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Drosophila miR-9a Targets the ECM Receptor Dystroglycan to Canalize Myotendinous Junction Formation

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SUMMARY

Establishment of intercellular interactions between various cell types of different origin is vital for organism development and tissue maintenance. Therefore, precise timing, expression pattern, and amounts of extracellular matrix (ECM) proteins must be tightly regulated. Particularly, the ECM is important for the development and function of myotendinous junctions (MTJs). We find that precise levels of the ECM receptor Dystroglycan (Dg) are required for MTJ formation in *Drosophila* and that Dg levels in this process are controlled by *miR-9a*. In the embryo, Dg is enriched at the termini of the growing muscles facing the tendon matrix and absent from *miR-9a*-expressing tendons. This gradient of Dg expression is crucial for proper muscle-tendon attachments and is adjusted by *miR-9a*. In addition to Dg, *miR-9a* regulates the expression of several other critical muscle genes, and we therefore propose that during embryogenesis, *miR-9a* specifically controls the expression of mesodermal genes to canalize MTJ morphogenesis.

INTRODUCTION

To achieve successful results, despite the extreme fluctuation of internal cues, genetic background, and external conditions, embryonic development must be stabilized. Coordinated transcription factor networks are prominent regulatory features of cell fate establishment during embryonic development and adult life. It is now becoming evident that, in conjunction with transcription factors, at least three epigenetic elements help to form a reciprocal regulatory circuit to maintain cell identity and differentiation: chromatin structure, DNA methylation, and microRNAs (miRNAs) (Gaspar-Maia et al., 2011; Lindeman et al., 2011; Nguyen and Frasch, 2006). miRNAs, based on their paradoxical properties, e.g., being highly evolutionarily conserved, but not essential, have been proposed to play a role in generating biological robustness as canalization factors to buffer gene expression against perturbation or variability (Hornstein and Shomron, 2006; Waddington, 1942). As canalization factors, miRNAs have previously been shown to liquidate transcripts resulting from aberrant gene expression (Ebert and Sharp, 2012; Herranz and Cohen, 2010; Wu et al., 2009) or leaky splicing (Stark et al., 2005; Weng et al., 2013). The previously described in vivo cases of miRNA-based regulation mostly are examples of simple pairs, in which one miRNA is targeting one gene. However, increasing evidence suggests that functionally related genes are clustered at the level of DNA sequence, histone modifications, chromatin loops, or chromosome territories (Hurst et al., 2004) and are under similar transcriptional control. Taking into account that, first, the gene expression in general is a noisy process that incidentally allows leaky expression of "neighboring" genes (Arias and Hayward, 2006; Macneil and Walhout, 2011) and, second, that one miRNA can regulate multiple genes, it is logical to propose that as a canalization factor one miRNA should be capable of regulation of multiple genes that are involved in the same signaling network. Therefore, we studied whether this type of miRNA-based regulation, employed to confer robustness of embryonic development, actually takes place.

Assembly of muscle tissue requires communication between mesoderm-derived myotubes and ectoderm-originated epidermal muscle-attachment cells or tendons. Since tendon cells invaginate into mesoderm, some mechanism that reassures the robustness of their identity must exist. Initially, the pretendon cells send signals to the myotubes and direct myotube attraction and adhesion to their target cells; subsequently, the muscle cells communicate a reciprocal signal to the epidermal muscle attachment cells, initiating their terminal differentiation into tendon-like cells (Frommer et al., 1996). This suggests the necessity of a microenvironment that will allow for both a rapid and precise signal transduction between these ectodermally and mesodermally derived cell types.

Importantly, the process of muscle guidance and attachment in *Drosophila* is remarkably similar to that of vertebrates, as both are greatly dependent on the extracellular matrix (ECM) gradient that is established through differential recruitment and clustering of transmembrane receptors by extracellular-presented signaling molecules (Martin-Bermudo and Brown, 2000; Snow and Henry, 2009). During *Drosophila* embryonic development, the initial determination of myoblast fate is controlled by high expression of the basic-helix-loop-helix protein Twist; after the myoblast division and fusion, multinucleated myotubes are formed (Baylies and Bate, 1996). At stage 12–14, myotubes undergo a substantial transformation: not only do they continue

to grow through cell fusion, but they also change their shape and form elongated filopodia at the leading edge that help to find their proper tendon cells in the epidermis (Schnorrer and Dickson, 2004). At the same time, the tendon cells also undergo a series of cell shape rearrangements, including apical constriction and apical-basal elongation, which results in the formation of epidermal furrows. When myotubes reach their targets, the surface of the myotube facing the tendon cells loses filopodia and multiple adhesion complex molecules accumulate at the muscle attachment site toward the tendon cell in order to form a stable adhesion complex (Martin-Bermudo and Brown, 2000). While the signaling crosstalk between these cell types has been extensively studied, it is not clear whether a genetic program exists that would aid cells that are subjected to similar spatiotemporal signaling to undergo distinct developmental programs. The role of miRNAs in this process, vital for muscle physiology, has not yet been analyzed; however, vigorous cell rearrangements and cell fate specifications that take place during establishment of the muscle attachment suggest a need for a mechanism that enhances robustness of the process by attenuating leaky transcripts.

In our study, we found that *Drosophila miR-9a* is involved in canalization of myotendinous junction (MTJ) assembly. Deficiency of *miR-9a* affects embryonic survival, a phenotype that can be rescued by specific expression of this miRNA in tendon cells. The survival of *miR-9a* mutants depends on the speed of embryonic development that reciprocally correlates with transcriptional noise. *miR-9a* is expressed in epidermally derived tendon cells, while many *miR-9a* predicted targets are essential muscle genes that are misregulated due to *miR-9a* loss and gain of function. Moreover, exogenous expression of *miR-9a* in mesoderm completely abolishes muscle formation. Therefore, we put forward a hypothesis that *miR-9a* adjusts tendon cell differentiation by preventing misexpression of muscle genes resulting from stress or aberrant transcription. To prove this hypothesis, we misexpressed putative *miR-9a* targets in tendon cells and found that ectopic *heartless* (*htl*), *wishful thinking* (*wit*), and *Dystroglycan (Dg)* in tendons cause muscle attachment and embryonic lethality phenotypes similar to those found in *miR-9a* mutants. In particular, we found that the muscular-dystrophy-associated ECM receptor, Dg, is regulated posttranscriptionally via the miRNA, *miR-9a.* During the early embryonic stages, Dg is present in all epidermal cells; however, for proper assembly of muscle attachment sites it is essential that Dg is eliminated from epidermally derived tendon cells, with *miR-9a* modulating the precision of this expression. Dg establishes a specific ECM gradient that influences muscle-tendon signaling; therefore, its differential localization is crucial for proper muscle-tendon attachments and is adjusted by *miR-9a.* When Dg is misexpressed in tendon cells, the composition of the tendon matrix is affected, resulting in aberrant muscle attachments and embryonic death.

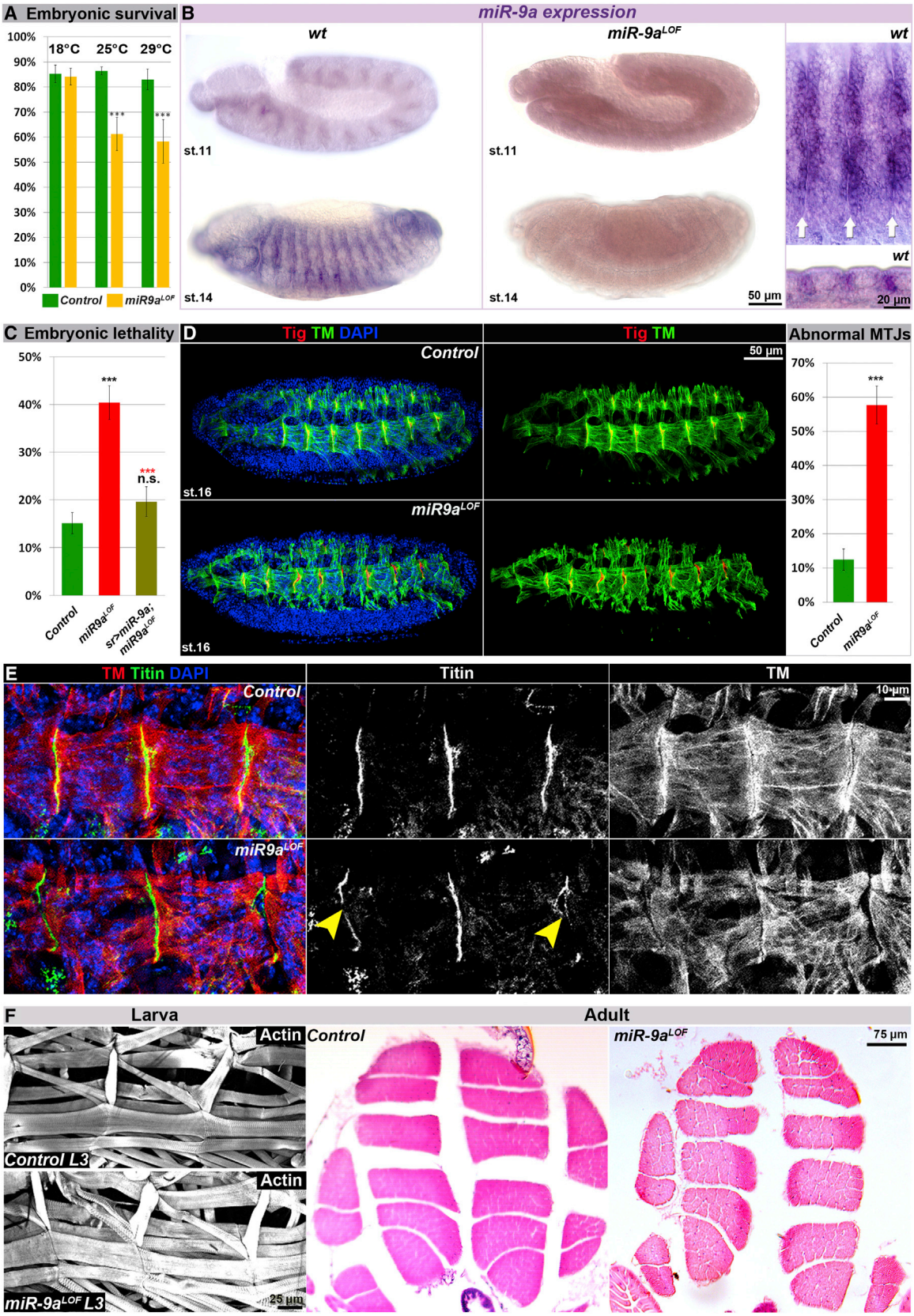
RESULTS

Embryonic *miR-9a* Modifies the Process of Muscle-Tendon Attachment

Since miRNAs have been suggested to act as canalization factors to protect the developing organism from transcriptional noise that increases due to unfavorable conditions in the natural environment (Arias and Hayward, 2006; Hornstein and Shomron, 2006), we searched for a miRNA that would play this role in *Drosophila* embryogenesis. *miR-9a* caught our attention because previous data indicated that this miRNA acts as a canalization factor controlling the ''bistable'' circuitry that sets the threshold for the formation of sense organ precursors versus neighboring cells (Bejarano et al., 2010; Li et al., 2006). *Drosophila* belongs to ectothermic animals; the speed of its development is directly proportional to the ambient temperature. Since accelerated development may cause errors and additional fluctuations in transcriptional status, we tested whether *miR-9a* would influence embryogenesis under different temperature conditions. In particular, we tested if lower and higher temperatures would have an effect on embryonic lethality of *miR-9a* mutants. We observed that at higher temperatures (25°C and 29°C), when the developmental processes are accelerated, the frequency of embryonic survival was significantly decreased in *miR-9a* loss-of-function mutants ($miR\text{-}9a^{LOF}$; Figure 1A; Table S1 available online), suggesting that *miR-9a* is a candidate miRNA conferring biological robustness of embryonic development. Therefore, next we focused on the following question: what process during embryogenesis does *miR-9a* have a role in?

First, we analyzed the *miR-9a* expression pattern in the developing embryo using the locked nucleic acid (LNA) in situ hybridization assay and observed that, starting from developmental stage 11, *miR-9a* was detected in parasegments (Figure 1B). At embryonic stage 13–14, *miR-9a* shows an obvious striped expression pattern and was localized in the epidermal cells of segmental grooves (Figure 1B, arrows). The grooves contain invaginations of epidermal cells that accept a smooth cuticle cell fate and differentiate into tendon-like cells upon induction of the expression of the epidermal-growth-factor-like transcription factor Stripe (Sr; Frommer et al., 1996). Importantly, the embryonic lethality caused by *miR-9a* deficiency was rescued by specific expression of exogenous *miR-9a* in tendon cells in *miR-9a* loss-of-function background (Figure 1C; Table S1). These results together with highly specific *miR-9a* embryonic expression pattern suggest that this miRNA functions predominantly in the tendon cells.

Tendons are connected to the muscle via the specialized tendon matrix, forming the muscle-tendon attachment called the MTJ. The architecture of the MTJ can be easily visualized by Tiggrin (Tig), an ECM component of the tendon matrix (Fogerty et al., 1994) Titin, that outlines the end of the myotube facing the tendon matrix (Fabian et al., 2007), and TropoMyosin (TM), which contours muscles fibers (Bullard et al., 1988). In *Control*, at embryonic stage 16, the ends of all muscles were opposed and attached to the corresponding tendons (Figures 1D and 1E). In *miR-9a* mutants, myotubes elongated; however, more than half of the muscle ends were disintegrated from their attachment sites (Figures 1D and 1E; Table S2). Not only was the process of muscle attachment disturbed, but also the formation of tendons was affected (Figure 1E, arrowheads). Most of the MTJs of *miR-9a* embryos were shorter in length or contained splits and gaps as visualized by Titin staining (Figure 1E). These data show that the presence of *miR-9a* is critical for proper establishment of muscle-tendon attachments.



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Consistent with the highly defined temporal and spatial expression pattern of *miR-9a* during embryogenesis and its specific role in establishment of MTJs, *miR-9a* deficit led to high embryonic, but not postembryonic, lethality. Approximately 60% of the *miR-9a*-deficient embryos hatched, and *miR-9a* larvae and adults showed no gross morphological defects in muscle organization (Figure 1F), confirming that *miR-9a* does not play an essential role but rather a refining role during embryogenesis.

Many Key Muscle Genes Are Putative *miR-9a* Targets

We analyzed possible *miR-9a* targets using TargetScan, PicTar, miRanda, and TarBase miRNA target prediction algorithm databases (Enright et al., 2003; Garcia et al., 2011; Grün et al., 2005; Kheradpour et al., 2007; Vergoulis et al., 2012) and, interestingly, found that more than 15% of all putative *miR-9a* targets are key regulators of muscle development (Figure 2A; Table S3). If *miR-9a* were capable of downregulating multiple genes essential for muscle maintenance and differentiation, then it is probable that misexpression of this miRNA in the muscle tissue would cause substantial defects. To test this assumption, we exogenously expressed *miR-9a* using different tissue-specific *Gal4* drivers. As expected, we found that *miR-9a* should not be present in muscle cells, since exogenous expression of *miR-9a* in mesodermal cells using different muscle-specific drivers resulted in embryonic lethality with the same frequency as with ubiquitous drivers (Figure 2B; Table S1). On the contrary, when *miR-9a* was overexpressed in the nervous system or in tendon cells, it did not affect embryonic development and the frequency of embryonic survival was comparable to that of *Control* (Figure 2B; Table S1). This implies that *miR-9a* does not have targets fundamental for neuronal and tendon cell fate determination and that the endogenous levels of *miR-9a* effectively obliterate its targets in those tissues, therefore increasing *miR-9a* levels there does not impact embryogenesis.

Consistent with the idea that multiple *miR-9a* targets are essential muscle genes, the causes for the lethality upon mesodermal *miR-9a* expression were severe abnormalities in musculature development. The severity of this phenotype depended on the expression strength and developmental timing of used mesodermal driver (Figure S1G). The *mef2-Gal4/UAS-miR-9a* embryos showed defects in myoblast fusion and muscle attachment processes (Figure 2C, arrowheads). Even though some *twi-Gal4/UAS-miR-9a* escapers could survive until the larval stage, they could not molt and died at the second instar (Figures S1A and S1B). Molting requires strong muscle contractions, but in the mutant larvae, muscles were underdeveloped, rounded, and lacked proper attachments to their tendons when compared to *Control* (Figures S1A′ and S1B′). Even at the later preadult stages, initiation of *miR-9a* expression in mesoderm (*mhc-Gal4/UAS-miR-9a*) caused substantial muscle phenotypes: most of the indirect flight muscles were significantly smaller or totally absent (Figures S1C and S1D), showing that the presence of *miR-9a* in muscle tissue is fatal. Notably, *miR-9a* misexpression led to severe muscle phenotypes only in developing, but not in terminally differentiated adult muscles (Figures S1E and S1F), suggesting that *miR-9a* predominantly targets genes important for muscle development.

Next, we tested if any muscle-related candidates are misregulated in *miR-9a* mutants. Due to high embryonic lethality in *miR-9a* mutants (Table S1) and an observation that *miR-9a* is also expressed postembryonically (Figure S2A; Table S4), we analyzed in adult *miR-9a*^{LOF} survivors the mRNA levels of predicted *miR-9a* target genes involved in muscle and neuromuscular junction development. We observed that the mRNA levels of *wit*, *myoblast city* (*mbc*), *htl*, *Dg*, *derailed* (*drl*), *kettin*, and *SCAR* were significantly increased due to loss of *miR-9a* (Figure 2D; Table S5). Next, we overexpressed this miRNA using preadult *mhc-Gal4* and adult induced *24B-Gal4*^{ts} mesodermal drivers to test the effect of exogenous *miR-9a* expression in muscles on predicted targets. Since exogenous expression of *miR-9a* with *mhc-Gal4* causes severe muscle loss and degeneration (Figure S1D), levels of the most tested muscle genes were reduced (Figure 2D; Table S5). Postdevelopmentally induced muscle expression of *miR-9a* did not affect muscle formation (Figure S1E), but it still led to significantly reduced levels of *wit*, *Dg*, *CG9849*, and *upheld* (*up*; Figure 2D; Table S5). Levels of *drl* were always upregulated, while *up* was always downregulated in *miR-9a* loss- and gain-of-function mutants. Since levels of *wit*, *mbc*, *htl*, *Dg*, and *kettin* were reciprocally affected, it is conceivable that *miR-9a* can directly target their mRNA. Improper mRNA expression levels of other genes imply that *miR-9a* can also indirectly affect multiple factors regulating muscle development. Since *miR-9a* muscle target genes are

Figure 1. *miR-9a* Is Expressed Dynamically during Embryogenesis and Is Required for Embryonic Survival and MTJ Formation

(A) Loss of *miR-9a* decreases embryonic survival at higher (25°C and 29°C) but not at the lower (18°C) temperatures.

(B) At embryonic stage 11, *miR-9a* is expressed at low levels in the ectodermal cells of parasegmental grooves. At stage 14, *miR-9a* is clearly expressed in the cells surrounding the parasegmental grooves. No *miR-9a* expression is detected in mutant *miR-9a* embryos. Right upper panel shows enlarged lateral view of the stage 13 epidermis with *miR-9a* expression in the two- to three-cell row surrounding epidermal invagination sites (white arrows). Right lower panel is an enlarged ventral view of the stage 14 epidermis showing *miR-9a* expression in epidermal cells at the location of MTJs.

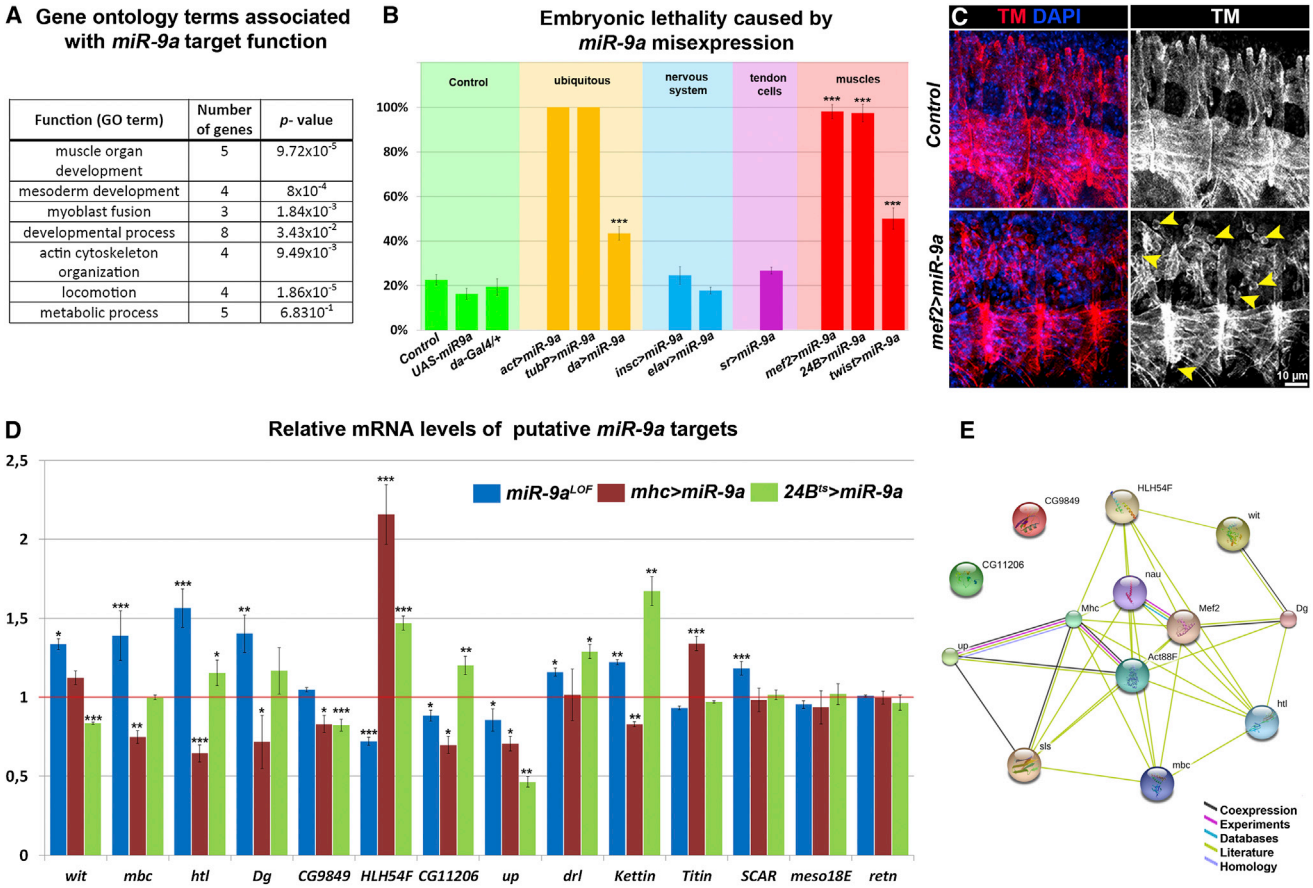
(C) Loss of *miR-9a* (*miR-9a*^{LOF}) leads to high embryonic lethality, which can be fully rescued by exogenous expression of *miR-9a* in tendons (*sr-Gal4/UAS-miR-9a; miR-9a*^{LOF}).

(D) The lateral view of embryo at stage 16 stained with antibodies against Tig that marks the MTJs and TM to visualize muscles. In *miR-9a* embryos, both MTJs (Tig; red) and muscles (TM; green) appear disorganized in comparison to *Control*.

(E) Titin is not properly localized (arrowheads) in *miR-9a* embryos when compared to *Control*. Note the appearance of shorter MTJs that also have gaps and splits. In addition, ventral longitudinal muscles (marked by TM) are not properly attached.

(F) Both *miR-9a* hatched larvae and eclosed adults do not exhibit any significant muscle architecture defects in comparison to *Control*. The frequency of muscle defects counted from muscle sections of adult flies is similar in *Control* and *miR-9a* mutants (*Control* 5.3% ± 4%, n = 107 muscles; *miR-9a*^{LOF} 7.5% ± 3%, n = 101).

Lateral views of embryos are shown with anterior to the left (B, D, and E). Panels in (B) and (F) (adult) are single-plane views. Panels in (D)–(F) (larva) are maximum intensity projections of confocal Z-stacks. Data presented as average (AVE) ± average deviation (AD) (A and C) and AVE ± SEM (D). ***$p < 0.001$, calculated by a two-tailed Student's t test. See also Tables S1 and S2.



Function (GO term)	Number of genes	*p*- value
muscle organ development	5	$9.72x10^{-5}$
mesoderm development	4	$8x10^{-4}$
myoblast fusion	3	$1.84x10^{-3}$
developmental process	8	$3.43x10^{-2}$
actin cytoskeleton organization	4	$9.49x10^{-3}$
locomotion	4	$1.86x10^{-5}$
metabolic process	5	6.8310^{-1}

Figure 2. *miR-9a* Affects Muscle Gene Expression and Leads to Increased Embryonic Lethality When Ectopically Expressed in the Mesodermal Tissue

(A) A table of GO terms of putative *miR-9a* targets shows enrichment within the groups associated with muscle biology (see also Table S3 for target gene function).

(B) Ectopic expression of *miR-9a* using ubiquitous (*tub-Gal4*, *act-Gal4*, and *da-Gal4*) and muscle (*24B-Gal4*, *mef2-Gal4*, and *twist-Gal4*), but not nervous (*insc-Gal4* and *elav-Gal4*) and tendon (*sr-Gal4*) specific drivers leads to increased embryonic lethality when compared to *Control*.

(C) Ectopic expression of *miR-9a* with the *mef2-Gal4* driver results in myoblast fusion defects of lateral and ventral muscles (round cells, arrowheads; TM, red; DAPI, blue).

(D) Relative expression levels of multiple putative *miR-9a* targets important for muscle differentiation are altered in *miR-9a* adults or flies exogenously expressing *miR-9a* in muscle tissue with the preadult *mhc-Gal4* and adult induced *24B-Gal4/tub-Gal80^{ts}* drivers. Note that *titin* and *kettin* are two isoforms of the *sallimus* gene, but only *kettin* contains *3′UTR* with *miR-9a* binding site and responds to *miR-9a* levels.

(E) A map of known and predicted protein interactions built using STRING database, which includes predicted *miR-9a* targets that are shown to be misregulated due to *miR-9a* deficiency and overexpression in the muscle tissue.

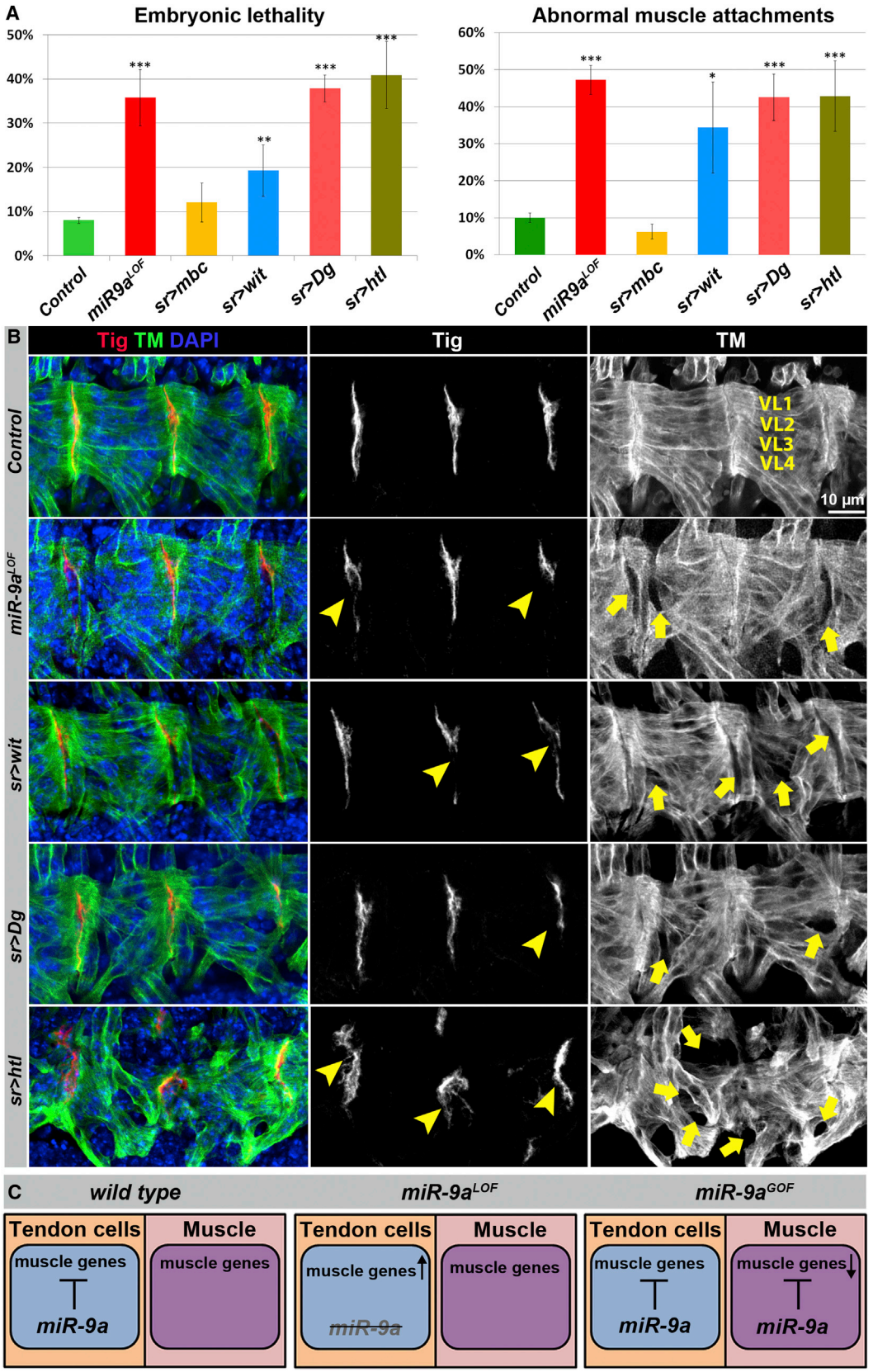
Panels in (C) are maximum intensity projections of confocal Z-stacks. Data presented as AVE ± SD; *p < 0.05, **p < 0.005, ***p < 0.001, calculated by a two-tailed Student's t test. See also Figures S1 and S2 and Tables S1, S3, and S5.

interconnected in the regulatory circuitry controlling muscle development and maintenance (Figures 2E), *miR-9a* misexpression destabilizes this developmental network. These results allow us to propose that the miRNA, *miR-9a* can regulate various genes crucial for muscle development and that some of them could potentially be its direct targets.

Also, we analyzed expression levels of some *miR-9a* putative target genes and found that one-third of them were significantly upregulated at higher temperature and half of them were significantly downregulated at lower temperature (Figure S2C; Table S5), while *miR-9a* levels did not show any temperature dependence (Figure S2D; Table S4). Fluctuation in expression levels of *miR-9a* targets reveals differential requirements for this miRNA at different conditions. This result helps to get insight into the molecular basis as to why *miR-9a* mutants have different survival rates at various temperatures and suggests that *miR-9a* acts as one of the factors that prevent aberrant muscle gene expression in tendon cells upon fluctuating external conditions (Figure S2B).

Misexpression of Predicted *miR-9a* Muscle Targets in Tendons Causes MTJ Defects

To test this assumption, we analyzed misexpression of some of the potential "direct" *miR-9a* targets in tendon cells (*mbc*, *wit*, *Dg*, and *htl*). We found that overexpression of *wit*, *Dg*, and *htl* in tendons caused embryonic lethality (Figure 3A; Table S1). Even more, upon tendon-specific overexpression of these putative *miR-9a* targets, the occurrence of aberrant and



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discontinuous MTJs was significantly increased and was comparable to the frequency of muscle attachment defects of *miR-9a* mutants (Figure 3A; Table S2). Similar to *miR-9a* MTJs, muscle attachment sites were underdeveloped and some myotube ends were separated from their attachment sites (Figure 3B; Table S2), showing that the presence of these genes in the tendon is disastrous for MTJ development (Figure 3C).

Interestingly, all candidates for *miR-9a* targeting in the tendon (*wit*, *Dg*, and *htl*) are transmembrane proteins implicated in extracellular signaling. Wit is the *Drosophila* homolog of the vertebrate bone morphogenetic protein type II receptor (Aberle et al., 2002), Htl is the *Drosophila* homolog of the vertebrate fibroblast growth factor receptor (Gisselbrecht et al., 1996), and evolutionarily conserved Dg is a major nonintegrin ECM receptor involved in cell-ECM adhesion (Deng et al., 2003). Since the ECM plays a crucial role for MTJ assembly, our data suggest that loss of *miR-9a* may affect MTJ formation due to alteration in the tendon matrix.

The ECM Receptor Dg Is a *miR-9a* Target

In general, cells can attach to the ECM via integrins or proteoglycan (e.g., Dg) complexes that do not contain integrins. While the role of integrins in MTJs has been extensively studied, Dg involvement in the process has not been documented in *Drosophila*. The ECM receptor Dg is best known as a key component of the Dystrophin glycoprotein complex (DGC) that is responsible for development of a variety of muscular dystrophies. Like in integrins, the extracellular part of the Dg protein binds to essential ECM components and plays a role in ECM constitution, while the cytoplasmic tail connects to the actin cytoskeleton via the cytosolic protein Dystrophin (Figure 4A).

First, we tested whether Dg is a bona fide *miR-9a* target. Analysis of *Dg 3′UTR* using the TargetScan database showed the presence of multiple miRNA binding sites, including *miR-9a* (Figure 4B). To validate whether Dg is regulated by this miRNA, we performed an in vitro luciferase assay with an ~300 bp fragment of *Dg*'s *3′UTR* and observed a 2.7-fold decrease in luciferase activity upon overexpression of *miR-9a* (Figure 4C). To test if *miR-9a* regulates Dg in vivo, we examined if Dg protein levels are changed due to miRNA overexpression in follicle epithelial cells, where Dg has been shown to have a distinct pattern and where clonal cells can be easily introduced and analyzed (Deng et al., 2003). We found that the levels of Dg decreased by almost two times as a result of exogenous expression of *miR-9a* in the follicular epithelium (Figure S3). Together, these data imply that *miR-9a* can regulate Dg in vitro and in vivo.

Second, we analyzed the expression pattern of the ECM receptor, Dg, in the developing *Drosophila* embryo and found that at earlier stages, the Dg protein is uniformly present in all epidermal cells (Figure 4D); however, starting from stage 13–14, Dg is detected in a striped pattern (Figure 4D). It is absent from the epidermal cells of the segmental grooves that will give rise to the tendon cells. At stage 16, the Dg expression pattern becomes even more defined, where Dg is present in multiple cell types (Shcherbata et al., 2007); however, it is excluded from tendon cells (Figure 4D). Importantly, the onset of *miR-9a* expression coincides with the time period when Dg is eliminated from these epidermal clusters (compare enlarged panels in Figures 1B and 4D). These data show that *miR-9a* and Dg are expressed in a mutually exclusive pattern, suggesting that Dg is regulated by *miR-9a* in the developing embryo.

Third, to prove that *miR-9a* targets Dg in tendons, we analyzed Dg expression levels at the sites of muscle-tendon interactions in *Control* and *miR-9a* embryos. Careful analysis of the Dg protein expression levels demonstrated that Dg expression at the tendon matrix was muscle specific; moreover, higher amounts of Dg are concentrated at the periphery of muscle or at the myotube ends, while tendon cells were devoid of Dg (Figures 4E and 4G). Upon *miR-9a* deficiency, Dg could be detected not only in muscles at muscle attachment sites, but also on the membrane of tendon cells (Figures 4E and 4G). This improper expression of Dg resulted in disorganization of musculature assembly and alteration of contacts between muscle cells and tendons, demonstrating that *miR-9a*-mediated targeting of Dg in tendon cells is one of the prerequisites for accurate development of MTJs. In agreement with this hypothesis, the embryonic lethality rate was significantly, but not fully, rescued upon reduction of Dg by one copy in the *miR-9a*-deficient background (Figure 4F; Table S1), implying that other factors are managed by this miRNA.

Fourth, to investigate if MTJ phenotypes observed in *miR-9a* mutant embryos and embryos with ectopic expression of Dg in tendon cells are not caused by abnormalities in tendon or muscle founder cell fates, we analyzed the distribution of specific myoblast and tendon cell identity markers (Figure S4). We could not observe any gross defects, indicating that these cell identities were not changed upon *miR-9a* deficiency or Dg misexpression.

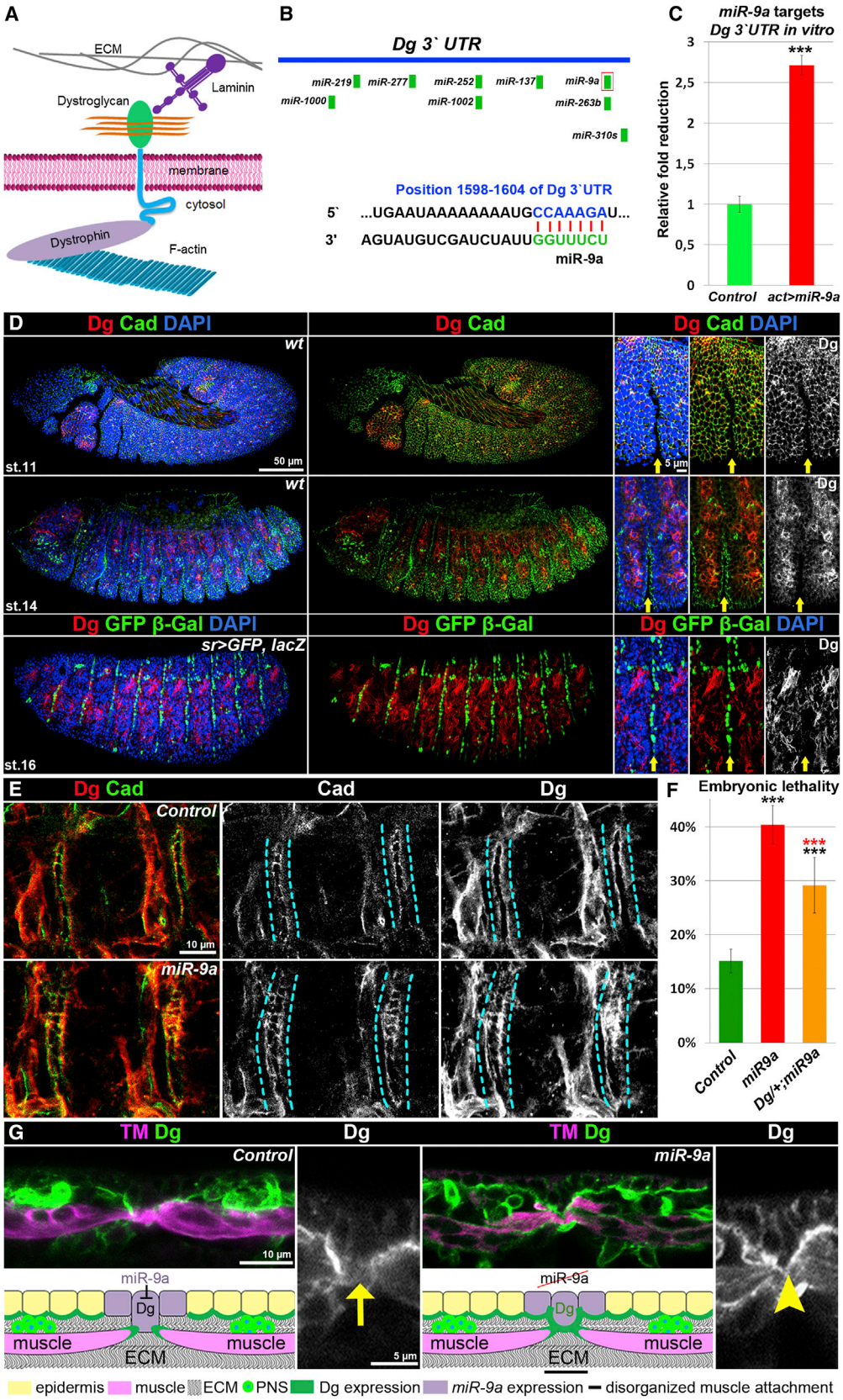
Together, these data show that during embryogenesis, both *miR-9a* and Dg have dynamic expression patterns that become mutually exclusive in the regions of muscle-tendon connections. Dg protein is present in all ectodermal cells, except for the ones that are differentiating into epithelial tendon cells and are also expressing *miR-9a*. Interestingly, a detailed analysis of *Dg mRNA* isoforms during *Drosophila* embryogenesis showed that *Dg mRNA* can be detected in some of the tendon cells (Schneider and Baumgartner, 2008), while Dg protein is not present in these cells, which necessitates its posttranscriptional regulation. Our data demonstrate that elimination of Dg from tendon precursor cells is required for proper muscle attachment

Figure 3. Ectopic Expression of Predicted *miR-9a* Targets in the Tendon Cells Leads to High Embryonic Lethality and Affects MTJ Morphology

(A) Ectopic expression of potential *miR-9a* targets (*wit*, *Dg*, and *htl*) leads to increased embryonic lethality (AVE ± AD) and a higher percentage of abnormal MTJs per embryo (AVE ± SEM); *p < 0.05, **p < 0.005, ***p < 0.001, calculated using a two-tailed Student's t test. See also Tables S1 and S2.

(B) In contrast to *Control*, the ECM protein Tig is not properly localized in *miR-9a*^{LOF} or embryos ectopically expressing *wit*, *Dg*, or *htl* in tendon cells (*sr>wit; sr>Dg*; *sr>htl*; arrowheads). In addition, ventral longitudinal muscles VL1–VL4 (marked by TM) are not properly attached in these embryos (arrows). Panels shown are maximum intensity projections of confocal Z-stacks.

(C) Models showing the consequence of *miR-9a* differential expression in muscle and tendon cells.



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assembly, and *miR-9a* helps to ensure that Dg is not misexpressed in these precursors. This suggests that *miR-9a* acts as one of the factors that buffer Dg gene expression in tendon cells.

Dg Misexpression Affects the Tendon Matrix

Finally, we wanted to unravel how misexpression of the ECM receptor Dg could affect MTJ assembly. The major players that have been shown to play an essential role in the MTJ establishment are integrins. Since Dg and integrins bind some of the same ECM components, for example Laminin (Lan), we hypothesized that misexpression of Dg affects the tendon matrix composition. To assay whether Dg affects the ECM and to avoid tissue-specific (muscle versus tendon) control of differential gene expression, we turned our attention to the ovarian follicular epithelium, where all cells are of the same origin and their differentiation is synchronized in each egg chamber. According to the genotype, Dg overexpression marked by GFP resulted in very high levels of Dg in the follicle cells (Figure S5A). Notably, Dg-overexpressing cells displayed appreciably lower levels of βPS Integrin (Figure S5B), while the levels of the known Dg ECM binding partner Lan (Figure S5C) were significantly increased. This demonstrates that levels of Dg can modulate amounts of certain ECM components, e.g., Lan, which is a trimeric protein interconnected to form a cross-like structure that binds to other cell membrane and ECM molecules. Lan molecules join to make net-like sheets that spread over the epithelial cells, and amounts of Lan determine 3D structure and composition of the ECM. We observed that an increase in Lan levels was seen not only around the cells overexpressing Dg, but also around the wild-type cells touching the clone (Figure S5, yellow arrows), suggesting that Lan enrichment does not stop at the clone border but is gradually decreasing over the adjacent cells. The increase in Lan amount was coincident with the increased βPS Integrin levels found at the membrane of neighboring cells, which demonstrates that higher levels of Lan can stimulate βPS Integrin levels and suggests that ECM receptor levels can be readjusted via modulation of the ECM gradient (Figure S5D).

Since tendons and muscles share the ECM at the MTJ, next we wanted to understand how misexpression of Dg would affect the ECM constitution at the muscle-tendon site and cause abnormal MTJs. Based on our analysis in follicular epithelium, which demonstrated that Dg levels can influence the amount of βPS Integrin cell autonomously (since cells overexpressing Dg have decreased βPS Integrin levels) and cell nonautonomously (since neighboring cells have increased βPS Integrin levels), we hypothesized that due to Dg misexpression in tendons, the ECM assembly at the MTJ would be changed. This, in turn, would alter the ECM receptor βPS Integrin levels in the muscle and tendon cells. To test this assumption, we analyzed the distribution of the ECM receptor βPS Integrin upon Dg overexpression in tendon cells and found that the levels of βPS Integrin were significantly reduced in tendon cell bodies (Figures 5A, red rectangles, and 5B). At the same time, amounts of βPS Integrin in muscles (yellow rectangles) and MTJs (cyan rectangles) were increased by ~30% (Figure 5B). As a result of this misexpression, the length and area of MTJs were significantly reduced (Figure 5B). Additionally, more Lan could be found at the mutant tendon matrix resulting from ectopic Dg expression (Figure 5C, yellow arrowheads) in comparison to *Control,* where Lan is present at the MTJs and in the basement membranes of muscles, being evenly enriched at the edge where muscles are connected to tendons (Figure 5C, arrows).

Thus, we propose two mechanisms, which are not mutually exclusive, of how misregulation of Dg can be influencing MTJ assembly. First, Dg competes with other ECM receptors for membrane localization, which would change the ratio and/or composition of ECM receptors at the cell membranes and result in changes in cell-ECM adhesion. Second, Dg misexpression alters the ECM, which in turn signals to the cells to readjust accordingly to the altered ECM composition, the expression levels of the cell adhesion proteins at the transcriptional level. To test these hypotheses, we used different transgenic constructs encoding for full-length or truncated Dg protein. Interestingly, only when transgenes that contained the extracellular domain of Dg (Dg full-length:*UAS-Dg-FL* and Dg C-terminal end deletion:*UAS-Dg-C1*) were misexpressed in tendon cells, embryonic survival was affected (Figure 5D; Table S1). Contrarily, ectopic expression of Dg with a truncated extracellular domain (*Dg-ΔExD*) did not lead to a significant increase in embryonic lethality (Figure 5D; Table S1). Since this construct still contains the transmembrane domain, the first hypothesis that upon overexpression Dg simply outcompetes integrins for the

Figure 4. *miR-9a* Targets Dg in Embryo MTJs

(A) Schematic drawing of *Drosophila* DGC.

(B) Localization and the sequence of *miRNA* binding sites in the *Dg*'s *3′UTR*.

(C) Ectopic expression of *miR-9a* in S2 cells downregulates *Dg-3′UTR* luciferase reporter (relative downregulation of luciferase activity [AVE ± SEM]: *Control*, 1.00 ± 0.10; *act>miR-9a*, 2.71 ± 0.12).

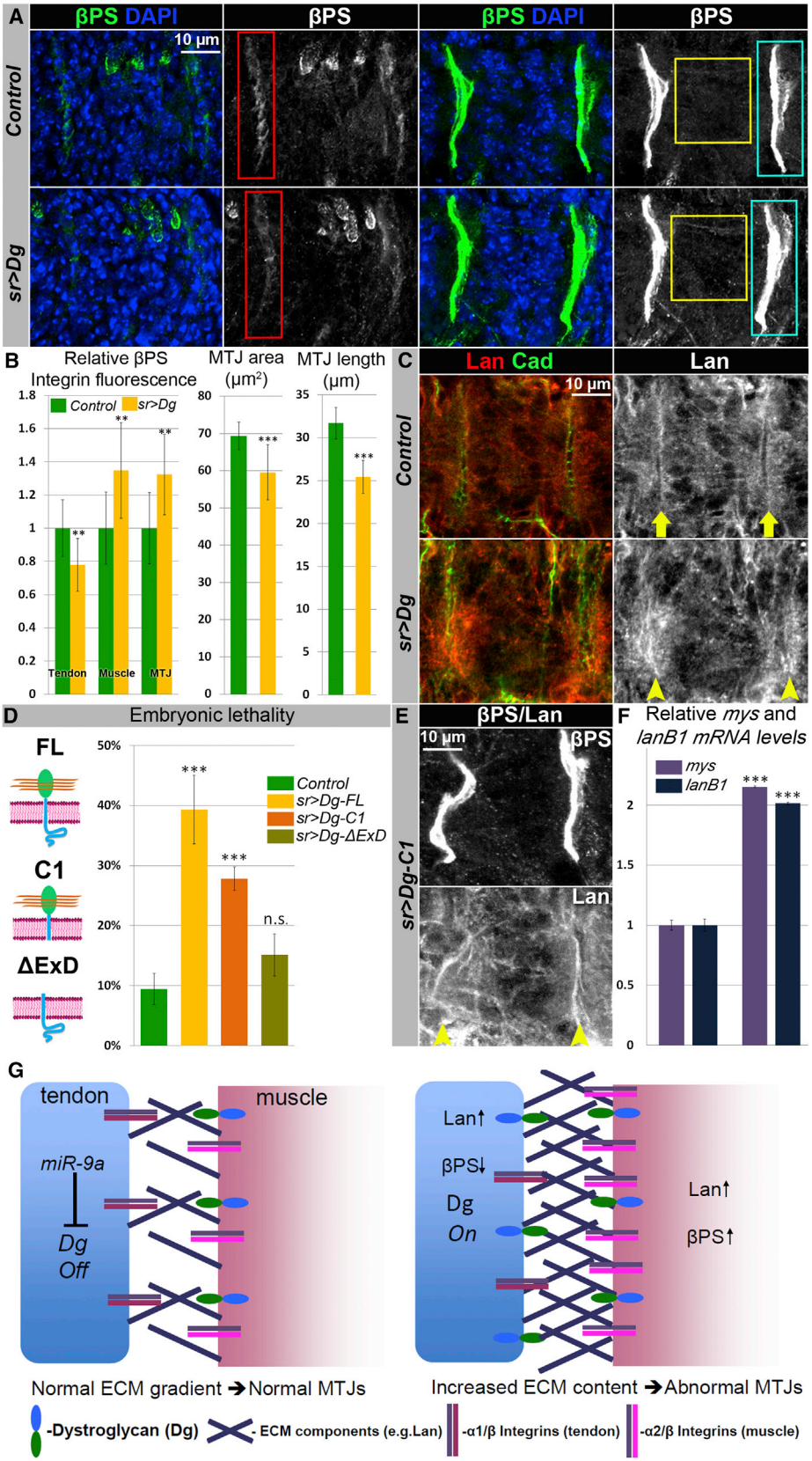
(D) At embryonic stage 11, Dg protein (red) is present uniformly in the ectoderm marked by DE-Cad (green). At stage 14, higher levels of Dg protein are detected in the epidermis in the middle of parasegments but not in the epidermal cells surrounding the grooves. At stage 16, Dg staining does not overlap with the tendon cell marker Sr visualized by expression of GFP and lacZ driven by *sr-Gal4*. Right panels show the enlarged view of embryo segments of the corresponding stages shown in the left panels. Lateral views of embryos are shown with anterior to the left.

(E) In wild-type embryo, Dg is enriched in the muscle fibers at the MTJ site, stained with Cad (green) that marks the membrane of tendon cells (cyan dashed lines). In *miR-9a* embryos, higher levels of Dg can be observed at the muscle attachments and in the membrane of tendon cells (note the yellow, ladder-like pattern in tendons resulting from Dg and Cad colocalization).

(F) The lethality caused by *miR-9a* loss can be partially rescued by reducing one copy of *Dg* (Dg^{086}/+; $miR\text{-}9a^{LOF}$). Data presented as AVE ± AD, ***p < 0.001, calculated by a two-tailed Student's t test.

(G) Ventral view of the embryonic MTJ showing higher levels of Dg protein in *miR-9a* tendons (arrowhead) when compared to *Control* (arrow). Schematic drawings of the ventral view of embryonic MTJs showing that the role for *miR-9a* in tendon cells is to downregulate the muscle gene Dg, which allows for proper assembly of MTJs.

Panels in (D)–(G) are maximum intensity projections of confocal Z-stacks. See also Figures S3 and S4 and Table S1.



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membrane localization cannot fully explain the βPS Integrin mislocalization phenotype. Importantly, misexpression of Dg that contains the extracellular, but lacks the intracellular, domains (*Dg-C1*) affected MTJ formation and caused abnormal βPS Integrin and Lan distribution at the MTJs, similarly to tendon-specific overexpression of full-length Dg (Figure 5E). This supports the hypothesis that the presence of the ECM-interacting domain of Dg is sufficient to modulate the expression of proteins at the tendon matrix. Therefore, next we tested whether abnormal ECM caused by Dg misexpression would affect βPS Integrin and Lan expression at the transcriptional level. We measured the levels of βPS Integrin (*myospheroid* [*mys*]) and Laminin B1 (*LanB1*) mRNAs and found that, upon Dg overexpression in tendon cells, mRNA levels of *mys* and *LanB1* are significantly increased (Figure 5F), which is consistent with the increased amounts of βPS Integrin protein seen in muscles and Lan detected at the MTJs. These results demonstrate that Dg misexpression in tendons cell autonomously influences LanB1 and βPS Integrin levels and attracts more ECM proteins to the tendon matrix, which cell nonautonomously enhances βPS Integrin expression in the attached muscle cells (Figure 5G). Collectively, these data support the idea that the transmembrane receptor Dg is involved in regulation of the ECM constitution at the muscle-tendon site and is capable of readjusting the levels of ECM receptors in both tendon and muscle cells at the level of transcription. Therefore, managing the precision of Dg's differential expression in muscle versus tendon cells is key for normal tendon matrix constitution and signaling, and *miR-9a* plays an important role in this process.

DISCUSSION

Here we show that the muscular-dystrophy-associated ECM receptor Dg can be posttranscriptionally regulated by *miR-9a*. During embryogenesis, both *miR-9a* and Dg have dynamic expression patterns that become mutually exclusive in the regions of muscle-tendon connections. Dg protein is present in all ectodermal cells, except for the ones that are differentiating into epithelial tendon cells and are also expressing *miR-9a*. Our data show that the elimination of Dg from tendon precursor cells is required for accurate muscle-tendon matrix assembly. The *miR-9a* ensures that Dg is not misexpressed in tendon precursors due to leaky transcription, as these epidermal cells invaginate into and reside within the mesoderm.

Embryonic development is an extremely dynamic process in which rapid cell specifications and rearrangements take place, features indicative of the need for stabilization. miRNAs have been implicated in stabilization of biological robustness in different animal systems (Ebert and Sharp, 2012). We now found that miRNAs are involved in the stabilization of the process of muscle-tendon attachment in the developing *Drosophila* embryo. In particular, our data imply that *miR-9a* acts as a backup mechanism in tendons to diminish the effects of leaky expression of a group of muscle genes. When two adjacent cells have different cell fates, evolutionarily it would make a lot of sense for a canalization factor in one cell type to regulate multiple genes critical for the differentiation of the other cell type. Apparently, many essential muscle differentiation genes are *miR-9a* predicted targets, and their ectopic expression in ectodermal tendon cells causes embryonic lethality and abnormal MTJs. Moreover, exogenous expression of *miR-9a* in the mesoderm completely abolishes muscle formation. With this in mind, we hypothesize that *miR-9a* specifically acts as a guardian to prevent aberrant muscle gene expression in the epidermal tendon precursor cells.

It has already been shown that *miR-124* (Smirnova et al., 2005; Sun et al., 2012; Weng and Cohen, 2012) and *miR-9a* (Bejarano et al., 2010; Biryukova et al., 2009; Li et al., 2006, 2013) act to canalize nonneuronal versus neuronal fates. Genes expressed in the nervous system are highly enriched for *miR-9a* binding sites (Stark et al., 2005), and our data show that multiple muscle genes also have *miR-9a* target sites. Since *miR-9a* is ectoderm specific and genes expressed in ectodermal tissues avoid *miR-9a* sites, previous findings and our findings insinuate that *miR-9a* can act as the key ectodermal canalization factor that protects ectodermal cell fate by repressing genes of the sibling tissues (such as muscle and nervous). This should reinforce the robustness of ectodermal cell differentiation. It would be interesting to find in the future if miRNAs that canalize mesodermal or endodermal fate exist and to address the question of

Figure 5. Dg Affects βPS Integrin Localization and ECM Composition at the Embryonic MTJs

(A) Ectopic expression of Dg in tendons (*sr>Dg*) decreases βPS Integrin levels in the tendon cell bodies (red rectangles); however, it increases βPS Integrin levels at the MTJs (*sr>Dg*; cyan rectangles) and in muscles (*sr>Dg*; yellow rectangles) in comparison to *Control*.

(B) Relative levels of βPS Integrin fluorescence in tendons (*Control*, 1.00 ± 0.17, n = 19 areas; *sr>Dg*, 0.78.4 ± 0.16, n = 15), in muscles (*Control*, 1.00 ± 0.21, n = 15 areas; *sr>Dg*, 1.35 ± 0.29, n = 17), and in MTJs (*Control*, 1.00 ± 0.22, n = 18 MTJs; *sr>Dg*, 1.32 ± 0.24, n = 15). Ectopic expression of Dg in tendon cells affects the MTJ's length (*Control*, 31.7 ± 1.8 μm, n = 18 MTJs; *sr>Dg*, 25.4 ± 1.8 μm, n = 15) and area (*Control*, 69.4 ± 3.7 μm^2, n = 18 MTJs; *sr>Dg*, 59.6 ± 7.4 μm^2, n = 15).

(C) Ectopic expression of Dg in tendon cells (*sr>Dg*) leads to accumulation of Lan (arrowheads) when compared to *Control* (arrows).

(D) Ectopic expression in tendons of the full-length and truncated Dg protein without the intracellular domain (*sr>Dg-FL* and *sr>Dg-C1*) leads to increased embryonic lethality compared to *Control*; however, ectopic expression of Dg without the extracellular domain (*sr>Dg-ΔExD*) does not significantly affect the embryonic survival.

(E) Ectopic expression of the truncated Dg containing the ECM-binding domain (*sr>Dg-C1*) causes abnormal accumulation of βPS Integrin and Lan (arrowheads) at the MTJ, which is similar to the defects seen at the MTJs of embryos overexpressing the full-length Dg (*sr>Dg-FL*; A and C). The frequency of disorganized MTJ in *sr>Dg-C1* mutants is 57%, n = 14 MTJs.

(F) Ectopic expression of Dg in tendon cells affects the levels of *mys* (*Control*, 1.00 ± 0.04; *sr>Dg*, 2.15 ± 0.01; $p = 6.4 \times 10^{-3}$) and *lanB1* (*Control*, 1.00 ± 0.05; *sr>Dg*, 2.01 ± 0.01; $p = 1.5 \times 10^{-7}$) mRNAs measured by qRT-PCR.

(G) Scheme describing the potential impact of Dg misexpression in the tendon cells observed in *miR-9a* mutants and its effect on ECM composition.

Measurements of βPS Integrin fluorescence were done from Z-stack projections (~1–2 μm thick for tendon cell membranes; ~9–11 μm for MTJs and muscle areas).

Panels in (A), (C), and (E) are maximum intensity projections of confocal Z-stacks. Data presented as AVE ± AD in (B) and (D) and AVE ± SD in (F); *p < 0.05, **p < 0.005, ***p < 0.001, calculated by a two-tailed Student's t test. See also Figure S5 and Table S1.

whether there are more examples of the miRNAs that target multiple genes from the same functional network.

One of the muscle genes that we show is a bona fide *miR-9a* target is the ECM receptor, Dg. The transmembrane protein Dg has a distinct expression pattern at the MTJ. It is present at the membrane of the developing muscle and is enriched at the myotube ends; however, it is absent from the tendon cell membranes. Similarly, a restricted expression pattern of Dg is required for neuromuscular junction (NMJ) establishment in vertebrates (*Xenopus*), with Dg being present at the entire muscle membrane and showing enrichment at the NMJ site, where it acts as a sink for the ECM component agrin, preventing its binding to muscle-specific kinase (MuSK). At the site of nerve contact, in the absence of Dg, agrin can bind to MuSK, allowing acetylcholine receptor aggregation and synaptic development (Heathcote et al., 2000). Thus, due to the distinct expression patterns in cells that form connections via the ECM, Dg is able to establish the ECM gradient, which, as we show, is also essential for proper formation of MTJs in the developing *Drosophila* embryo. It would be interesting to investigate if there is a regulatory molecule that is differentially distributed between muscle and tendon due to specific binding to Dg at the MTJ.

The extracellular environment of the cell is a complex organization of ECM receptors, matrix proteins, and the regulatory molecules that reside in it. Also, it continuously changes during development and allows rapid communication between different cells to coordinate tissue formation (Edeleva and Shcherbata, 2013). Therefore, changes in the composition of the ECM can have a profound effect on an organism's development. Our study shows that *miR-9a*-based regulation of Dg is needed to adjust the ECM composition at the MTJ. Regulation of the affinity of the transmembrane adhesion receptor integrins has a key role during development as it generates strong adhesion of cells to the insoluble ECM (Pines et al., 2011). Now we show that, at the MTJ, Dg also acts as a receptor regulating ECM gradient at the tendon matrix, since Dg levels affect the amount of the ECM protein Lan. In addition, Dg can modulate expression of a key ECM receptor, βPS Integrin. These data are consistent with previous findings revealing a regulatory pathway between the DGC and integrin receptors and lends the idea that Dg is involved in selective regulation of integrin gene expression (Côté et al., 2002; Hodges et al., 1997). Moreover, integrin overexpression alleviates the development of muscular dystrophy phenotypes in *mdx* mice (Burkin et al., 2001), supporting the possibility that Dg and integrin compensate for each other in mediating cell-ECM adhesion. Additionally, we show that this regulation can be cell nonautonomous, since abnormal Dg levels, through modification of Lan amounts, affect integrin expression in the neighboring cells. In intestinal epithelial cells, the DGC coprecipitates with β1-integrin, suggesting a possible direct interaction among these proteins where the strength of this interaction depends on the Lan type (Driss et al., 2006). Moreover, it has been shown that increased Lan expression can ameliorate muscular dystrophy in mice (Goudenege et al., 2010; Van Ry et al., 2014). These results, on the one hand, support our findings that alterations in Lan levels influence the expression of the ECM receptors but, on the other hand, pose an interesting question of why, depending on the animal's genetic background (dystrophic or not), the increased levels of Lan have positive or negative effects on MTJs and muscles. The beneficial influence of Lan was seen so far only when it was upregulated in dystrophic animals: muscular dystrophy *mdx* mouse (dystrophin deficient), $dy^{w-/-}$ merosin-deficient congenital muscular dystrophy mouse model (Lan deficient), and muscular dystrophy zebrafish model (Dg reduced). All above muscular-dystrophy-related components per se are required for accurate Lan localization and distribution, suggesting that restoration of Lan levels in the ECM has favorable effects on dystrophic muscle maintenance. Previous studies did not address what would happen if Lan were overexpressed in the otherwise normal background or they were differentially upregulated in muscle or tendons. Since elegant studies on the role of Lan-111 in muscle development and maintenance (Van Ry et al., 2014) propose Lan protein therapy as a treatment option for muscular dystrophy patients, it would be important in the future to study, using different models, the effects of differential Lan expression on muscle-tendon attachments during development and to determine the levels that can be tolerated without induction of deleterious effects on muscle maintenance and tendon attachments during adulthood.

Taken together, previous studies and our findings show that the amounts and types of the ECM receptors affect ECM constitution and govern its remodeling, and then via "dynamic reciprocity" the ECM readjusts intracellular signaling, gene expression, and morphology of the cells and tissues. We show that the crosstalk between tendons and muscles depends on differentially expressed ECM receptor Dg that, together with integrins, helps to establish the ECM gradient. The information about the tendon matrix composition is communicated back to the muscles and tendon cells that readjust their ECM receptor expression profiles in order to reinforce and stabilize the MTJ. Providing a link between the ECM and cytoskeleton, Dg acts as a vital signal-transducing element that allows for communication between the cell's outer environment and inner milieu. In vertebrates, Dg is implicated in multiple biological processes: for example, formation of spatiotemporally regulated microenvironments necessary for muscle fiber morphogenesis at the MTJ (Snow and Henry, 2009). In *Drosophila*, in addition to its function in muscle maintenance (Kucherenko et al., 2011; Shcherbata et al., 2007), Dg is involved in control of neuron behavior; modulation of the concentration of postsynaptic and synaptic proteins, in particular the ECM component Lan at the neuromuscular junction (Bogdanik et al., 2008; Marrone et al., 2011a, 2011b); and regulation of miRNA expression profiles (Marrone et al., 2012; Marrone and Shcherbata, 2011). The currently increasing amount of research on the diverse roles of Dg during development demonstrates its critical role in multiple developmental circuits, suggesting that there is a necessity for precise and dynamic regulation of Dg levels. Despite the vast data about posttranslational regulation of Dg activity, Dg posttranscriptional regulation has not been studied. Our data show that Dg can be regulated by miRNAs and this regulation has an important functional role at the MTJ. Since the human homolog of *Drosophila* Dg (Dag1) also contains multiple predicted miRNA biding sites, it would be important to study if miRNAs also play a role in regulation of Dg in mammals. Even though there are numerous studies in vertebrate models indicating that MTJ assembly affects muscle development, the role of aberrant MTJ in muscle maintenance

and function in muscular dystrophy patients is greatly underappreciated; therefore, understanding of the miRNA-based mechanisms controlling the ECM assembly at the MTJ may suggest new directions for muscular dystrophy research.

EXPERIMENTAL PROCEDURES

Drosophila melanogaster stocks were raised on standard medium at 25°C unless stated otherwise. To analyze the specificity of the loss of function phenotypes and to eliminate the possibility of a second site mutation, heteroallelic flies were analyzed. All mutants and transgenes used in the experiments had homogeneous w^{1118} genetic background. Flies were kept on standardized medium and in controlled environment (25°C, constant humidity and the light-dark cycle). The following flies were used: *Control* (*OregonR* crossed to $white^{1118}$) and $miR\text{-}9a^{LOF}$ ($miR\text{-}9a^{J27}$ crossed to $miR\text{-}9a^{E39}$) (both lines were a gift from Fen-Biao Gao) and Dg^{086}/*CyO* (gift from Robert Ray). For ectopic expression the following lines were used: *tub-Gal4/TM3*; *actin-Gal4/CyO*, *twist-Gal4*; *mhc-Gal4*; *mef2-Gal4*; *da-Gal4*; *insc-Gal4*; *elav-Gal4*; *24B-Gal4*, $tub\text{-}Gal80^{ts}$; *UAS-htl* and *UAS-mbc* (Bloomington *Drosophila* Stock Center); *sr-Gal4* (gift from Gerd Vorbrüggen); *UASt-miR-9a* (gift from Eric Lai); *UASt-Dg* and *UASt-Dg-ΔExD* (Deng et al., 2003); *UASp-Dg-Fl* and *UAS-Dg-C1* (Yatsenko et al., 2009); and *UAS-wit* (gift from Michael O'Connor). For rescue experiments the following lines were used: *sr-Gal4*, *UAS-dsRed:miR-9a*; $miR\text{-}9a^{J27}/miR\text{-}9a^{E39}$ and Dg^{086}/*CyO*; $miR\text{-}9a^{J27}/miR\text{-}9a^{E39}$.

Immunofluorescent staining was done using the standard procedure (Shcherbata et al., 2007). The following primary antibodies were used: Titin (rabbit) 1:330 (gift from Deborah J. Andrew), Alien (rabbit) 1:1,000 (gift from Achim Paululat), Tiggrin (rabbit) 1:1,000 (gift from Andrew Simmonds), Nautilus (guinea pig) 1:200 (gift from Susan Abmayr), Kruppel (rabbit) 1:1,000 (gift from Herbert Jäckle), Tropomyosin (rat) 1:400 (Babraham Bioscience Technologies), betaPS (mouse) 1:20, beta-Gal (mouse) 1:20, DE-Cadherin (DE-Cad; rat) 1:20 (DSHB), beta-Gal (rabbit) 1:1,000 (Invitrogen), GFP (chicken) 1:1,000, LanB (ab47651; rabbit) 1:1,000 (Abcam), and Dg (rabbit) 1:1,000 (gift from Hannele Ruohola-Baker). The following secondary antibodies were used: Alexa 488, 568, or 633 goat anti-mouse; Alexa 488, 568, or 633 goat anti-rabbit or Alexa 488, 568, or 633 goat anti-rat; and Alexa 488 goat anti-chicken (1:500; Molecular Probes). To analyze muscle morphology in larva, we used Alexa Fluor 568 phalloidin (1:40; Molecular Probes). For visualizing cell nuclei, DAPI dye was used (Sigma). Analysis of indirect flight muscle morphology was done as previously described (Kucherenko et al., 2010). Images were obtained with a confocal laser-scanning microscope (Zeiss LSM 700) and processed with Adobe Photoshop software. To identify *miR-9a* predicted targets, Target Scan 6.0 (Kheradpour et al., 2007), PicTar (Grün et al., 2005), TarBase 6.0 (Vergoulis et al., 2012), and miRANDA (Enright et al., 2003) were used. To assess predicted protein-protein interactions and gene ontology (GO) term associated with their function, the String 9.05 database was used (Franceschini et al., 2013).

To determine mRNA expression levels, quantitative RT-PCR (qRT-PCR) was performed on total RNA derived from whole adult animals; to determine miRNA expression levels, TaqMan MicroRNA assay was used. LNA in situ and muscle sections were done as in Kucherenko et al. (2010, 2012) and Zimmerman et al. (2013). For detailed description of embryonic lethality, morphometric analyses of MTJs, in situ, RNA preparation and qRT-PCR, luciferase reporter assay, and ectopic expression of *miR-9a* and Dg in follicle cell epithelium, see Supplemental Experimental Procedures. Statistical analysis was done using Student's t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.01.004.

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