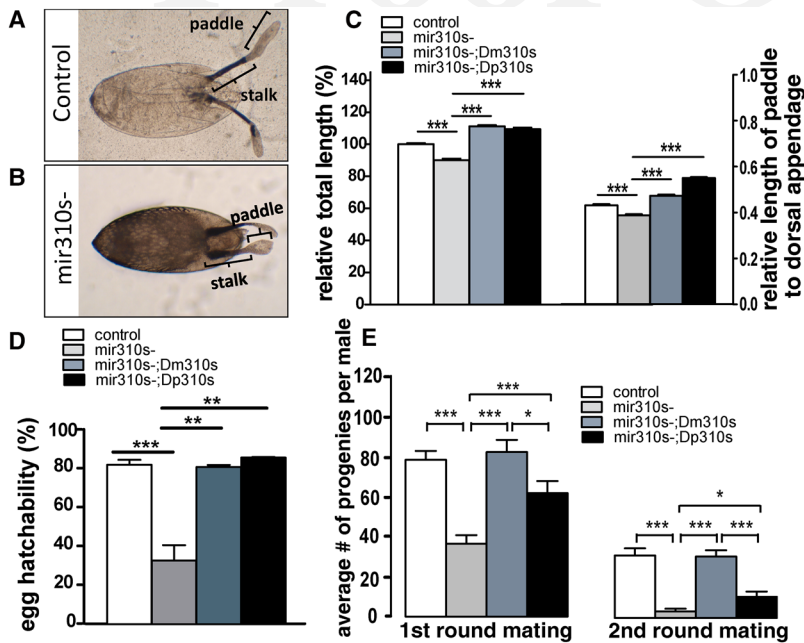


Figure 3. Three phenotypic defects in the *mir310s* flies rescued by *Dm310s* or *Dp310s*. The reintroduced *mir310s* of either *D. melanogaster* (*Dm310s*) or *D. pseudoobscura* (*Dp310s*) are used. (A,B) The dorsal appendage of eggshell consists of a stalk and a paddle. Note that the paddle of the *mir310s* egg is flattened and shortened. (C) Loss of *mir310s* significantly shortened the dorsal appendage (left), especially the paddle (right). Either *Dm310s* or *Dp310s* rescued the phenotypes (more than 200 eggs examined for each genotype). (D) Loss of *mir310s* significantly decreased egg hatchability, which could also be fully rescued by *Dm310s* or *Dp310s*. (Five replicates, each with more than 100 eggs for each genotype.) (E) Average number of progeny produced by each male in two consecutive mating. Mean progeny count of *mir310s* males was reduced by >50% in the first round of mating and 87% in the second round. *Dm310s* fully restored the fertility, but *Dp310s* rescue is partial. More than 15 males were individually tested for each genotype; error bars, SEs. (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ by Student's *t*-test.

All these genes contain conserved *mir310s* binding sites in their 3' UTRs (Fig. 4A). They are overexpressed in *mir310s* flies and are repressed in the [*mir310s*; *Dm310s*] and [*mir310s*; *Dp310s*] lines (Fig. 4B).

To stringently confirm the expected repression by *mir310s*, we cloned sequences with either the wild-type *mir310s* target site or the mutated site from each of the five genes (see Methods; Fig. 4A). Each of the cloned sequences carrying a luciferase reporter was then transfected into *Drosophila* S2 cells for the reporter assay (see Methods). The comparisons of luciferase activity in transfected cells are given in Figure 4C. Reporter activity is higher in cells with mutated target sites for each of the five genes, confirming the direct repression by *mir310s* through the target sites shown in Figure 4A.

Restoring wild-type expression by promoter-driven RNAi

Expression of target genes increases in *mir310s* knockout lines. As noted before, the restoration of the expression of a major target gene to its wild-type pattern should fully rescue the phenotype. We first established a *Gal4* driver under the control of the *mir310s* promoter. This *Gal4* construct drives UAS-RNAi in the same tissues as the native *mir310s*. We then use the *Gal4*/UAS-RNAi construct to repress each of the five verified target genes in *mir310s* flies. Measured by qRT-PCR, all five genes were significantly repressed by RNAi in ovaries (Fig. 5A).

To see whether expression of these target genes has been restored to the wild-type level, we combine expression increase

two target genes as shown in Table 1B, the comparison should be done in the knockout background. In reference to Table 1, A and B, it is likely that one approach may reveal more genes for the same phenotype than the other, and the two sets of targets may not overlap. Since strong epistasis requiring mutations at

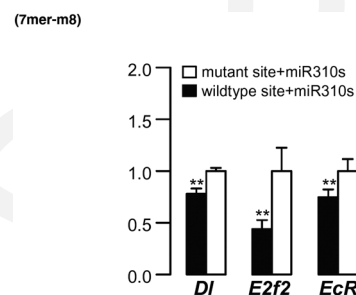


Figure 4. Validation of direct targets of *mir310s* by in vitro mutagenesis. (A) Putative target sites of *mir310s* on the 3' UTRs of *Dl*, *E2f2*, *EcR*, *Mad*, and *Mef2*. *mir310s* seed region, target sites, and mutant sites were shaded in red. The mutant site of each gene contained altered base pairs, which disrupt pairing with *mir310s*. The 3' UTR of *Mef2* has two target sites, both of which were mutated. (B) The expressions of the five target genes are repressed in *mir310s* flies (in ovaries of 3-d-old females) and repressed again by either *Dm310s* or *Dp310s*. (Relative expression were measured by qRT-PCR in triplicate, using *rp49* as endogenous control.) (C) The luciferase reporter with wild-type *mir310s* target sites is significantly inhibited by the cotransfected *mir310s* vis-à-vis those carrying mutated target sites. Five replicates in *Drosophila* S2 cells. (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ by Student's *t*-test.

due to *mir310s* deletion and its decrease due to RNAi in ovaries. As summarized in Figure 5B, each target gene is up-regulated by a percentage in *mir310s* ovaries (x ; second column), and then down-regulated by a percentage by RNAi (y ; third column). The restored value relative to the wild type would be $(1 + x)(1 - y)$, as shown in the last column. The observations fall in the range of 83%–122% for the five genes. The expression rescue is then used for evaluating the phenotypic effect of each target gene.

Phenotypic effects of individual gene expression rescue

With the expression rescue confirmed, we can now address miRNA effects on phenotypes. We follow the common approach, which rescues the phenotypes in the miRNA knockout background (e.g., Hunter et al. 2013). Another approach is to carry out the rescue in the wild-type background (e.g., Ecsedi et al. 2015). The appropriate strategy depends on the genetic interactions underlying the phenotypes. If knocking out a miRNA would result in a phenotype through two target genes independently as shown in Table 1A, then the basis of comparison should be the wild type. On the other hand, if the knockout phenotype requires the epistatic actions of

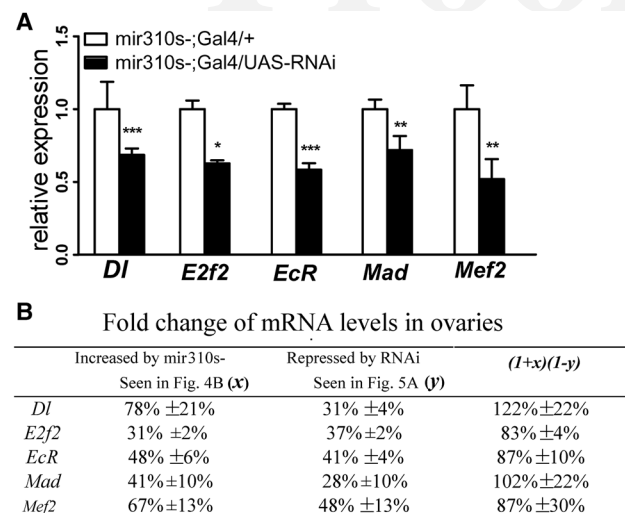


Figure 5. Restoration of target gene expression by RNAi in the *mir310s*-line. (A) Relative expression of the five targets is measured by qRT-PCR in the ovaries of 3-d-old females. In *Gal4/UAS-RNAi* flies, genes that are repressed in the *mir310s*-line are repressed by RNAi. Assays were taken in triplicate. (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ by Student's *t*-test. (B) The restoration of target gene expression in the *mir310s*-line by RNAi. The second column shows the up-regulation of the five targets due to *mir310s* knockout, and the third column shows the down-regulation due to RNAi of individual targets. The last column gives the restored level relative to the wild-type expression. Note that the tissue distribution of RNAi is under the control of a *Gal4*, which is itself driven by the *mir310s* promoter.

multiple loci to yield a phenotype is common in evolution (Palopoli and Wu 1994; Sun et al. 2004), the approach based on Table 1B is appropriate. The considerations above also dictate the methodology suitable for testing the models of Figure 1. In Supplemental Text S3, a detailed discussion of competing methods is presented.

F6 The phenotypic rescues are shown in Figure 6, A through C, where red lines indicate the *mir310s*- phenotype (0% rescue) and black lines reflect the wild-type values, or 100% rescue. For the dorsal appendage, RNAi experiments show strong rescue by restoring expression of four of the five genes (Fig. 6A). Dorsal appendage length is reduced by 10% in *mir310s*-lines. *Dl* and *Mef2* RNAi's rescue the phenotypes by 15% and 12%, respectively. Similarly, *E2f2* and *EcR* rescue the phenotype by ~7%. Hence, all four genes can be considered "major genes" with 0.7-, 1.2-, and 1.5-fold rescue ($P < 10^{-3}$ for all; for detail, see legends), and *mir310s* regulate the dorsal appendage through several target genes redundantly (Fig. 6A; Supplemental Fig. S7).

Incoherent control of egg hatchability and male fertility

Mad RNAi could restore the hatchability defect of *mir310s* knockout to the wild-type level, suggesting that this is the single major gene in the conventional sense. In addition, *Dl* RNAi increases hatchability by 30%, but the variability is too large for the result to be statistically significant (Fig. 6B). The remaining three genes are intriguing. RNAi experiments not only fail to show rescue but in fact aggravate the phenotype below the red dashed line by 58% (*E2f2*, $P = 0.007$), 57% (*EcR*, $P = 0.0082$), and 69% (*Mef2*, $P = 0.0032$), respectively. We note that the dorsal appendages and hatchability both pertain to the properties of eggs but have divergent genetic interactions.

The control of male fertility by *mir310s* is similar to that of egg hatchability. *Mad* RNAi males produced 75.2 progeny (Fig. 6C), very close to the number of wild-type males (78.0) (Fig. 3E). Since another *mir310s* target, *Arm* (*armadillo*), has been shown to be a major gene in previous studies (Pancratov et al. 2013), there is redundancy for male fertility. The reverse rescue observed in Figure 6B is frequently observed in Figure 6C with the exception of *Dl* RNAi, which goes in the opposite direction for the two phenotypes (although the differences are not statistically insignificant in either direction). RNAi experiments aggravate the phenotype by 55% (*E2f2*, $P = 0.006$), 38% (*EcR*, $P = 0.05$), and 58% (*Mef2*, $P = 0.03$), respectively.

Discussion

Many previous studies supporting the "simple regulation" model of Figure 1A reach the conclusion by identifying a target gene with near 100% rescue (Karres et al. 2007; Varghese and Cohen 2007; Hyun et al. 2009; Luo and Sehgal 2012). Most did not test other genes as redundancy is assumed to be absent. The issue of redundant control is unsettled even for the same miRNA (*let-7*) and the same phenotype (vulva rupture) in the same species (*C. elegans*) (Hunter et al. 2013; Ecsedi et al. 2015; Rausch et al. 2015). Hunter et al. (2013) concluded that a large number of target genes (more than 20) participate in vulva regulation using RNAi rescue for a large panel of target genes. In contrast, Ecsedi et al. (2015) concluded that LIN-41 is the sole target of *let-7* driving the vulval rupture phenotype. The stark contradiction is usually explained in technical terms, which are summarized in the Supplement Text S2. Briefly, the RNAi experiments (Hunter et al. 2013) may not have restored wild-type expression, and the "double mutant" assay (Ecsedi et al. 2015) effectively introduced an artificial miRNA for rescue. Either approach might be interpreted to be flawed, but as noted in Table 1, A and B, they may both be correct. Rescue experiments in the wild type (Ecsedi et al. 2015) versus miRNA knockout background (Hunter et al. 2013) may yield complementary, rather than overlapping, results.

To our knowledge, ours may be the first study that restores the expression of each of many target genes to (near) wild-type patterns. Every miRNA phenotype analyzed in this study is indeed governed by multiple target genes, thus supporting the conclusions of previous reports (Grosshans et al. 2005; Subramanyam et al. 2011; Hunter et al. 2013; Lee and Hyun 2014). The claim of

Table 1. Modes of target gene interactions

Target expression	Y ₁	y ₁
(A) Independent control of mutant phenotype		
Y ₂	Normal	Defects
y ₂	Defects	Defects
(B) Epistatic control of mutant phenotype		
Y ₂	Normal	Normal
y ₂	Normal	Defects

In both A and B, miRNA target genes Y₁ and Y₂ are under the control of the same miRNA as shown in Figure 1. Y₁ and Y₂ (in red) represent the wild-type expression repressed by miR-X, while y₁ and y₂ (in black) represent the elevated expression when miR-X is absent. In A, either y₁ or y₂ can yield the mutant phenotype, whereas in B, both y₁ and y₂ are needed. Therefore, the detection of the underlying genes would depend on the mode of action. In order to see the phenotypic difference (normal vs. defect), experiments are done in the background of either wild type (A) or miR-X knockout (B).

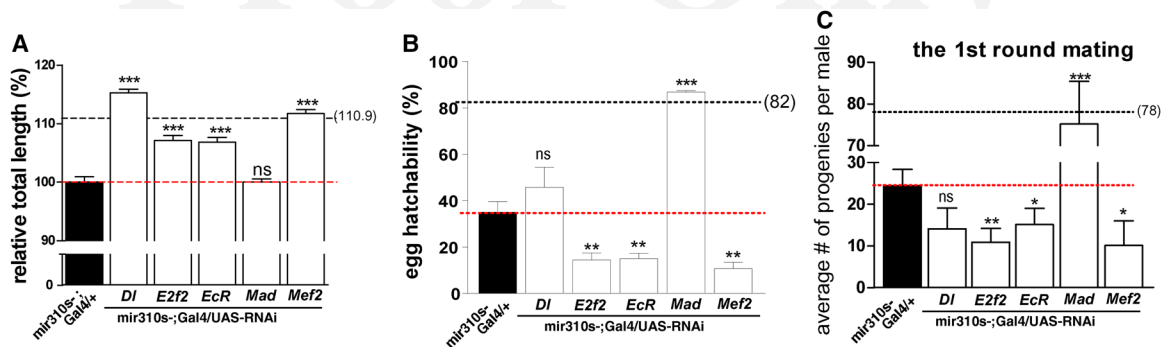


Figure 6. Phenotypic rescue by restoring the expression of individual target genes. The red dotted line, indicating the phenotypic value of *mir310s*-flies, demarcates positive and negative rescue. The black line indicates the wild-type value, hence denoting full rescue. (A) Dorsal appendages: Four genes with their expression restored by RNAi rescue the phenotypic defect. More than 200 eggs were examined for each genotype. (B) Egg hatchability: Only one gene (*Mad*) shows full rescue, and a second one (*DI*) shows partial but insignificant rescue. The remaining three genes (*E2f2*, *EcR*, and *Mef2*) show negative rescue. (C) Male fertility (progeny count for males in the first round of mating). Only one gene (*Mad*) shows full rescue. The rest show negative rescue, which is statistically significant for three genes (*E2f2*, *EcR*, and *Mef2*) and insignificant for one (*DI*). More than 15 individual males were tested for each genotype. (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ by Student's *t*-test.

the “simple regulation” hypothesis that miRNAs in animals, like those in plants, have few target genes connected to phenotypes is unsupported by these studies. Simple regulation essentially means that the bulk, rather than a small fraction, of miRNA repressions are biologically irrelevant (Pinzon et al. 2017).

The question is then whether miRNAs evolve gene targeting that coherently governs the phenotypes. In this study, the control is incoherent for two of the three phenotypes (Fig. 6B,C; Supplemental Fig. S8). Since only five target genes are analyzed, all phenotypes could be incoherently regulated if more target genes were assayed. Such incoherent control is also evident in the results presented by Hunter et al. (their Fig. 4). The pleiotropic effects of target genes further complicate the task of coherent control. For example, RNAis of *E2f2*, *EcR*, and *Mef2* rescue dorsal appendages positively but rescue egg hatchability negatively (see Table 2), even though both phenotypes pertain to female fertility.

With redundancy and, especially, incoherence, *mir310s*' actions in driving phenotypic changes seem to be self-canceling (Figs. 1C, 6). Available data from other miRNAs suggest that this conclusion may be general (Subramanyam et al. 2011; Hunter et al. 2013). When a regulator controls the output via two mutually opposing pathways, as in an incoherent feed-forward loop (Hornstein and Shomron 2006; Wu et al. 2009), the control is often indicative of stability and homeostasis (von Dassow et al. 2000; Tsang et al. 2007; Li et al. 2009; Wu et al. 2009). Regulation of the stabilizing kind is further interpreted to manifest stasis in phenotypes. Waddington (1942) first proposed the existence of mechanisms that “canalize” development (Waddington 1942), which are compared with water traveling in defined canals. The molecular basis of canalization has been suggested to be heat shock proteins (Rutherford and Lindquist 1998) and miRNAs, respectively, at the level of proteins and transcripts. The literature on miRNAs' possible roles in canalization has been quite extensive (Hornstein and Shomron 2006; Martinez et al. 2008; Li et al. 2009; Peterson et al. 2009; Wu et al. 2009; Ebert and Sharp 2012; Gursky et al. 2012; Cassidy et al. 2013; Posadas and Carthew 2014).

The view of miRNAs being the elusive canalizing agent is generally based on the analysis of motifs with two to four nodes (Hornstein and Shomron 2006; Wu et al. 2009). Recently, the analysis of small motifs has been extended to larger gene regulatory networks (GRNs). An interesting and intuitive model is the network of RNA cross-talks (Salmena et al. 2011; Ebert and Sharp

2012; Tay et al. 2014; Thomson and Dinger 2016). In a companion study (Chen et al. 2017), we apply the May-Wigner theory of network stability (May 1972, 2001; Wu et al. 2009; Allesina and Tang 2012; Tang et al. 2014), commonly used in the study of foodweb stability, to GRN stability.

Either at the motif or network level, the canalization view is about miRNAs' ability to stabilize the output. In this capacity, miRNAs' function depends on the connectivity and distribution of interaction strength among nodes. For that reason, the edges (i.e., connections between nodes) may often be exchangeable in evolution (Wu et al. 2009). Indeed, when *Dm310s* and *Dp310s* are inserted into *D. melanogaster*, 40% of their targets do not overlap (Tang et al. 2010). Nevertheless, the observed phenotypic rescues by *Dp310s* are comparable with those by *Dm310s* (Fig. 3C–E), suggesting a level of functional conservation associated with substantial divergence in targeting.

In conclusion, miRNAs often part of motifs such as incoherent feed-forward loops that stabilize expression output (Tsang et al. 2007; Osella et al. 2011). The observations on hatchability and male fertility (Fig. 6B,C; for summary, see Table 2) support a generalized model of incoherent feed-forward (Fig. 1C). Furthermore, miRNAs as a whole are abundant and stable relative to their target mRNAs (Miska et al. 2007; Alvarez-Saavedra and Horvitz 2010; Song et al. 2014; Sun et al. 2015). All these characteristics suggest a role in maintaining homeostasis (Wu et al. 2009; Herranz and Cohen 2010; Vidigal and Ventura 2015).

Table 2. Summary of phenotypic rescue by *Dm310s*, *Dp310s* and RNAi's

Genotype	Dorsal appendage	Egg hatchability	Male fertility
Dm310s	+	+	+
Dp310s	+	+	+
DI-RNAi	+	0(+)	0(–)
E2f2-RNAi	+	–	–
EcR-RNAi	+	–	–
Mad-RNAi	0	+	+
Mef2-RNAi	+	–	–

(+) Full phenotypic rescue ($P < 0.05$); (–) negative rescue (i.e., aggravated phenotype, $P < 0.05$); [0(+)] positive or negative rescue with high variability ($P > 0.05$).

Methods

Fly stocks and genetics

The *mir310s* knockout strain of *D. melanogaster* (w^{1118} ; *mir310s*;- denoted as *mir310s*- here) through P-element excision and the knockout control strain of precise P-element excision was obtained as previously described (Tang et al. 2010). To rescue the *mir310s* knockout, w^{1118} flies were transformed with pUAST vector expressing *mir310s* from *D. melanogaster* (denoted as *Dm310s*) or *D. pseudoobscura* (denoted as *Dp310s*) as previously described (Tang et al. 2010). The resultant flies with genotype w^{1118} ; UAS-*Dm310s* or w^{1118} ; UAS-*Dp310s* and the *mir310s*- flies were crossed with w^{1118} ; *Cyo/Sp*; TM3, *Ser/Sb*, following the cross scheme showed in Supplemental Figure S2A, to obtain the rescue strains of w^{1118} ; *mir310s*;- UAS-*mir310s* (also denoted as *mir310s*;- *Dm310s* and *mir310s*;- *Dp310s*, respectively).

The GAL4/UAS-RNAi system was used to rescue individual targets in the *mir310s*- background. To capture the native expression pattern of *mir310s* in *D. melanogaster*, we constructed the *mir310s*-*Gal4* strain by transforming the putative promoter region of *mir310s* into w^{1118} flies. A ~2.0-kb fragment upstream *mir310s* were amplified from the genomic DNA of w^{1118} with forward primer 5'-TTAGATCTGAGCAGCAATTGTCTCGCAGTA-3' and reverse primer 5'-TGGGAATTCGGCTAGAACGATGTCGGGTA-3', and the amplicon was cloned into BglII and EcoRI sites of pCaSpeR-*Gal4* vector after gel purification and restriction digestion. The germline transformation of w^{1118} flies was conducted using a pTURBO (Flybase. ID:FBmc0002087) helper plasmid, and standard procedures (Rubin and Spradling 1982) finally obtained the flies with genotype w^{1118} ; *mir310s*-*Gal4*. Five UAS-RNAi strains of the *mir310s* targets were used, including UAS-*DI*-RNAi (Bloomington Stock Center, hereafter, stock no. 28032, genotype: y^{11} v^{11} ; P[y[+t7.7] v[+t1.8]=TRiP.JF02867]attP2), UAS-*E2f2*-RNAi (stock no. 36674, genotype: y^1 sc^+ v^1 ; P[y[+t7.7] v[+t1.8]=TRiP.HMS01562]attP2), UAS-*Ecr*-RNAi (stock no. 29374, genotype: y^1 v^1 ; P[y[+t7.7] v[+t1.8]=TRiP.JF02538]attP2), UAS-*Mad*-RNAi (stock no. 35648, genotype: y^1 sc^+ v^1 ; P[y[+t7.7] v[+t1.8]=TRiP.GLV21013]attP2/TM3, *Sb*), and UAS-*Mef2*-RNAi (stock no. 28699, genotype: y^1 v^1 ; P[y[+t7.7] v[+t1.8]=TRiP.JF03115]attP2). The UAS-RNAi (y^1 sc^+ v^1 ; +; UAS-RNAi), *mir310s*-*Gal4* (w^{1118} ; *mir310s*-*Gal4*), and *mir310s*-(w^{1118} ; *mir310s*-) flies were crossed with w^{1118} ; *Cyo/Sp*; TM3, *Ser/Sb*, following the cross scheme showed in Supplemental Figure S2B to obtain the target rescue strains of *mir310s*;-*mir310s*-*Gal4*/UAS-RNAi and the control strain of *mir310s*;-*mir310s*-*Gal4*/+. All flies were reared on cornmeal-sucrose-yeast medium at 25°C and a 12-h light-dark cycle.

Egg dorsal appendage, hatchability, and male fertility assays

We measured the length of egg dorsal appendages, hatchability, and male fertility for the *mir310s* knockout, control, and rescue flies. For the length of dorsal appendages, eggs laid within 1-h time bin were collected, aligned on cover slips, and examined under a Leica DMI4000B microscope (Leica Microsystems). Pictures of dorsal appendages were analyzed using the Leica Application Suite (version 3.8.0) to measure the length of dorsal appendages for the paddle and stalk region separately. Dorsal appendages of more than 200 eggs were measured for each genotype. For egg hatchability, seven pairs of virgin females and males were allowed to mate for 24 h in an egg-laying vial, and then the grape juice plate was removed and replaced with a fresh plate. Five vials were set up for each genotype. After 12 h, the eggs laid on the fresh plate were collected and kept in 25°C to allow hatching for 24 h. Hatchability was calculated as the mean proportion of the number of hatched eggs relative to the total number of collected eggs

across the five replicates. For male fertility, each 5-d-old male was mated with five 3- to 5-d-old virgin females of w^{1118} for 48 h and then transferred to a new vial to mate with another five virgin females for 48 h. More than 15 males were examined for each genotype. Male fertility was measured as the average number of progenies per male in each of the two consecutive rounds of mating. A two-tailed *t*-test was used to determine the statistical significance of phenotypic differences between genotypes.

Quantitative miRNA and mRNA analysis by qRT-PCR

We measured the relative expression levels of individual *mir310s* members in the strains of *mir310s*-, the knockout control, and *mir310s*;-UAS-*mir310s* using qRT-PCR. Total RNA was extracted from ovaries or testes of 3-d-old flies using TRIzol reagent (Thermo Fisher Scientific, catalog no. 15596026) and subject to the removal of genomic DNA using TURBO DNA-free Kit (Thermo Fisher Scientific, catalog no. AM1907) following the manufacturer's protocols. qRT-PCR of miRNAs was conducted using stem-loop reverse transcription (Chen et al. 2005) followed by Taqman PCR analysis using the miRNA UPL (Roche Diagnostics) probe assay protocol (Varkonyi-Gasic et al. 2007; He et al. 2016). Three biological replicates were conducted for each genotype, and 2S RNA was used as an endogenous control (primers are listed in Supplemental Table S1).

Total RNA was extracted from ovaries at the early and late stages of oogenesis for qRT-PCR analyses of the 47 predicted target genes or from ovaries of 3-d-old females for the analysis of the five targets involved in UAS-RNAi with Trizol (Thermo Fisher Scientific, catalog no. 15596026). To collect samples of early oogenesis, young females (<6 h after eclosion) were dissected to collect ovaries, which only contain egg chambers before stage 5. To collect samples of late oogenesis, 3-d-old virgin females were dissected in FBS solution, and egg chambers during stage 9–12 were collected. Total RNA was treated with a TURBO DNA-free kit (Thermo Fisher Scientific, catalog no. AM1907) and reverse-transcribed into cDNA with a PrimeScript first strand cDNA synthesis kit (TAKARA Bio, catalog no. 6110A). qPCR was performed using a SYBR Premix Ex Taq II kit (TAKARA Bio, catalog no. RR82WR) following the manufacturer's instructions. The housekeeping gene *rp49* was used as an endogenous control. The experiments were performed in triplicates. Relative expression levels of miRNAs or target genes were obtained by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) and were statistically analyzed using the two-tailed Student's *t*-test. All primers used in qRT-PCR were listed in Supplemental Table S3.

Target prediction and identification of functional-relevant targets

To select *mir310s* candidate targets for experimental validation, targets in *D. melanogaster* were predicted using three computational tools (Grun et al. 2005; Lewis et al. 2005; Kertesz et al. 2007; Kheradpour et al. 2007; Ruby et al. 2007): (1) TargetScan (TargetScanFly release 6.2, 2012 (the prediction was download from the webpage http://www.targetscan.org/cgi-bin/targetscan/fly_12/targetscan.cgi?gid=&mir_c=mir-92%2F310%2F311%2F312%2F313&mir_nc=&mirg=); (2) PicTar (the *mir310s*/311/312/313 targets were predicted, chosen data set: single miRNA target prediction with the setting S1: high sensitivity; the results were download from http://pictar.mdc-berlin.de/cgi-bin/new_PicTar_fly.cgi?species=fly for each member and then combined); and (3) PITA version 6, which is based on miRbase r11.0 miRNAs and on the *Drosophila* genome version dm3. The prediction results were downloaded from the webpage https://genie.weizmann.ac.il/pubs/mir07/mir07_data.html, selecting "All" catalogs with no

flank, yielding 546 candidate genes. The predicted targets were filtered by requiring they (1) are known to be expressed in the late stage of oogenesis (Yakoby et al. 2008; Tootle et al. 2011); (2) are involved in the KEGG signaling pathways critical for oogenesis or spermatogenesis, such as the *BMP*, *MAPK*, *Hh*, *Notch*, *JAK/STAT*, and *Wnt* signaling pathways (Xie and Spradling 1998; Kiger et al. 2001; Roth 2001; Suzanne et al. 2001; Tulina and Matunis 2001; Chen and McKearin 2003; Kawase et al. 2004; Kirilly et al. 2005; Song et al. 2007; Michel et al. 2012); and (3) produce mutant phenotypes associated with terms “germline stem cells,” “follicle cells,” or “dorsal appendage” as reported in FlyBase (<http://flybase.org>). This gave a list of 58 predicted targets, which are functionally relevant to the miR310s phenotypes. We further excluded 13 genes expressed at low levels in ovaries according to FlyBase and added another two experimentally validated targets of miR310s, *Khc-73* and *imd* (Robins et al. 2005; Tsurudome et al. 2010), as positive controls. By doing so, we finally obtained a list of 47 genes for further analysis (Supplemental Table S2).

To examine target overlap among the four miR310s members, which share a conserved seed region, we predicted their targets using miRanda and DIANA-microT-CDS (Enright et al. 2003; Betel et al. 2010; Reczko et al. 2012; Paraskevopoulou et al. 2013). Predicted targets were downloaded from <http://www.microrna.org/microrna/getDownloads.do> (version: August 2010 Release or searched on http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index (version: 5.0)). Venn diagrams were produced by R package “VennDiagram.”

Luciferase reporter assay

A dual-luciferase reporter assay was used to validate the predicted miR310s targets in S2 cells. Full-length 3' UTRs of five targets, as described in *D. melanogaster* genome release r6.11, were amplified from *w¹¹¹⁸* flies. The miR310s target sites at these 3' UTRs were mutated using fusion PCR (Supplemental Fig. S6). Amplicons of the wild-type or mutant 3' UTRs were cloned into 5'NotI and 3'XhoI sites of psiCHECK-2 vector (Promega Biotech, catalog no. C8021) and used to transfect S2 cells in 96-well plates with 100 ng of ub-Gal4 vectors, 200 ng of pUAST-miR310s (Tang et al. 2010), and 200 ng of the wild-type or mutant 3' UTR reporter vectors using Lipofectamine 2000 (Thermo Fisher Scientific, catalog no. 12566014). Luciferase activities were measured 60 h after transfection using the Dual-Glo luciferase assay system (Promega Biotech, catalog no. E2920) following the manufacturer's instruction. Experiments were performed in four replicates for each gene. Primers used are listed in Supplemental Table S4.

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Queries

Zhongqi Liufu et al.

- Q1 As outlined in our Instructions to Authors, you must use approved nomenclature for gene and protein names and symbols, as it applies for each organism, in text, tables, and figures. In addition, it is the journal's style to set gene symbols, alleles, and loci in italic, and proteins in Roman type. Please verify that all have been properly set throughout the manuscript.
- Q2 In the paragraph that begins, "The question is then whether miRNAs evolve ...": For "Hunter et al.", are you referring to Hunter et al. 2013?
- Q3 In the Methods subsection "Fly stocks and genetics", 2nd paragraph: For "A ~2.0-kb fragment upstream *mir310s* were amplified", is "A ~2.0-kb *mir310s* fragment upstream was amplified" or "A ~2.0-kb fragment upstream of *mir310s* was amplified" meant?

