The E3 ubiquitin ligase Nedd4/Nedd4L is directly regulated by microRNA 1

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ABSTRACT

miR-1 is a small noncoding RNA molecule that modulates gene expression in heart and skeletal muscle. Loss of Drosophila miR-1 produces defects in somatic muscle and embryonic heart development, which have been partly attributed to miR-1 directly targeting Delta to decrease Notch signaling. Here, we show that overexpression of miR-1 in the fly wing can paradoxically increase Notch activity independently of its effects on Delta. Analyses of potential miR-1 targets revealed that miR-1 directly regulates the 3′UTR of the E3 ubiquitin ligase Nedd4. Analysis of embryonic and adult fly heart revealed that the Nedd4 protein regulates heart development in Drosophila. Larval fly hearts overexpressing miR-1 have profound defects in actin filament organization that are partially rescued by concurrent overexpression of Nedd4. These results indicate that miR-1 and Nedd4 act together in the formation and actin-dependent patterning of the fly heart. Importantly, we have found that the biochemical and genetic relationship between miR-1 and the mammalian ortholog Nedd4-like (Nedd4L) is evolutionarily conserved in the mammalian heart, potentially indicating a role for Nedd4L in mammalian postnatal maturation. Thus, miR-1-mediated regulation of Nedd4/Nedd4L expression may serve to broadly modulate the trafficking or degradation of Nedd4/Nedd4L substrates in the heart.

KEY WORDS: miR-1, Nedd4, Nedd4L, Nedd4-2, Heart

INTRODUCTION

Notch signaling regulates many aspects of cell differentiation and specification. In the developing Drosophila wing, Notch cooperates with the Hedgehog (Hh) pathway to form and pattern the anterior-posterior (AP) boundary (Casso et al., 2011). Several E3 ubiquitin ligases assure that Notch and its ligands properly migrate from the cell surface to various endocytic compartments for activation (e.g. Deltex, Mindbomb1). By contrast, the E3 ubiquitin ligase Nedd4 (neural precursor cell-expressed, developmentally downregulated 4) negatively regulates Notch by directing it to the lysosome (Ingham et al., 2004; Mindbom et al., 2001). miR-1 targets revealed that miR-1 directly regulates the 3′UTR of the E3 ubiquitin ligase Nedd4. Analysis of embryonic and adult fly heart revealed that the Nedd4 protein regulates heart development in Drosophila. Larval fly hearts overexpressing miR-1 have profound defects in actin filament organization that are partially rescued by concurrent overexpression of Nedd4. These results indicate that miR-1 and Nedd4 act together in the formation and actin-dependent patterning of the fly heart. Importantly, we have found that the biochemical and genetic relationship between miR-1 and the mammalian ortholog Nedd4-like (Nedd4L) is evolutionarily conserved in the mammalian heart, potentially indicating a role for Nedd4L in mammalian postnatal maturation. Thus, miR-1-mediated regulation of Nedd4/Nedd4L expression may serve to broadly modulate the trafficking or degradation of Nedd4/Nedd4L substrates in the heart.

RESULTS

Overexpression of miR-1 in the wing can increase Notch signaling

Using the decapentaplegic-GAL4 driver (dpp-GAL4) system, we expressed Drosophila miR-1 specifically in the AP organizer of the wing imaginal disc (dpp>miR-1) (Brand and Perrimon, 1993; King et al., 2011). Capitalizing on the temperature responsiveness of the GAL4-UAS system, we placed the dpp>miR-1 line at 20°C or 22°C and scored the wings for changes in vein patterning and L3/L4-intervein distance. As miR-1 expression increased, the wing veins thickened and intervein distance decreased progressively, consistent with miR-1 repressing the Notch ligand Delta in the AP organizer. Unexpectedly, at 22°C, when miR-1 expression is highest, a significant number of flies were missing the distal region of L3, a wing phenotype similar to that produced by increased Notch signaling (Fig. 1A, lower panel; Fig. 1B).

Next, we focused on the molecular mechanism of this truncation. To determine whether the distal loss of L3 under conditions of high miR-1 expression reflected increased Notch signaling, we genetically crossed alleles of positive and negative regulators of the Notch pathway. All parental lines had wild-type L3-vein morphology (data not shown). When mated to dpp>miR-1 flies, fly lines containing loss-of-function alleles of positive regulators of Notch (i.e. Notch, deltex, Suppressor of Hairless) showed a variable loss of intervein distance, indicating a genetic interaction with miR-1 (Fig. S1A).
However, none of these alleles generated flies that lacked the distal region of L3, suggesting that reductions in the gene dose of Notch effectors were not responsible for the shortening of L3.

**Dysregulation of Delta ubiquitylation or expression does not reduce L3 length**

To determine whether dysregulation of Delta caused the loss of L3 structures, we manipulated the relative amounts of active Delta with mutants of mindbomb1 (mib1) and UAS lines expressing wild-type or dominant-negative Delta (UAS-Delta and UAS-Delta\(^{DN}\) respectively). Mib1 facilitates the activation of Delta through ubiquitylation and subsequent endocytosis of the ligand (Itoh et al., 2003). Interestingly, reduced levels of mib1 did not affect the dpp\(><\)miR-1 phenotype, suggesting that the loss of L3 is not sensitive to the level of Mib1 expression in this system (Fig. S1B). Likewise, when crossed with the dpp\(><\)miR-1 line, neither the UAS-Delta nor the UAS-Delta\(^{DN}\) lines produced offspring with L3 truncation (Fig. S1B). Wings overexpressing both miR-1 and Delta\(^{DN}\) had a thickened and tortuous L3; however, the most distal region of L3 could not be reliably visualized, as the wings were globally deformed (data not shown). These results suggest that miR-1-mediated reduction of Delta or gross perturbation of Delta expression is insufficient to inhibit L3 formation.

**Reductions in the dose of Notch co-repressors lead to L3 truncation**

The Notch receptor associates with its DNA-binding partner Suppressor of Hairless [Su(H)] to activate several downstream genes, some of which form a negative-feedback loop (Fig. 1C). When we crossed transgenic lines containing loss-of-function alleles of Notch co-repressors (i.e. groucho, hairless or goosecoid) with the dpp\(><\)miR-1 line, we observed an enhanced loss of intervein distance and a truncated L3 – phenocopying the wings of dpp\(><\)miR-1 flies raised at 22°C (Fig. 1D). These results were obtained at 18°C, where miR-1 expression is below the usual threshold for suppression of L3 formation. From these results, we concluded that the distal loss of L3 results from de-repression of the Notch pathway and reflects an epistatic relationship between miR-1 and Notch repressors.

**Truncation of L3 in dpp\(><\)miR-1 flies is not due to phosphorylation of Groucho by receptor tyrosine kinases**

Loss-of-function alleles of groucho, hairless or goosecoid could impair L3 formation by genetically interacting with a separate genetic pathway that is also required for vein formation in the wing. For example, epidermal growth factor receptor (EGFR) signaling modulates Notch activity through phosphorylation of Groucho mediated by tyrosine receptor kinase (Hasson et al., 2005). To determine whether the length of L3 is sensitive to EGFR activity, we tested effectors of EGFR signaling, both positive [e.g. Spitz (spti), Star (S), Rhomboid (Rho), Vein (vn) and Roughoid (ru)] and negative [e.g. Knot (also known as Collier) (kn), Argos (aos)]. Isolated alleles of S, Rho, ru, spti, vn, aos, or kn were crossed with the dpp\(><\)miR-1 line. Scoring of the genetically relevant progeny did not reveal a genetic interaction between the EGFR pathway and miR-1 (Fig. S2). However, their interaction was genetically enhanced when fly lines were deficient in ED207 and BS2C289. These lines are haploinsufficient at both the Rho and ru loci, and produce a variation in the intervein distance between L3 and L4 (the fourth long vein). However, in no instance did a reduction in gene dose of positive or negative regulators of EGFR signaling cause loss of the distal region of L3, nor did alleles of constitutively active EGFR (Elp\(^{B1}\)/ellipse) affect L3 formation (Baker and Rubin, 1989) (Fig. S2). Thus, L3 shortening does not depend on EGFR signaling or EGFR-mediated phosphorylation of Groucho.

**miR-1 regulates the Notch pathway through conserved targeting of Nedd4**

To explore the possibility that miR-1 directly downregulates groucho, hairless or goosecoid, we interrogated the Targetscan
fly database (www.targetscan.org) and determined that no probable miR-1 sites exist in the 3′UTRs of these genes. By contrast, Su(H), mib1 and Nedd4 each contain one evolutionarily conserved miR-1 site (data not shown). Interestingly, in Drosophila Schneider 2 (S2) cells transiently transfected with miR-1, followed by immunoprecipitation of Argonaute1 (Ago1) and quantitative RT-PCR, Nedd4 mRNA was enriched (Essov et al., 2007). These results support the hypothesis that miR-1 and Nedd4 mRNA physically associate with each other within a miRNA-effector complex. These findings prompted us to focus on a possible genetic or biochemical relationship between miR-1 and Nedd4.

Nedd4 ubiquitylates the PPSY motif in the intracellular domain of Notch in a HECT domain-dependent fashion (Sakata et al., 2004). Ubiquitylation of this motif by Nedd4 or Suppressor of Deltex [Su(dx)] reduces Notch signaling by directing the receptor to late endosomes. Nedd4-like proteins are evolutionarily conserved from yeast to mammals. Nedd4 and its murine orthologue Nedd4L (also known as Nedd4-2) share a common domain structure (Fig. 2A). Nedd4 localizes to the cell membrane through its C2 domain and uses its WW domains to recognize substrate proteins. It negatively regulates Notch signaling by directing the Notch receptor towards endocytosis and lysosomal degradation. Interestingly, the predicted miR-1 binding site in the 3′UTR of Nedd4 and Nedd4L is widely conserved (Fig. 2B).

We hypothesized that the loss of L3 structures is due to miR-1-mediated downregulation of Nedd4. To test this hypothesis, we analyzed alleles of Nedd4 in the wing assay at both 18°C and 20°C. At 18°C, the dpp > miR-1 line had minimal miR-1 expression, and penetration of the L3 truncation phenotype was low but measurable with two Nedd4 alleles (Nedd4<sup>M007766</sup>, 4.7%, n = 103; Nedd4<sup>C153</sup>, 2.3%, n = 130; data not shown). At 20°C, where truncation of L3 is detected in the dpp > miR-1 line, all alleles of Nedd4 generated significant numbers of progeny with a shortened L3 (Fig. 2C, Fig. S3). The weakest allele, Nedd4<sup>T119FS</sup>, also generated progeny with L3 breaks (17%, n = 54; data not shown). Thus, reducing the Nedd4 dose enhances the effects of miR-1, resulting in loss of L3 structures. To verify that the loss of Nedd4 activity was responsible for the effects on L3 formation, flies harboring a wild-type Nedd4 construct (UAS-Myc-Nedd4) (Myat et al., 2002) were crossed to the dpp > miR-1 line. For all alleles tested, concurrent overexpression of wild-type Nedd4 robustly reduced the number of progeny lacking the distal region of L3 (Fig. 2D). To determine whether an intact HECT domain was necessary for rescue of the wing vein phenotype, we crossed a line containing a ubiquitin ligase inactive Nedd4 construct [UAS-Myc-Nedd4(C/A)] with the dpp > miR-1 line (Myat et al., 2002). Again, we observed marked suppression of the wing vein phenotype for all alleles except for Nedd4<sup>T119FS</sup> and Nedd4<sup>C153</sup> (Fig. 2E). These results suggest that the L3 truncation is attributable to reduced levels of Nedd4, though an intact HECT domain is not essential for this effect.

The genetic interaction between miR-1 and Nedd4 does not extend to other closely related E3 ubiquitin ligases

Like Nedd4, Su(dx) negatively regulates Notch. In wing discs, Nedd4 can compensate for the loss of Su(dx), implying functional redundancy (Wilkin et al., 2004). Therefore, we sought to determine whether miR-1 interacts genetically with other members of the Nedd4 family [i.e. Su(dx), Smurf] using L3 morphology as a readout. Su(dx)<sup>−/−</sup>, but not Su(dx)<sup>KG02902</sup> or Su(dx)<sup>−/−</sup>, generated flies with a shortened L3. Similarly, Smurf<sup>KG07014</sup>, but not Smurf<sup>M007104</sup> or Smurf<sup>−/−</sup>, genetically interacted with miR-1 to repress L3 formation (Fig. 2F). These results indicate that L3 shortening cannot be generalized beyond the interaction of miR-1 and Nedd4 and that Nedd4 family members have non-redundant functions in our dpp > miR-1-based system.

Reduced Nedd4 activity is due to a functional miR-1-binding site in the 3′ UTR of Nedd4

The 3′UTRs of fly Nedd4 and murine Nedd4l are bioinformatically predicted to contain miR-1-binding sites (Fig. 2B). To assess the biological relevance of these putative sites, we cloned the 3′UTRs of Nedd4 or Nedd4l into luciferase-reporter constructs. Introduction of the miR-1 mimic into the murine C2C12 myoblast cell line significantly reduced luciferase activity, suggesting that miR-1 negatively regulates Nedd4 mRNA in mammalian cells (Fig. 2G, left). Deletion of the putative miR-1 seed sequence within the predicted target site prevented the repressive effects, demonstrating that silencing is miR-1 dependent (Fig. 2G, right). Parallel studies also performed in the C2C12 myoblast cell line using the Nedd4l 1′ UTR produced similar results (see Fig. 6A), suggesting that the regulation of Nedd4 and Nedd4l by miR-1 is evolutionarily conserved.

Nedd4-null flies have abnormal heart specification and patterning that result in early lethality

To determine whether the genetic and biochemical relationship between miR-1 and Nedd4 was relevant in tissues where miR-1 is endogenously expressed, we examined the cardiac phenotypes of the two Nedd4 mutant alleles (Nedd4<sup>T119FS</sup> and Nedd4<sup>M007766</sup>) in a Hand-GFP (Han and Olson, 2005) background, which supports visualization of cardioblasts and pericardial cells in the developing fly heart. Analysis of these animals revealed that Nedd4 is necessary for normal heart formation in the embryonic period and first instar stage (Fig. 3). Homozygous null Nedd4 embryos were distinguished using a GFP-positive balancer chromosome and stained with antibodies against Mef2, a marker of somatic and heart muscle. We observed that most homozygous Nedd4 mutants displayed reduced numbers of cardioblasts and pericardial cells at stage 15, resulting in gaps among the two rows of cardioblasts (Fig. 3A-C). Homozygous mutants of either of the two alleles die around the late first-instar larval stage. Although cardiac structures such as ostia (hemolymph inflow tracts) still form, the number of cardioblasts is reduced, reflected by fewer than four non-ostia cardioblasts in each hemisegment (Fig. 3D-F). These results indicate that Nedd4 is necessary to generate the expected numbers of cardioblasts.

Misexpression of Nedd4 disrupts the patterning of cardioblasts and ostia cells in the adult fly hearts

As Nedd4 and miR-1 are both expressed in the developing fly heart, we speculated that miR-1 might modulate the absolute levels of Nedd4 within the fly heart. To determine the sensitivity of the fly heart to Nedd4 activity, we overexpressed Nedd4 in the heart using twist-GAL4 or Hand-GAL4 drivers (Han and Olson, 2005). Interestingly, using these UAS-GAL4 systems (Brand and Perrimon, 1993) to misexpress wild-type (UAS-Myc-Nedd4) and ubiquitin-ligase mutant forms of Nedd4 [UAS-Myc-Nedd4(C/A)], as well as Nedd4-RNAi, within the developing mesoderm permitted the recovery of adult flies (Fig. 4). Close examination of first-instar larvae revealed mild defects in the arrangement of the four pairs of Tinman-positive cardioblasts separated by two pairs of ostial cells (Fig. S4A,B). At 29°C, Hand > UAS-Myc-Nedd4, Hand > UAS-Myc-Nedd4(C/A) and Hand > UAS-Nedd4-RNAi animals had mildly reduced survival to eclosion and reduced adult life expectancy compared with control lines (n = 60 for each genotype; Fig. S4C,D).
Altered Nedd4 expression caused pericardial cells to detach from mature fly hearts

To determine whether these modest disruptions in the normal patterning of first instar larvae resulted in morphological defects in the adult heart, we collected newly eclosed (adult) mutant fly hearts harboring the Hand>UAS-Myc-Nedd4, Hand>UAS-Myc-Nedd4 (C/A) or Hand>UAS-Nedd4-RNAi constructs. Individual hearts were fixed and stained with phalloidin to visualize the structure of the cardiac actin filaments. Pericardial cells were marked with Hand-GFP; an anti-pericardin antibody labeled the extracellular...
matrix of the heart tube. Interestingly, compared with wild-type controls, animals with supraphysiological expression of Nedd4 or the catalytically inactive form of Nedd4 [Nedd4(C/A)] showed a dramatic increase in the number of detached pericardial cells, generalized disorganization of cardiac actin filaments (Fig. 4A-D) and a loss of cardiac muscle-fiber density (Fig. 4E). This phenotype is strikingly similar to other cardiac extracellular-matrix mutants, such as Lonely heart (loh) (Drechsler et al., 2013). To determine whether pericardial cells detached in the context of abnormal extracellular-matrix formation, we stained the flies for pericardin, a type IV collagen that is crucial for maintaining cardiac integrity in Drosophila. Consistent with a role for Nedd4 for normal cardiac extracellular-matrix formation, we observed increased pericardin deposition in flies overexpressing Hand>UAS-Myc-Nedd4(C/A), which expresses catalytically inactive Nedd4 (Fig. 4C’). These results indicate that Nedd4 may help maintain the normal myofibrillar structure of the heart tube and its associated pericardial cells. We propose that some of its effects on heart structure may be secondary to changes in pericardin deposition.

Changes in muscle fiber-associated actin filaments induced by overexpression of miR-1 can be rescued by increasing expression of Nedd4 during larval stages

To determine whether abnormalities in heart structure or function induced by overexpression of miR-1 could be genetically rescued by concurrent Nedd4 overexpression, we generated animals that concurrently overexpress miR-1 and Nedd4 or Nedd4(C/A) [Hand>UAS-Myc-Nedd4+UAS-miR-1, Hand>UAS-Myc-Nedd4(C/A)+UAS-miR-1]. Animals were raised at 20°C and pertinent progeny evaluated. We did not recover adult animals when miR-1 was overexpressed, regardless of Nedd4 expression, indicating that overexpression of Nedd4 does not completely rescue abnormalities induced by excessive miR-1 expression. By contrast, we were able to detect viable larvae, suggesting that genetic rescue may occur at earlier developmental stages. To visualize larval hearts, individual animals were fixed and stained with phalloidin to visualize the structure of the cardiac actin filaments. Overexpression of miR-1 universally resulted in profound disorganization of the actin structures compared with wild type (Fig. 5B). Surprisingly, we found that, at L3, overexpression of Nedd4 and, to a lesser extent, Nedd4(C/A) was able to reduce the myofibrillar disorganization induced by overexpression of miR-1. These results indicate that, in larval heart, increasing Nedd4 expression can normalize actin fiber organization induced by excessive miR-1 expression.

Nedd4L and miR-1 levels are inversely proportional in mammalian hearts

These in vivo results prompted us to determine whether miR-1 and Nedd4l interact genetically and biochemically in the mammalian heart. As with the luciferase assays testing the 3′UTR of Nedd4 for...
miR-1 sensitivity, the 3' UTR of Nedd4L was also sensitive to miR-1 regulation (Fig. 6A). To determine whether the expression of Nedd4L was inversely proportional to miR-1 levels in murine hearts, we took advantage of the fact that miR-1 levels rise after birth as part of the transition from the fetal to the postnatal circulation (Fig. 6B). We postulated that during this postnatal period, as miR-1 levels physiologically increase, Nedd4L levels would fall. To test this hypothesis, we performed western blot analysis on whole-heart lysates obtained from wild-type mice at postnatal days 2 and 21 (P2 and P21) using anti-Nedd4L antibodies (Bethyl). Each postnatal period was represented by a minimum of five individual hearts, and the amounts of Nedd4L were normalized to the GAPDH loading control.
control. We found that the levels of Nedd4L expression were ~2.5-fold higher in P2 hearts compared with P21 hearts. Based on these results, we investigated whether Nedd4L expression was perturbed in \textit{miR-1} null (miR-1-1\textsuperscript{-/-}; miR-1-2\textsuperscript{-/-}) mice. We performed a targeted analysis of data from RNA sequencing of heart lysates from late embryonic (E18) \textit{miR-1} null (miR-1-1\textsuperscript{-/-}; miR-1-2\textsuperscript{-/-}) mice versus wild-type controls (Heidersbach et al., 2013). Consistent with a model in which \textit{miR-1} directly targets Nedd4L, \textit{miR-1}-null hearts had significantly increased levels of Nedd4L mRNA (Fig. 6D). To determine whether this increase in Nedd4L RNA altered protein expression, we analyzed lysates of P2 murine heart cells by western blot (Fig. 6E). In wild-type P2 hearts, Nedd4L levels were low but detectable. By contrast, \textit{miR-1}-null mice had significantly increased Nedd4L levels at the predicted molecular weight, plus two additional bands (n=3 per genotype), as seen in the wild-type hearts. Nedd4L levels in \textit{miR-1}-null mice at P21 could not be determined, because the \textit{miR-1} null state is lethal by P7 owing to heart failure. In summary, these results support the hypothesis that \textit{miR-1} regulates Nedd4L to affect the physiology of the mammalian heart.

**DISCUSSION**

Unexpectedly, we found that overexpression of \textit{miR-1} in the anterior-posterior (AP) organizer of the wing disc results in a dose-dependent loss of L3 vein structures, consistent with de-repression of Notch or weakening of a regulatory mechanism that dampens the Notch signal. Using genetic techniques, we determined that the loss of Notch or weakening of a regulatory mechanism that dampens the positive effectors of the Notch receptor (e.g. Deltex) or perturbations in Delta-mediated cis-inhibition, contributed to the de-repression of Notch in our wing-based assay system.

Our findings in the mammalian heart indicate that the genetic and biochemical interaction between \textit{miR-1} and Nedd4L is physiologically relevant and may provide developmental or tissue-specific regulation of Nedd4L in the myocardium. We speculate that the additional bands observed on western blots of heart lysates using an anti-Nedd4L antibody might result from post-translational modifications, because Nedd4L can autoregulate its stability through ubiquitylation of its HECT domain (Bruce et al., 2008). Alternatively, they might represent heart-specific splice variants, because tissue-specific isoforms of Nedd4L have been found in the heart and the liver (Chen et al., 2001; Fu et al., 2013).

Importantly, although \textit{miR-1}-mediated reductions in Nedd4 activity caused wing-vein phenotypes induced by Notch, \textit{miR-1}-mediated dysregulation of Nedd4L in the heart likely affects proteins outside the Notch pathway. Indeed, protein microarrays comparing human Nedd4 with human Nedd4L, suggest that Nedd4L (also known as Nedd4-2) preferentially targets ion channels, whereas Nedd4 targets are enriched for signaling pathways (Persaud et al., 2009). Thus, in the heart, where \textit{miR-1} and murine Nedd4L are both expressed, their genetic and biochemical interaction might influence the excitability and connectivity of cardiomyocytes. Indeed, susceptibility to cardiac arrhythmias and sudden death in humans is associated with six genes that encode ion channels (SCN5A, KCNQ1, KCNH2, KCNE1, KCNE2 and RYR2) (reviewed by Keating and Sanguinetti, 2001). Murine Nedd4L regulates the cell-surface densities of the sodium channel, the voltage-gated type V alpha subunit (Scn5A) (Abriel et al., 2000; Rougier et al., 2005; van Bemmelen et al., 2004), the potassium voltage-gated channel, KQT-like subfamily member 1 (Kcnn1) (Jespersen et al., 2007; Krzystanek et al., 2012) and the human Ether-a-go-go-related (KCNH2, previously hERG) channel (Albesa et al., 2011; Guo et al., 2012). Furthermore, \textit{miR-1} directly regulates human KCNJ2, a channel that maintains cardiac resting potential (Yang et al., 2007). These findings suggest that...
the regulation of murine Nedd4l by miR-1 contributes to some of the electrophysiological abnormalities seen in miR-1 null mice (Heidersbach et al., 2013; Wei et al., 2014; Zhao et al., 2007). It would be interesting to determine whether Nedd4L is dysregulated in the heart after an infarction or under ischemic conditions, when miR-1 is upregulated and fatal cardiac dysrhythmias are common.

MATERIALS AND METHODS

Fly stocks
Drosophila lines were obtained from Bloomington Stock Center (NIH P40OD018537). The following fly lines were generously provided by G. Tear (King’s College London, UK): Nedd4T119FS, Nedd4N121FS and UAS-Nedd4 (Myat et al., 2002). W1118 flies served as wild-type controls. Transgenes were overexpressed with the UAS-GAL4 system (Brand and Perrimon, 1993). The following GAL4 and UAS lines were used: dpp-GAL4 (Bloomington Stock Center) and UAS-miR-1 (C. Kwon, Johns Hopkins University, Baltimore, MD, USA) (Kwon et al., 2005). The Ellipse allele (ElpB1) was provided by G. Rubin (HHMI Janelia Research Campus, VA, USA) (Baker and Rubin, 1989).

Wing imaging
Adult Drosophila were anesthetized with CO2, placed in isopropanol for 1 min and euthanized. Flies were air dried, their wings were removed and embedded in Canada Balsam (Sigma), and covered with a glass coverslip. Images were taken on a LeicaMZ16F microscope at 5× magnification with a Leica DFC310FX camera and the Leica Application Suite (LAS) program. Images were processed using Adobe Photoshop CS6 and the white set-point function was used for background lightening.

Drosophila embryonic imaging
Embryos were collected and stained with various antibodies as previously described (Han and Olson, 2005). Rabbit anti-Dmef2 was used at 1:1000 dilution. Cy3, Cy5 or biotin-conjugated secondary antibodies (from Jackson Labs) were used. Adult flies were dissected and fixed for 10 min in 4% paraformaldehyde in phosphate-buffered saline (PBS). Alexa Fluor R555 phalloidin was obtained from Thermo Fisher. Mouse anti-pericardin antibody (EC11) was used at 1:500 dilution, followed by Cy3-conjugated secondary antibodies (Jackson Labs). Confocal imaging was performed with a Zeiss ApoTome.2 microscope using a 20× Plan-Apochromat 0.8 N.A. air objective. For quantitative comparisons of intensities, common settings were chosen to avoid oversaturation. ImageJ Software Version 1.49 was used to process images.

Drosophila larval and adult heart imaging
Larvae and adult flies were dissected and fixed for 10 min in 4% paraformaldehyde in phosphate-buffered saline (PBS). Alexa Fluor 555 phalloidin was obtained from Thermo Fisher. Mouse anti-pericardin antibody (EC11) was used at 1:500 dilution, followed by Cy3-conjugated secondary antibodies (Jackson Labs). Confocal imaging was performed with a Zeiss ApoTome.2 microscope using a 20× Plan-Apochromat 0.8 N.A. air objective. For quantitative comparisons of intensities, common settings were chosen to avoid oversaturation. A minimum of 6 larvae or adults per genotype were visualized. ImageJ Software Version 1.49 was used to process images.
Software Version 1.49 was used for image processing. For quantitative comparisons of cardiac muscle fiber density, cardioblast cell numbers and pericardin deposition we analyzed six control flies and six flies of each experimental genotype. Sample size determinations were based upon extensive previous experience of mutant fly heart morphological analysis.

Fly survival assay

On day 6 after egg laying, Drosophila larvae were transferred from 25°C to 29°C to enhance the temperature-sensitive UAS-transgene expression. Adult male flies were subsequently maintained at 29°C in vials containing 15 or animals. Sixty flies were assayed per genotype.

Luciferase assay

Approximately 200 base pairs surrounding the predicted miR-1-target sites in the untranslated regions (UTRs) of Nedd4 or Nedd4L were amplified directly from cDNA generated from W1118 flies or wild-type mouse hearts, respectively, and subcloned with XhoI into the PGL3 (Promega) firefly luciferase vector at the 3’ end of the reporter gene. Correct insertion was confirmed by sequencing.

For transfection of the C2C12 murine myoblast cell line (ATCC) with luciferase constructs and a Renilla normalization vector, we used Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions. Briefly, cells in 12-well plates were transfected at 60% confluency and analyzed 20 h later. Each well received 3 μl of Lipofectamine 2000, 800 ng of PGL3-Target and 200 ng of Renilla vector. Experimental wells received 10 pmol of miR-1 mimic (Ambion/Life Technologies), and control wells received 10 pmol of a non-targeting control mimic (Ambion/Life Technologies).

Firefly and Renilla luciferase activities in lysates were quantified with the Dual Luciferase Reporter Assay kit (Promega) and a Victor 1420 Multilabel Counter (PerkinElmer). Firefly luciferase values were normalized to Renilla to control for transfection efficiency.

RNA sequencing

Whole hearts from embryonic day 18 (E18) wild-type and miR-1 double-knockout mice were isolated and analyzed as described previously (Heidersbach et al., 2013).

Quantitative PCR

Postnatal hearts were obtained from C57BL/6J pups after timed matings, with n=3 at each time point. Animals were sacrificed by CO2 asphyxiation followed by decapitation. Hearts were snap frozen in liquid nitrogen and disassociated in a TissueLyser II (Qiagen) at 30 Hz for 2 min. Left ventricular cardiac tissue was also isolated from adult mice and homogenized in a Bullet Blender using 1 mm ZrSiO beads according to the manufacturer’s instructions. RNA was isolated using the Direct-zol RNA MiniPrep kit (catalog number R2052) from Zymo Research. cDNA was prepared using a High Capacity cDNA Reverse Transcription Kit (catalog number 4368814) and miR-1 TaqMan probes (catalog number 4429795) from Thermo Fisher. miR-1 levels were normalized to SnoRNA-202 (catalog number 427975) according to manufacturer’s recommendations. Mice of both sexes were housed under the UCSC Assurance of Compliance with PHS Policy on Humane Care and Use of Laboratory Animals by Awardee Institutions, and their use was approved by the UCSC Institutional Animal Care and Use Committee.

Western blots

Hearts were isolated from mice on postnatal days 2 and 21 (P2, P21) and rinsed with 1× PBS. The tissue was resuspended in RIPA buffer and disassociated in a Bullet Blender using 2 mm ZrSiO beads according to the manufacturer’s instructions (Next Advance). After clarification and sonication, the lysates were loaded onto a 4-12% SDS-PAGE gel (Biorad), blotted onto polyvinylidene fluoride (PVDF) membranes by standard protocols, and probed with primary antibodies against GAPDH (Abcam; 1:5000) and Nedd4L (Bethyl A302-512A; 1:2000). Blots were visualized and quantified with a Licor Odyssey system and fluorescently conjugated secondary antibodies (Licor, Lincoln, NE), according to the manufacturer’s instructions.

Statistics

The rates of genetic enhancement or truncation of long vein 3 (L3) were analyzed with the chi-square test. Mean, s.d. and s.e.m. were calculated for luciferase assays. qRT-PCR of relative miR-1 levels were performed with GraphPad Prism (version 6.0) for t-tests. Differences between two means were assessed by two-tailed t-tests, unless otherwise stated. Error bars represent s.e.m. Null hypotheses were rejected at P<0.05.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

I.N.K. designed and performed the Drosophila wing assay and conducted western blotting on wild-type mouse hearts. J.-y.Z. and Z.H. performed the misexpression analysis, rescue studies of the fly heart and fly mortality studies. A.H., K.N.I. and D.S. generated the miR-1-null mice. A.H. performed the western blot on the miR-1-null heart cell lysates and the luciferase assays. I.S.K. and B.I.G. obtained and determined the miR-1 levels from wild-type murine hearts. Z.H. and I.N.K. wrote the manuscript.

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Supplementary information

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