# MicroRNA1 influences cardiac differentiation in *Drosophila* and regulates Notch signaling

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**MicroRNAs (miRNAs) are genomically encoded small RNAs that hybridize with messenger RNAs, resulting in degradation or translational inhibition of targeted transcripts. The potential for miRNAs to regulate cell-lineage determination or differentiation from pluripotent progenitor or stem cells is unknown. Here, we show that microRNA1 (*miR-1*) is an ancient muscle-specific gene conserved in sequence and expression in *Drosophila*. *Drosophila miR-1* (*dmiR-1*) is regulated through a serum response factor-like binding site in cardiac progenitor cells. Loss- and gain-of-function studies demonstrated a role for *dmiR-1* in modulating cardiogenesis and in maintenance of muscle-gene expression. We provide *in vivo* evidence that *dmiR-1* targets transcripts encoding the Notch ligand Delta, providing a potential mechanism for the expansion of cardiac and muscle progenitor cells and failure of progenitor cell differentiation in some *dmiR-1* mutants. These findings demonstrate that *dmiR-1* may "fine-tune" critical steps involved in differentiation of cardiac and somatic muscle progenitors and targets a pathway required for progenitor cell specification and asymmetric cell division.**

Delta | microRNA | progenitor cells | stem cells | cardiogenesis

Deciphering the mechanisms by which specific cell lineages arise from pluripotent stem cells and subsequently differentiate is a fundamental challenge in stem cell and developmental biology. MicroRNAs (miRNAs) are 21- to 22-nt noncoding RNAs that are sometimes expressed in a lineage-specific fashion and thus have the potential to control cell fate decisions (1–3). There are >300 known miRNAs, and each is thought to target numerous messenger RNA (mRNA) transcripts for either degradation or, more often, translational inhibition. miRNAs typically bind to 3′ UTRs of mRNAs through inexact sequence matching. The lack of precise sequence homology between miRNA and targets has made target prediction difficult, although it does appear that sequence matching of the 5′ end of the miRNA and a permissive secondary structure of target mRNA are important features (4, 5). Despite recent advances in target prediction, only a handful of miRNA targets have been validated thus far, resulting in limited knowledge of biological roles for most miRNAs.

miRNAs may play a role in regulation of stem cell fates (6–8), but direct experimental evidence and a mechanistic understanding of miRNA regulation of cell lineages have been lacking. In *Drosophila,* the dorsal vessel, a primitive heart, is composed of distinct cell types, each arising from progenitor cells that follow stereotypic lineage decisions (9), providing a tractable system in which to study the possible involvement of miRNAs in cell fate decisions. We previously demonstrated that *miR-1-1* and *miR-1-2* (miR-1, microRNA1) are redundant muscle-specific mammalian miRNAs that play a role in cardiogenesis (5). Mouse *miR-1-1* and *miR-1-2* were regulated by serum response factor (SRF), a central transcriptional regulator of muscle differentiation, and excess *miR-1 in vivo* resulted in premature withdrawal of cardiomyocytes from the cell cycle. However, whether *miR-1* is required for cardiac determination or differentiation is unknown.

In this study, we used the *Drosophila* system to investigate whether *miR-1* is necessary for determination or differentiation of cardiac or somatic muscle progenitor cells. We found that the cardiac expression of the single orthologue of *miR-1* in *Drosophila* (*dmiR-1*) is transcriptionally regulated through a conserved SRF-like binding site, and that overexpression of *dmiR-1* in cardiac mesoderm results in fewer cardial cells. Loss of *miR-1* was uniformly lethal, with a spectrum of severity ranging from embryonic death to later demise in the larval stages after hatching. During the course of our work, Sokol and Ambros (10) reported similar loss-of-function effects of *dmiR-1* and described milder defects present in hatched larvae. Here, we focused on the *dmiR-1* mutant flies that did not escape the early lethality and that died during embryogenesis and hatching. We demonstrate that in these embryos, *dmiR-1* is involved in maintaining muscle gene expression and in some cases determination of specific cardiac cell types from pluripotent progenitors. In addition, we provide *in vivo* evidence that *dmiR-1* targets the Notch ligand, Delta, for translational inhibition. Although *dmiR-1* likely targets multiple mRNAs, regulation of the dosage of Notch signaling, which is involved in distinguishing cell types among equivalency groups, is consistent with the lineage defect observed in some *dmiR-1* mutants.

## Materials and Methods

***Drosophila* Strains.** The *dmiR-1* locus deletion was generated by using piggyBac insertion lines (f03931 and f03249 from the Exelixis collection at Harvard Medical School) and by following reported methods (11). The following fly lines were used: *dmiR-1 4.6KB-GFP*, *2.5KB-GFP*, *0.72KB-GFP*, *0.72KB-SRFmut-GFP*, *UAS-miR-1*, *twi-Gal4*, *5.1KB-rescue 24B-Gal4*, *Daughterless (Da)-Gal4*, *UAS-DSRF*, *UAS-DMRTF*, *dpp-Gal4*. Overexpression of *dmiR-1* was accomplished by using the *UAS-Gal4* system (12). Oregon-R was used as the wild-type reference strain.

**Immunohistochemistry, *in Situ* Hybridization, and Microscopy.** Embryos from different lines were collected and stained with various antibodies as described (13). The following primary antibodies were used: mouse anti-β-galactosidase 1:300 (Promega); rabbit anti-myosin heavy chain 1:100 (from D. Kiehart); rat anti-Eve 1:200, guinea pig anti-Odd 1:300 (from D. Kosman); rabbit anti-Tinman 1:500 (from R. Bodmer); rabbit anti-Dmef2 1:1,000 (from B. Paterson); rabbit anti-GFP 1:2,000 (Abcam, Inc., Cambridge, MA); mouse anti-GFP 1:1,000 (Invitrogen); rabbit anti-Twist 1:500 (R. Cripps); mouse anti-Delta 1:400 (DSHB); and Cy3, Cy5, biotin- or horseradish peroxidase-conjugated (with TSA plus Fluorescent Systems, PerkinElmer)

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Abbreviations: mRNA, messenger RNA; SRF, serum response factor; miRNA, microRNA; miR-1, microRNA1; dmiR-1, *Drosophila* miR-1; Eve, Even-skipped.

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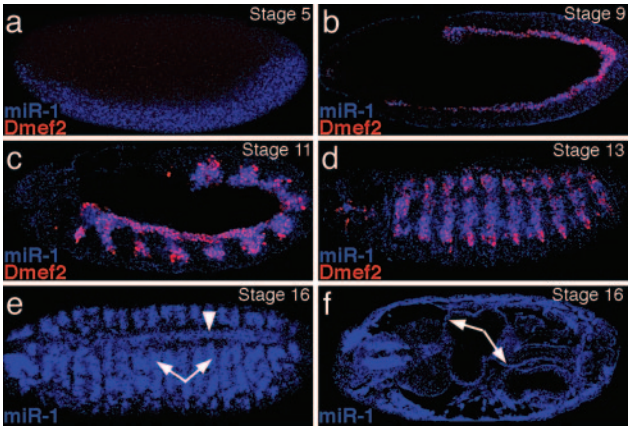


**Fig. 1.** *dmiR-1* transcripts mark mesodermal cells and derivatives. (*a–f*) Whole-mount *in situ* hybridization of *miR-1* (blue) at stages 5 (*a*), 9 (*b*), 11(*c*), 13 (*d*), and 16 (*e* and *f*) embryos. *dmiR-1* is expressed in the presumptive mesoderm (*a*), mesodermal cells (*b–d*) and derivatives such as the heart tube (arrowhead in *e*), and somatic and visceral muscles (arrows in *e* and *f*, respectively). Embryos in *a–d* were costained with anti-Dmef2 (red). (*a–f*) Anterior left, posterior right views; (*a–d*) lateral views; (*e*) dorsolateral view; (*f*) interior confocal view of *e*.

secondary antibodies (The Jackson Laboratory). These antibodies were used to recognize the primary antibodies. *In situ* probe synthesis and hybridization were performed as described (14). Fluorescent images were obtained with a Zeiss LSM510-meta confocal microscope.

**Construct Generation, Transformation, and Transfection Assays.** GFP and 5.1-kb-rescue transgenes were generated by cloning the corresponding genomic DNA into the pH-Stinger vector (15). The SRF-like-binding site mutation was generated by substituting GCTATTTATG to GgTAccTATG (altered bases are shown in lowercase). For UAS-*miR-1* generation, 552 bp around *dmiR-1* (from 310 bp upstream to 220 bp downstream of *dmiR-1*) were cloned into pUAST. These constructs were introduced into flies by P-element-mediated germline transformation. The *in situ* probe was PCR-amplified by using the following primers: TGGCCATGTGGCGCGAAGTATGCGC and TCATCTAGAGCCTGTGGTGGAATGGTATTTGTG. The *dmiR1*-luciferase, *dmiR1*-mut-luciferase, Delta 3′UTR, and Delta 3′UTRmut were generated by cloning the corresponding enhancers and three copies of a wild-type or mutant *dmiR-1* target site present in the Delta 3′UTR into the pGL3 vector (Promega). Cell transfection and luciferase assays were performed as described (13). Luciferase activities are expressed as mean ± standard deviation from three experiments with constitutive activity of luciferase set at 100%.

## Results

**Expression and Regulation of *dmiR-1*.** The single orthologue of *miR-1* in *Drosophila*, *dmiR-1*, is nearly identical in sequence to mouse and human *miR-1* (5). *In situ* hybridization revealed *dmiR-1* transcripts in presumptive mesodermal cells as early as stage 5 (2.2–2.8 h) of *Drosophila* development (Fig. 1*a*). This pattern changed dynamically throughout gastrulation, but *dmiR-1* consistently marked mesodermal cells (Fig. 1 *b–d*). Transcripts persisted in later stages of cardiac and somatic (body wall) muscle differentiation (Fig. 1*e*), as in mice, and were also found in visceral muscles of the gut (Fig. 1*f*). *dmiR-1* expression overlapped, but preceded, that of *dmef2*, a transcriptional regulator of muscle precursors (ref. 16, Fig. 1 *a–d*).

To determine whether transcriptional regulation of *miR-1* was evolutionarily conserved, we aligned 10 kb of genomic DNA surrounding *Drosophila melanogaster* and *Drosophila pseudoobscura miR-1* genes to find regions of sequence conservation (Fig. 2*a*). Transgenic flies containing conserved islands 4.6 kb upstream of *dmiR-1* adjacent to the gene encoding nuclear GFP (nGFP) recapitulated the endogenous *dmiR-1* expression in all muscle types (Fig. 2 *b* and *c*). Cardiac nGFP expression coincided with *dmef2* expression in cardial cells and was present in pericardial cells, which do not express *dmef2*. The basic helix–loop–helix transcription factor twist is essential for mesoderm specification and regulates *dmiR-1* in certain domains (10, 17), so we directly compared the expression of nGFP driven by the *dmiR-1* enhancer with twist expression. We found that, whereas there was considerable overlap, the *dmiR-1* enhancer directed expression in many areas of low twist expression, including cardiac and visceral muscle progenitors, suggesting twist-independent regulation in these domains (Fig. 2 *d–g*).

Deletion analyses indicated that a 2.5-kb region was sufficient for expression in all domains of *dmiR-1* expression except pericardial cells (Fig. 2 *h* and *i*). Within this domain, a 720-bp genomic region containing a highly conserved SRF-like binding site recapitulated the expression directed by the 2.5-kb fragment (Fig. 2*j*). SRF, which is closely related to MEF2, controls the expression of genes involved in muscle differentiation, cell migration and proliferation (18). Our prior studies showed that SRF was an obligate activator of *miR-1* expression during cardiac development in the mouse (5). Mutation of the SRF-like site in flies abolished nuclear GFP expression in cardiac and visceral muscle cells but not somatic muscle (Fig. 2 *k* and *m*). *In vitro*, *Drosophila* SRF (DSRF) weakly activated transcription of a luciferase reporter through the SRF-like binding site (Fig. 2*n*). Addition of the potent cardiac and smooth muscle-specific SRF cofactor myocardin-related transcription factor (13, 18) robustly activated luciferase activity dependent on an intact SRF-like binding site (Fig. 2*n*). This observation is consistent with regulation of *miR-1* in mice, but we cannot rule out the possibility that Dmef2 also regulates cardial expression of *dmiR-1* through this site independently or cooperatively with SRF.

**Deletion of *dmiR-1* Affects Cardiac Development.** To begin to define the functions of *dmiR-1 in vivo*, we used two Exelixis lines (11) of *Drosophila* containing *FRT* sites surrounding the *dmiR-1* gene and generated a FRT-FLP-based deletion of the *dmiR-1* locus (Fig. 3). Successful excision of *dmiR-1*, the only known or predicted gene in the 31-kb deleted interval, was confirmed by sequence analysis and RT-PCR (not shown). Homozygous *dmiR-1* deletion was 100% lethal, but a spectrum of severity was observed, with approximately one-third dying at embryonic stages, one-third around hatching, and the remaining at larval stages. Homozygous mutant larvae were abnormally lethargic compared with their heterozygous siblings before death. The embryonic and larval lethality was fully rescued by overexpression of *dmiR-1* by using a mesoderm specific *twi-Gal4* driver (Fig. 3*b*) or by a 5.1-kb transgene encompassing the *dmiR-1* genomic locus including the 4.6-kb enhancer and the sequence encoding *dmiR-1* (not shown), consistent with *dmiR-1* being the sole gene within the deleted region responsible for the lethal phenotype. The variability in phenotype may be related to previously described maternal *dmiR-1* transcripts (19, 20), redundancy with other miRNAs or may simply reflect the role of *dmiR-1* in "fine-tuning" whether cells achieve the thresholds of critical proteins to initiate critical developmental events.

Because one-third of all *dmiR-1* mutants died around the time of hatching and another one-third at larval stages with poor mobility, we investigated whether there might be a discernable muscle defect. We found that nearly half of all *dmiR-1* mutant embryos displayed severe defects in muscle gene expression with down-regulation of sarcomeric genes such as *myosin heavy chain* (*MHC*) (Fig. 3 *c* and *d*), indicating a late requirement for *miR-1*

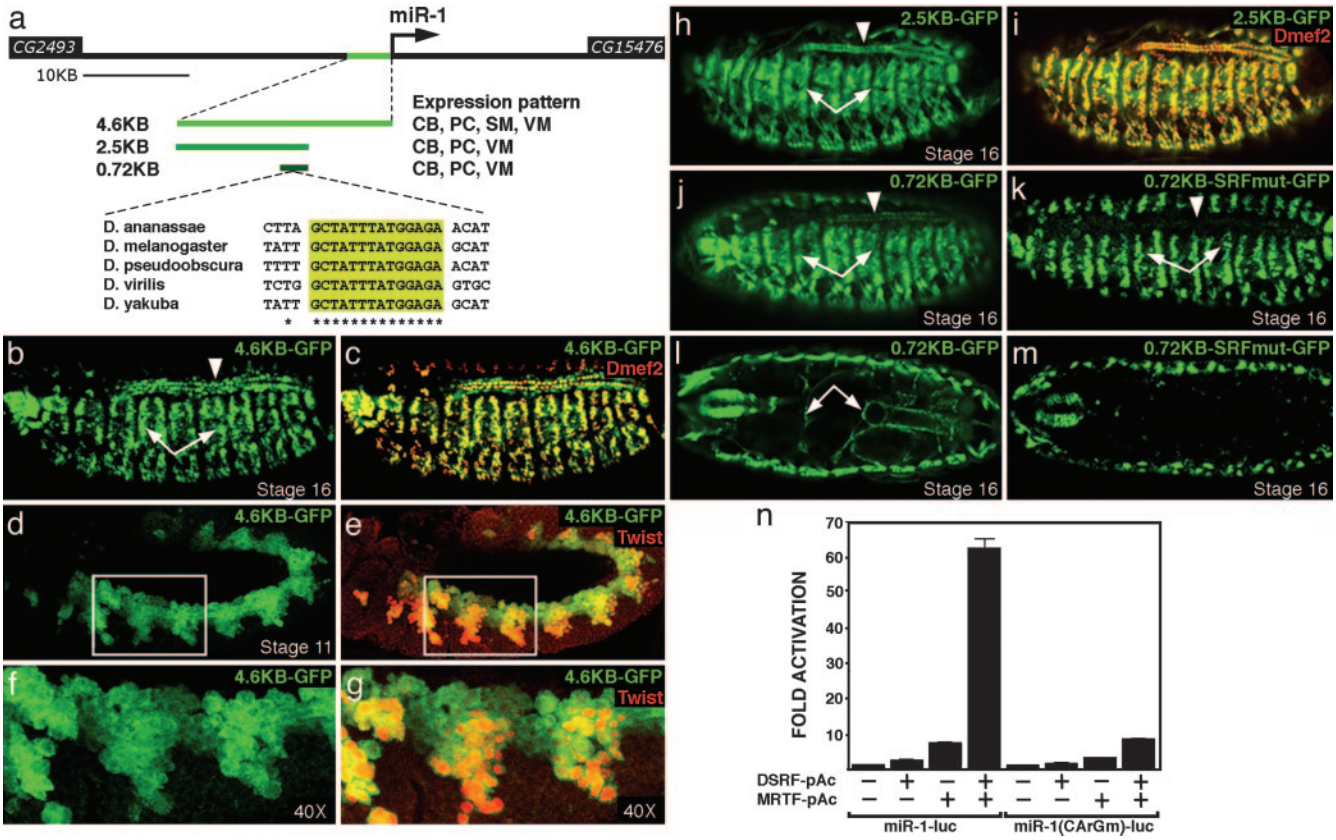


**Fig. 2.** Regulation of *dmiR-1* in cardioblast and visceral muscle cells. (*a*) Map of the *dmiR-1* locus showing the position of the 4.6-kb *dmiR-1* enhancer (green) and subfragments, with expression domains summarized as follows: SM, somatic muscle; VM, visceral muscle; CB, cardioblast; PC, pericardial cell. An A/T-rich SRF-like-binding site conserved in other *Drosophila* species is highlighted. (*b–m*) GFP expression in embryos carrying the 4.6-kb (*b–g*), 2.5-kb (*h* and *i*), 0.72-kb (*j* and *l*), or SRF-like site-mutated 0.72-kb (*k* and *m*) element. Embryos were costained with anti-Dmef2 (*c* and *i*) or anti-Twist (*e* and *g*). (*n*) Luciferase (luc) activity determined with luciferase reporters linked to the 0.72-kb element or the SRF-like site mutated (CArGm) 0.72-kb element in *Drosophila* S2 cells in the presence or absence of *Drosophila* SRF and myocardin-related transcription factor. Error bars indicate standard deviations. (*b*, *c*, *h–k*) Dorsolateral views of stage 16 embryos. (*d–g*) Lateral views of stage 11 embryos. *f* and *g* are ×40 images of *d* and *e*, respectively. *l* and *m* are inside views of *j* and *k* embryos, respectively, focusing on visceral muscles. Arrowheads indicate the presence (*b*, *h*, and *j*) or absence (*k*) of the heart tube, and arrows indicate somatic (*b*, *h*, *k*, and *j*) and visceral muscles (*l*).

to maintain muscle differentiation. This phenotype was also uniformly rescued by *dmiR-1* under the control of the *twist* driver, indicating that the muscle differentiation defect was due to loss of *dmiR-1* and not other sequences within the deleted region.

Analysis of the more severely affected embryos revealed abnormal cardiac and somatic muscle patterning. To determine whether the absence of *dmiR-1* affected early muscle cell determination or differentiation, we took advantage of several unique markers of cardiac and somatic muscle cell fates. The transcription factor Tinman is initially expressed in all cardiac progenitors, because they arise from a group of equivalent precursors through lateral inhibition (21). The cardiac progenitors undergo symmetric and asymmetric cell divisions, resulting in the generation of cardioblasts, pericardial cells, and dorsal muscles (DA1) (22). In particular, progenitor cells expressing the transcription factor Even-skipped (Eve) undergo asymmetric cell division, giving rise to $Eve^+$ pericardial cells or DA1 somatic muscles, whereas Dmef2-expressing cells aligned at the dorsal edge of the mesoderm become cardioblasts. Expression of Tinman becomes restricted into a subset of cardioblasts and pericardial cells after their differentiation. Lateral inhibition induced by the transmembrane receptor Notch is essential for lineage decisions and for the formation of proper number of cardiac progenitors (21, 22).

At stage 11, wild-type flies had a well defined segmental pattern of $Eve^+$ cardiac progenitors (three to four cells per cluster) (Fig. 3*e*). In contrast, 5–10% of all *dmiR-1* mutants had an overabundance of poorly patterned $Eve^+$ progenitors at this stage (Fig. 3*f*). By stage 12 in wild-type embryos, $eve^+$ progenitor cells normally differentiate in a defined pattern into two pericardial ($Eve^+Dmef2^-$) cells and one DA1 muscle ($Eve^+Dmef2^+$) per hemisegment (Fig. 3*g*). A row of $Dmef2^+$ cardioblasts also aligns at the dorsal edge of the mesoderm, separated from $Dmef2^+$ somatic muscle progenitors cells by rows of pericardial cells including $Eve^+$ pericardial cells (EPCs) (Fig. 3*g*). At stage 12, Tinman expression is normally restricted to cardioblasts and the EPCs but is absent in the DA1 muscles (Fig. 3*g*). However, in the subset of *dmiR-1* mutants described above, the overabundant progenitor pool at stage 12 appeared arrested in development, similar to stage 11, and failed to differentiate into $Eve^+Dmef2^-$ pericardial cells (Fig. 3*h*). Tinman was ectopically expressed in the expanded pool of $Eve^+$ and/or $Dmef2^+$ cells, indicating that these were cardioblasts and muscle progenitors that failed to terminally differentiate in the absence of *dmiR-1* (Fig. 3 *i* and *j*).

Although the majority of embryos displaying early defects died early during embryogenesis, some survived to later embryogenesis and revealed varying degrees of gaps in the rows of cardial cells that constitute the dorsal vessel, consistent with the requirement of *dmiR-1* for determination and/or differentiation of cardiac cells (Fig. 3 *k* and *l*). These flies had morphologic

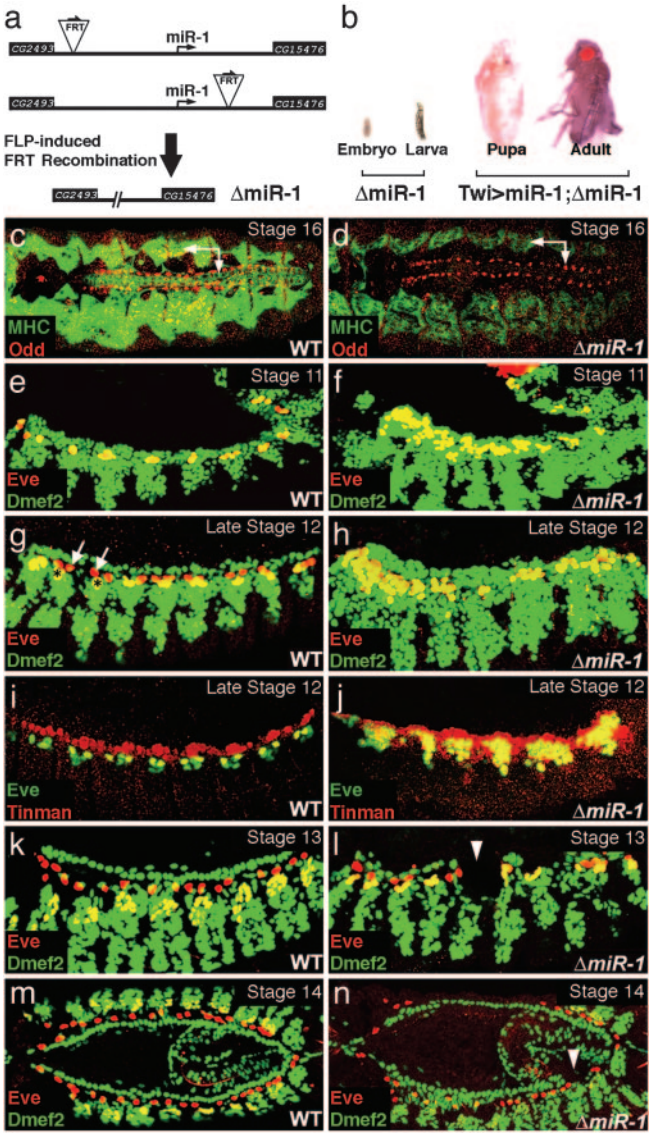


**Fig. 3.** Loss of *dmiR-1* causes abnormal heart and muscle development. (*a*) Schematic of FRT-FLP-mediated *dmiR-1* locus deletion and locations of neighboring genes. Embryonic and larval lethality was rescued by *UAS-miR-1* with *twi-gal4* driver (*b*) or by a transgene containing the 5.1-kb *miR-1* locus (not shown). (*c*, *e*, *g*, *i*, *k*, and *m*) WT embryos. (*d*, *f*, *h*, *j*, *l*, and *n*) Homozygous *miR-1* mutant (Δ*miR-1*) embryos. Expression of *MHC* (*myosin heavy chain*) was dramatically reduced both in the heart and muscles (arrows in *c* and *d*) in half of all Δ*miR-1* embryos compared with WT, whereas expression of a pericardial cell marker, *odd-skipped*, was not affected (*c* and *d*). Δ*miR-1* embryos with the early defect had ectopic *Eve* progenitor cells at stage 11 (*f*). The ectopic progenitor cells failed to differentiate into pericardial (arrows in *g*) and dorsal muscles (asterisks) and maintained expression of both *Dmef2* and *eve* at late-stage 12 (*h*). Ectopic *Dmef2*$^+$ cardioblasts, identified based on their dorsal location relative to the *Eve* progenitors, were also observed (*h*). *tinman* was expressed in most of the ectopic cardiac progenitors that expressed *eve* (*j*). (*k*–*n*) Another subset of embryos that fail to hatch showed reduced numbers of cardiac and muscle cells, indicated by gaps in the row of cardioblasts and missing dorsal muscles in Δ*miR-1* embryos (arrowheads in *l* and *n*). (*e*–*l*) Lateral views; (*c*, *d*, *m*, and *n*) dorsal views.

defects in somatic muscle and heart formation, including frequent loss of cardioblasts and DA1 dorsal muscles (Fig. 3 *m* and *n*).

***dmiR-1* Overexpression Disrupts Dorsal Vessel Patterning.** Because *dmiR-1* loss of function led to decreased muscle gene expression

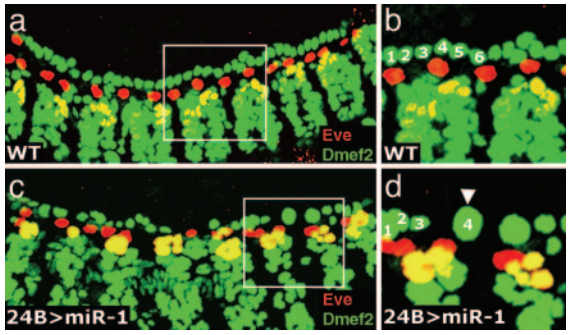


**Fig. 4.** *dmiR-1* overexpression in the mesoderm affects heart and muscle cell morphology. (*a*–*d*) Lateral views of stage 13 WT (*a* and *b*) or *dmiR-1* overexpressed embryos with *24B-Gal4* driver (*c* and *d*). *b* and *d* are ×40 images of white boxes in *a* and *c*, respectively. On average, four rather than six cardioblasts per hemisegment were observed in transgenic flies with occasional enlarged cardioblasts (arrowhead).

and an increased pool of undifferentiated muscle progenitors in a subset of embryos, we asked whether excess *dmiR-1* might result in a decreased number and/or premature differentiation of precursor cells into muscle. Ubiquitous expression of *dmiR-1* using the *Daughterless (Da)-Gal4* or late mesoderm expression using the *24B-Gal4* driver resulted in 100% embryonic lethality at various stages of development. Twenty percent of *dmiR-1* overexpressing flies showed disrupted patterning of cardiac and skeletal muscle with insufficient numbers of cardioblasts (Fig. 4). The dorsal vessel was reliably patterned with six cardioblasts per hemisegment in wild-type flies but had only three to four cardioblasts per hemisegment, with frequent enlargement of cardioblasts, in *dmiR-1*-overexpressing flies. Thus, excess *dmiR-1*, when sufficiently affecting the threshold of critical events, may result in early diversion of cardiac progenitors into an alternative cell fate or may cause premature differentiation of precursors, resulting in an insufficient pool of progenitors.

***dmiR-1* Can Target *Delta* for Translational Inhibition.** Because miRNAs typically target numerous mRNAs, the phenotype of *dmiR-1* mutants is likely due to down-regulation of multiple critical proteins. Despite the likely complexity of targets, we sought to identify mRNA targets of *miR-1* in flies that might be involved in *dmiR-1*-dependent lineage determination and differentiation decisions. Although mouse *miR-1* targets transcripts encoding the cardiac-enriched basic helix–loop–helix transcription factor Hand2 (5), we did not identify any *miR-1*-binding sites in the 3′-UTR of *Drosophila hand*, suggesting alternative targets in flies. Because the more severe *dmiR-1* gain- and loss-of-function phenotypes were reminiscent of progenitor defects induced by altering Notch signaling, we searched the 3′-UTRs of genes involved in the Notch pathway for potential sequence matching and accessibility to *dmiR-1*.

Several conserved putative *miR-1*-binding sites were found in the 3′-UTR of the gene encoding Delta, a membrane-bound ligand for Notch (Fig. 6, which is published as supporting information on the PNAS web site). Upon interaction with Delta, Notch is cleaved, allowing the Notch intracellular domain to translocate into the nucleus and regulate gene expression (23). Signaling between neighboring Delta- and Notch-expressing cells is necessary for lateral inhibition and asymmetric cell fates during lineage determination (24–26) and involves repression of Delta in Notch-expressing cells and similar repression of Notch in adjacent Delta-expressing cells (27–33). Notch signaling also later regulates differentiation of numerous cell types, including cardial cells (22).

Introduction of one of the putative *dmiR-1*-binding sites from

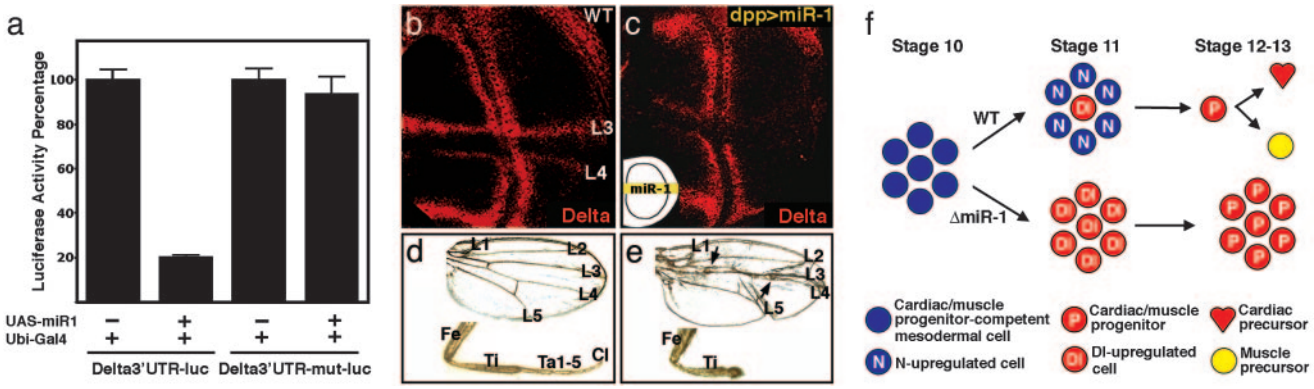


**Fig. 5.** *dmiR-1* can target the Notch ligand *Delta*. (*a*) Relative luciferase activity was determined by inserting part of the 3′-UTR target sequence from Delta, recognized by *dmiR-1*, into the 3′-UTR of luciferase (Delta 3′-UTR-luc) in the presence or absence of *dmiR-1* in Drosophila S2 cells. The basal level of Delta 3′-UTR-luc or Delta 3′-UTR-mut-luc activity in the presence of *Ubi-Gal4* but not *UAS-miR-1* was defined as 100%. Overexpression of *dmiR-1* inhibited activity of the luciferase reporter with WT Delta 3′UTR, but not with the *dmiR*–binding-site mutated Delta 3′UTR. Error bars indicate standard deviation. (*b*) *Delta* expression in the WT wing pouch using anti-Delta antibody (red). (*c*) Ectopic *dmiR-1* expression in the *dpp* expression domain (indicated by a yellow stripe in the wing pouch cartoon in *c*) resulted in severely compromised endogenous *Delta* protein expression along the *dmiR-1*-overexpressed domain. (*d*) WT adult wing and leg. (*e*) Overexpression of *dmiR-1* in wing and leg discs caused thickened wing veins (arrows) specifically in longitudinal veins 3 and 4 (L3 and L4) and shortened legs, similar to the loss of *Delta* phenotype. (*f*) Hypothetical model for early function of *dmiR-1* in mesodermal cells through regulation of Notch signaling. *dmiR-1* may be important in reinforcing selection of lineages from equivalency groups normally regulated by Notch. Disruption of the normal segregation of Delta- and Notch-expressing cells in the absence of *dmiR-1* may result in excess Delta-expressing cells and consequent down-regulation of Notch and failure of lineage determination. L1–5, longitudinal vein 1–5; Fe, femur; Ti, tibia; Ta1–5, tarsal segment 1–5; Cl, claw.

the Delta 3′-UTR into the 3′-UTR of luciferase resulted in dose-dependent and specific down-regulation of luciferase activity in the presence of *dmiR-1* in cultured fly S2 cells (Fig. 5*a*). Although the *in vitro* data supported Delta as a *dmiR-1* target, we sought to determine whether *dmiR-1* affected Delta protein levels *in vivo*. Based on our experience and the literature, available Delta antibodies are not sensitive enough to distinguish levels of Delta protein in embryonic muscle precursors. Therefore, we turned to an *in vivo* assay involving the well described role of Delta-Notch signaling in the developing wing disk, where disruption of *Delta* results in thickening of fly wing veins (30, 34). Delta protein is normally detectable and expressed in two perpendicular stripes in the wing pouch (Fig. 5*b*). We overexpressed *dmiR-1* along one of the two stripes using a *dpp-Gal4* driver and assayed the effects on Delta protein expression. Delta protein was markedly reduced exclusively in the domain of *dmiR-1* expression, providing *in vivo* support for Delta as a target of *miR-1* in flies (Fig. 5*c*). *dmiR-1*-induced loss of Delta in this specific subdomain of the wing resulted in thickening of wing veins, recapitulating the loss-of-Delta phenotype (Fig. 5 *d* and *e*). The shortened-leg phenotype upon *dmiR-1* overexpression (Fig. 5 *d* and *e*) provided further evidence of *dmiR-1*'s effects on the Notch pathway, because this, too, was similar to the phenotype of flies lacking Delta (32). Together, the *in vivo* experiments provided compelling evidence that *dmiR-1* can regulate Delta protein levels, providing a potential means to fine-tune cellular responses to Notch signaling. Given the recognized role of Notch signaling in asymmetric cell division of muscle progenitors (21, 22), *dmiR-1* regulation of Delta, along with other *dmiR-1* targets, may be important in cardiac lineage determination events.

## Discussion

We have shown that *dmiR-1* is an ancient gene that functions at multiple stages of *Drosophila* development, including regulation of specific cardiac and somatic muscle lineages from progenitor cells. Later in development, *dmiR-1* is also involved in muscle differentiation and maintenance of muscle gene expression. Expression of *dmiR-1* in cardiac and visceral cells depended on an A/T-rich DNA sequence that resembles a binding site for SRF and MEF2. Finally, we demonstrate that the Notch ligand, Delta, is a target of *dmiR-1*, providing *in vivo* evidence of miRNA-mediated regulation of Notch signaling.

The roles of some miRNAs have been revealed through genetic screens in *Caenorhabditis elegans* and *Drosophila*. The majority appears to be involved in fine-tuning biological processes by titrating precise dosages of regulatory proteins; however, targeted deletion of a specific miRNA in vertebrates has not yet been reported. The *dmiR-1* mutant phenotype we characterized in this report is similar to that described by Sokol and Ambros (10), in that ≈70–80% of homozygous mutants survive to around the period of hatching in both studies, with the remaining mutants dying during embryogenesis. However, we found that half of all mutants had a muscle differentiation defect marked by decreased muscle gene expression. In addition, our study revealed an interesting lineage defect in the subset that suffered embryonic lethality, suggesting that, in some cases, the dosage of proteins regulated by *dmiR-1* could reach a critical threshold affecting cell determination events. It is worth noting that, in contrast to the mild muscle defect described by Sokol and Ambros (10), injection of *dmiR-1* complementary 2′*O*-methyl oligoribonucleotides, which efficiently block miRNA function (35), resulted in significant embryonic lethality with substantial defects in embryonic morphology and few survivors (20). Although the discrepancy in phenotypes is difficult to resolve, the similar severity of defects in the *dmiR-1* mutants we described, flies described by Leaman *et al.* (20), and the successful rescue of the mutant phenotype by *dmiR-1* in our study suggest that the incompletely penetrant embryonic defects are due to loss of *dmiR-1*.

*dmiR-1* likely controls numerous mRNA targets that are important for muscle development and maintenance but, interestingly, the *Drosophila* orthologue of *Hand2*, a mammalian *miR-1* target (5), did not have any *miR-1*-binding sites. Previous reports that a reduction in Notch signaling results in excessive muscle progenitor cells and failure of pericardial cell formation (22, 26, 36), which is similar to severe *dmiR-1* mutants, led us to examine members of the Notch signaling pathway as potential *dmiR-1* targets. Our findings that *dmiR-1* could regulate protein expression through a target site in the *Delta* 3′ UTR *in vitro*, could down-regulate Delta protein *in vivo*, and could mimic the *Delta* loss of function in the wing and leg upon misexpression

together support *Delta* as a validated *dmiR-1* target. It will be interesting to determine whether any of the Delta-like Notch ligands are also targets of *miR-1* in mammals.

Whether targeting of Delta in cardiac progenitors by *dmiR-1* is important during normal development remains to be determined, because interrogation of Delta protein in muscle progenitors has been limited by technical considerations. However, along with other *dmiR-1* targets within the cardiac progenitors, it is worth considering that elevated levels of Delta protein in mesodermal cells upon loss of *dmiR-1* might result in disruption of the normal pattern of cells sending ($Delta^+$) or receiving ($Notch^+$) the Notch signal during the process of lateral inhibition. In this scenario, inappropriate levels of Delta in $Notch^+$ cells might result in feedback down-regulation of Notch expression in the same cells and consequent recruitment of excess progenitor cells that fail to undergo appropriate segregation into distinct lineages (Fig. 5*f*). Because *dmiR-1* may only be titrating dosage of protein, increased Delta in $Delta^+$ cells may not significantly affect the degree of Notch signaling in neighboring $Notch^+$ cells, particularly if they have down-regulated expression of the Notch receptor. This is consistent with previous observations that high levels of Delta in active Notch domains inhibit Notch signaling (24, 27–33). Future studies may more directly test this hypothesis within cardiac precursors and determine whether miRNAs target Notch signaling in other cell types to regulate asymmetric cell division and lineage determinations.

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