Cdc42 and formin activity control non-muscle myosin dynamics during Drosophila heart morphogenesis

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Introduction

The fruit fly Drosophila melanogaster is widely used as a genetically tractable model organism for studying the cellular and molecular mechanisms of tissue specification and organogenesis. In recent years, Drosophila has also been used to dissect the genetics of heart morphogenesis and function (Ocorr et al., 2010). During early heart development, cardiac precursor cells are specified through the well-characterized activity of signaling pathways (e.g., Wnt, Dpp/Bmp, PGF) and transcription factors (e.g., Tinman/Nkx2-5, Gata, Tbx). Later, a highly stereotypic and rather simple morphogenetic process leads to the formation of a single dorsal tube that differentiates into a beating heart. Many conserved members of the cardiac transcription factor network have been identified, and the extent to which factors and signaling pathways guide cardiac cell fate and differentiation, but the genetic mechanisms orchestrating heart assembly and lumen formation remain unclear. Here, we show that the small GTPase Cdc42 is essential for Drosophila melanogaster heart morphogenesis and lumen formation. Cdc42 genetically interacts with the cardiogenic transcription factor tinman; with dDAAM which belongs to the family of actin organizing formins; and with zipper, which encodes nonmuscle myosin II. Zipper is required for heart lumen formation, and its spatiotemporal activity at the prospective luminal surface is controlled by Cdc42. Heart-specific expression of activated Cdc42, or the regulatory formins dDAAM and Diaphanous caused mislocalization of Zipper and induced ectopic heart lumina, as characterized by luminal markers such as the extracellular matrix protein Slt. Placement of Slt at the lumen surface depends on Cdc42 and formin function. Thus, Cdc42 and formins play pivotal roles in heart lumen formation through the spatiotemporal regulation of the actomyosin network.

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molecules for Drosophila heart morphogenesis include Unc5 (Albrecht et al., 2011), integrins (Vanderplou et al., 2012), Lammin (Yarnitzky and Volk, 1995), and Syndecan (Knox et al., 2011). However, the underlying molecular and cellular events, like the role of molecular motors during heart formation are still unclear.

Filamentous actin- and nonmuscle myosin–based molecular motors are essential for cell movement and cell shape changes. For example, blebbistatin-mediated inhibition of nonmuscle myosin function (i.e., in an actin-detached state) during development can disrupt numerous morphogenetic events (Kovács et al., 2004; Köppen et al., 2006). In Drosophila, the gene encoding nonmuscle myosin II, zipper, is essential for embryonic development (Young et al., 1993). To exert their motor function, actin and myosin must assemble in a coordinated fashion, and both the assembly and activity of the actomyosin complex must be tightly controlled to achieve directed tissue morphogenesis. Actin and myosin can localize in a dynamic or static pattern, depending on the tissue and developmental process. In Drosophila, dynamic changes in actin occur during filopodia formation, whereas static actomyosin cables are formed during dorsal closure. Thus, regulation of actomyosin assembly is cell and context dependent.

Drosophila heart formation requires extensive changes in CB shape, which suggests that the actomyosin network may play a critical role in cardiac morphogenesis. Cytochalasin D, an actin-depolymerizing agent, is known to inhibit lumen formation (Haag et al., 1999), underscoring the important role of the actin cytoskeleton. However, little is known of how the cardiac actomyosin network is regulated in Drosophila or how the activity of the network is orchestrated during CB assembly and lumen formation.

To investigate the role of actomyosin in cardiac morphogenesis, in particular the regulation of nonmuscle myosin, we examined a possible involvement of Rho GTPases. These enzymes regulate specific cytoskeletal events, including actin polymerization, F-actin stabilization, and actomyosin assembly (Iden and Collard, 2008), by acting as molecular hubs to integrate signaling events that control cell shape and polarity (Etienne-Manneville and Hall, 2002). In the adult heart, Cdc42 genetically interacts with the cardiac determinant tinman/nkx2.5 in regulating cardiomyocyte function (Qian et al., 2011). To determine whether Cdc42 plays a role during embryonic heart morphogenesis, we analyzed the Cdc42 loss-of-function allele, Cdc42″ (Fehon et al., 1997). Approximately two thirds of Cdc42″ mutant embryo hearts, stained for Nmn1, a CB nuclear marker, showed a wild-type CB arrangement (Fig. 1 A and B; and see Table 1), but the remaining one third exhibited a range of CB alignment defects (Fig. 1 C and S1, A–A″), including occasional dorsal–ectodermal closure defects (Fig. S1 A″). Expression of a genomic fragment encompassing the Cdc42 locus (pCosMer; Fehon et al., 1997) reversed the dorsal closure and CB alignment defects (Fig. S1 A‴), including occasional dorsal–ectodermal closure defects (Fig. S1 A‴). Expression of a genomic fragment encompassing the Cdc42 locus (pCosMer; Fehon et al., 1997) reversed the dorsal closure and CB alignment defects (Fig. S1 A‴), which confirms that the observed phenotype was due to a defective Cdc42 gene. The lack of complete phenotypic penetrance is likely due to maternally supplied Cdc42 (see Lundström et al., 2004). Because maternal depletion of Cdc42 inhibits oogenesis, analysis of Cdc42 function is limited to the examination of zygotic mutants or animals with cardiac expression of dominant alleles (Genova et al., 2000).

To analyze the heart morphology of Cdc42″ mutants in detail, we analyzed the localization of Dystroglycan (Dg), which is a basement membrane marker, and of Slit, a prominent lumenal marker (Figs. 1, B′–C″). In wild-type embryos, the CB nuclei aligned in parallel, Dg is enriched at the basal and luminal domain, and Slit accumulated at the heart lumen (Fig. 1, B′ and B″). In contrast, Cdc42″ mutant embryos showed misaligned CB nuclei, and Dg and Slit no longer delineate a distinct luminal space and function in the adult Drosophila and murine hearts (Qian et al., 2011). Here, we study Cdc42’s role in embryonic heart morphogenesis. We found that CBs of Cdc42 mutant embryos failed to align properly at the dorsal midline, compromising lumen formation, likely due to aberrant cell adhesion and shape changes. In a genetic interaction screen, we identified an essential role for the nonmuscle myosin II–encoding zipper gene in mediating Cdc42’s cardiogenic function. Zipper exhibited a dynamic and highly polarized localization in CBs before alignment and assembly at the midline, and later during lumen formation. Inhibition of Cdc42 activity abolished the dynamic Zipper accumulation between contralateral CBs, and activation of Cdc42 (or the formins Diaphanous and dDAAM) resulted in ectopic lumen formation. Collectively, our findings suggest a new genetic program for orchestrating cardiac morphogenesis that is controlled by Cdc42 and dDAAM/Dia and mediated by Zipper nonmuscle myosin. In contrast, in mutants of Slit–Robo or Notrin–Unc5, which also function in heart tube formation and CB polarity, Zipper’s dynamic localization is unaltered. Thus, the Cdc42/Formin/Zipper program constitutes a novel mechanism in the control of distinct aspects of cardiac morphogenesis that includes CB contact and lumen formation.

Results

Cdc42 is required at multiple points during heart morphogenesis

Small GTPases of the Rho family, particularly RhoA, Cdc42, and Rac, are involved in cell migration, polarization, and adhesion (Etienne-Manneville and Hall, 2002). In the adult heart, Cdc42 genetically interacts with the cardiac determinant tinman/nkx2.5 in regulating cardiomyocyte function (Qian et al., 2011). To determine whether Cdc42 plays a role during embryonic heart morphogenesis, we analyzed the Cdc42 loss-of-function allele, Cdc42″ (Fehon et al., 1997). Approximately two thirds of Cdc42″ mutant embryo hearts, stained for Nmn1, a CB nuclear marker, showed a wild-type CB arrangement (Fig. 1, A and B; and see Table 1), but the remaining one third exhibited a range of CB alignment defects (Fig. 1 C and S1, A–A″), including occasional dorsal–ectodermal closure defects (Fig. S1 A‴). Expression of a genomic fragment encompassing the Cdc42 locus (pCosMer; Fehon et al., 1997) reversed the dorsal closure and CB alignment defects (Fig. S1 A‴), which confirms that the observed phenotype was due to a defective Cdc42 gene. The lack of complete phenotypic penetrance is likely due to maternally supplied Cdc42 (see Lundström et al., 2004). Because maternal depletion of Cdc42 inhibits oogenesis, analysis of Cdc42 function is limited to the examination of zygotic mutants or animals with cardiac expression of dominant alleles (Genova et al., 2000).

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Cdc42 is necessary and sufficient for CB shape changes in lumen formation

Tubular structures can be formed by several cellular processes, including the creation of a central lumen through changes in cell shape (i.e., by wrapping; Lubarsky and Krasnow, 2003). Loss of Cdc42 function seemed to be associated with a failure to undergo appropriate cell shape changes; i.e., CBs failed to make ventral contacts once dorsal contacts between contralateral CBs had been established (Fig. 1 D), thus forming a non-contiguous heart lumen, even when the CB arrangement was normal. We reasoned that if Cdc42 was mediating the localized cell shape changes, activation of Cdc42 might be sufficient to induce cell shape changes and thus ectopic lumina. We tested this by expressing constitutively active Cdc42V12 specifically in the developing heart. Indeed, such hearts showed ectopic structures reminiscent of heart lumina that formed between ipsilateral neighboring CBs (Fig. 1, F and H). To better visualize the CB cell shape, we expressed Discs-large 1 (Dlg1), which localizes to CB cell–cell interfaces (Dlg1::GFP; Koh et al., 1999) in wild-type and Cdc42V12 mutant hearts. In contrast to the columnar arrangement of wild-type CBs (Fig. 1 G), Cdc42V12 CBs appear rounded, and lumina-like structures were evident between ipsilateral cells (Figs. 1 H and S2 B). In addition to Dg, we found that other markers of the heart lumen such as Slit and the basement membrane marker Trol (Fig. 1 I) also localize to these ectopic sites (Fig. 1 J). This suggests that Cdc42 is necessary and sufficient to induce cell shape changes and direct heart lumen formation.

Cdc42 is not required for CB migration or filopodia formation

CBs of the developing heart are connected to the overlying epidermal cells and the underlying amnioserosa (AS) through cell–cell junctions (Rugendorff et al., 1994). CBs display an active migratory behavior, with a highly dynamic pattern of actin-rich filopodia formation, extension, and retraction (Fig. S2, D and E; and Video 1). To determine if Cdc42 plays a role in filopodia formation during CB migration to the midline, we expressed Cdc42V12 in the prospective heart-forming tissue. Despite the expected CB intermingling and loss of columnar shape (Fig. S2, arrows in the bottom of panel E), we observed no change in the number or directionality of filopodia in the migrating CBs (Fig. S2, arrowheads in top panels; and Video 1). Interestingly, abolishing or interfering with Slit–Robo signaling, known to also be involved in heart tube formation (Qian et al., 2005), also had no apparent effect on filopodia formation (Fig. S2 G). It therefore remains to be determined if and how CB filopodia contribute to cardiac morphogenesis, and which pathways control CB filopodia formation.

Cdc42 genetically interacts with nonmuscle Myosin II, Zipper

Embryos that are zygotic mutant for the Cdc42V12 loss-of-function allele display a hypomorphic phenotype, with only a third of the embryos having cardiac or dorsal closure defects. We reasoned that genetic interactors might significantly increase the frequency of these defects and therefore conducted a candidate gene approach that included cardiac transcription factors as well as cytoskeletal genes to test if they can change the number of cardiac defects when heterozygous in a Cdc42V12 background (Table 1). Of the cardiac transcription factors tested, we found that Cdc42V12 strongly interacted with loss-of-function alleles of tinman (tin), but not with tail-up (Islet-1) or zfh1. Of note, cardiac-specific tinABD; tin+/− mutant embryos not only have CB specification defects (Zaffran et al., 2006) but also fail to properly undergo heart morphogenesis, as indicated by the lack of luminal Slit localization (Fig. S3, A and B). We therefore hypothesize that a cardiac role of Tin is to control, directly or indirectly, heart morphogenesis, and that Cdc42 is part of this genetic program. Cdc42 did not show genetic interaction with slit or robot lea (robo2); however, when we tested genes that are implicated in regulation of the actomyosin network, we found a genetic interaction between Cdc42 and Abelson tyrosine kinase (Abl) and zipper, which encodes the Drosophila nonmuscle myosin II. This interaction was indicated by a significantly increased frequency of dorsal closure/myocardial closure defects (Table 1), which suggests that Cdc42 interacts with Abl and zipper during...
Figure 1. Cdc42 is necessary for cardiac alignment and lumen formation. (A and B) Wild-type Drosophila heart at the end of heart morphogenesis, st17. Cardiac nuclei (Nmr1, blue) are aligned at the dorsal midline. Basement membrane (Dg, red) and heart lumen (Slit, green) are properly localized to the respective domains (see schematic in A). (B and C) CB cell shape in wild-type (B–B') and Cdc423 mutant (C–C') hearts at embryonic early stage 17, stained with anti-Nmr1 (B and C), anti-Dg (B' and C'), and anti-Slit (B' and C'). Transverse cross sections (insets) were taken from the indicated positions (broken line in A–D). Wild-type hearts show aligned cardioblasts enclosing a single, central lumen (open arrowheads in B–B' and inset), with Slit enriched at the luminal surface (arrowheads). CBs in Cdc423 zygotic mutant hearts fail to form a central heart lumen (C' and C') or are mispositioned (curved arrowheads in C').

control

Cdc423

24Bnow > Cdc42N17

tinCΔ4 > Cdc42N17

tinCΔ4 > Cdc42V12

tinCΔ4 > dlg1GFP

tinCΔ4 > Cdc42V12
epithelial and cardiac morphogenesis. Interestingly, all these loci, Cdc42, Abl, and zip, exhibit enriched Tinman binding during embryonic development (Junion et al., 2012).

**Zipper plays heart autonomous and non-autonomous roles during cardiac morphogenesis**

Morphogenesis of the heart tube requires cell migration in addition to changes in cell–cell adhesion and in cell shape. Because Cdc42 mutant CBs are abnormally shaped, we analyzed the pattern of Zipper expression before CB alignment and during heart lumen formation (Fig. 2, A and A’). Previous studies showed that in the dorsal ectoderm of the embryo, Zipper localizes in a contiguous string at the leading edge of the closing epidermis (Young et al., 1993) and to the extra-embryonic AS cells that undergo histolysis (Toyama et al., 2008). Using immunodetection, we found that Zipper is also expressed in the developing heart (Fig. 2), accumulating in dynamic foci at the leading edge before alignment at stage 15–16 (Fig. 2, A–D). At each time point, only a subset of CBs showed such apical Zipper foci in a continuously changing pattern. This was strikingly different from the pattern at the epidermal leading edge, where Zipper (and actin) forms a contiguous and stable “purse string” structure during epidermal dorsal closure. After CB alignment at stage 17, cardiac Zipper localized toward the luminal side of the forming heart tube (Fig. 2, A’ and C), which indicates that this protein might also play a role during heart lumen formation.

To more closely examine the dynamic pattern of Zipper localization before and during CB alignment, we expressed GFP-tagged Zipper (Franke et al., 2005) in the developing heart and monitored the GFP signal at high resolution in real time. At stage 15, ZipperGFP was expressed in a highly dynamic pattern, forming foci at the leading edge of the CB and then dispersing in a wave-like pattern into the cytoplasm (Fig. 2 B and Video 2). This cycle was repeated several times within each CB until the cells established contralateral contacts and began to form the lumen. Staining of Dlg1 to delineate the CB membranes showed that ZipperGFP localization coincided with constriction of the apical side/leading edge, in contrast to the neighboring cell that did not accumulate Zipper (Fig. 2 D). Thus, it is possible that Zipper may function in facilitating shortening of the leading edge, thereby supporting dorsal mesodermal/cardiac closure in a manner similar to that seen during closure of the epidermis, but with a distinct and dynamic pattern. Coexpression of ZipGFP and RFP-tagged actin-linking protein MoesinRFP in CBs revealed that Moesin localizes laterally to apical Zipper and therefore is likely to be anchored at the apical membrane (Fig. S3, C and C’; and Video 3). After formation of the ventral CB contacts, intense Zipper staining was found at the luminal domain within the aorta and the heart proper (Fig. 2, A’ and C; and Video 4), which suggests a role for Zip during lumen formation or maintenance. In addition to CB-autonomous Zip, we also find that the cells of the AS, which can associate with the heart lumen (Rugendorff et al., 1994), also contribute to the observed signal (Fig. S4 D). In summary, we find that Zipper appears at distinct domains during heart morphogenesis: at the CB leading edge before dorsal closure, and at the luminal domain during lumen formation.

To determine whether Zipper might contribute to the change in CB shape that accompanies central luminal closure, we analyzed the hearts of zip mutant embryos. In wild-type or heterozygous zip mutant embryos, Slit was localized to the luminal

| Cdc42/FM7 x | n | Wild type | DC failure/MC failure | Heart defects | P-value |
|------------|---|----------|---------------------|---------------|---------|
| w1118      | 662| 60.7     | 8.5                 | 30.8          | 0.68    |
| ena111      | 27 | 66.7     | 7.4                 | 25.9          | 0.74    |
| zfh173,34   | 42 | 64.3     | 0.0                 | 35.7          | 0.27    |
| tup101      | 23 | 47.8     | 0.0                 | 52.2          | 0.001   |
| tinEC40     | 11 | 18.2     | 0.0                 | 81.8          | 0.009   |
| tinEC46     | 29 | 6.9      | 6.9                 | 86.2          | 10−4    |
| Ab2         | 28 | 19.9     | 25                  | 57.1          | 10−4    |
| tinG4,dia4CA| 19 | 0        | 5                   | 14            | 10−7    |
| Cdc42F2,dDAAM168 | 22 | 18.2 | 81.8               |              | 10−4    |

Embryos hemizygous for Cdc42 and heterozygous for the indicated alleles were phenotypically analyzed.

Fisher’s exact test was applied to test for significant differences in the expected number of wild-type hearts vs. mutant hearts.
side and the lumen could easily be seen in both the aorta and the heart proper (Fig. 2E). In contrast, zip2/zipII mutant embryos showed two types of heart defects: incomplete mesodermal closure at the dorsal midline and a failure to form the heart lumen (Fig. 2F). In the anterior region of the heart, where the epidermis and the CBs complete closure, Slit still accumulated at the luminal side of the CBs but no luminal space was detected (Fig. 2F compared with Fig. 2E), which suggests that Zipper activity is critically required for heart lumen formation.

**Cdc42 is required for Zipper localization**

To characterize the basis for the observed genetic interaction between Cdc42 and zip, we next examined Zipper localization and dynamics in Cdc42 mutant embryos. For this, we analyzed the spatiotemporal pattern of ZipperGFP in embryos with cardiac-specific expression of either dominant-negative or constitutively active Cdc42 (Cdc42N17 and Cdc42V12, respectively; Fig. 3 and Video 5). In contrast to wild-type embryos, Cdc42V12 expression abolished not only localization of ZipperGFP to the CBs’ leading edge but also the dynamic wave-like movement (Fig. 3, A and B). Conversely, upon expression of activated Cdc42V12, we still find apically accumulated Zipper that was more broadly distributed along the leading edge of the CBs (Fig. 3C). The same phenotypes were observed when we monitored endogenous Zipper protein in situ (Fig. 3, D–F). These findings are consistent with a model in which Cdc42 is required to direct Zipper localization to the CB’s leading edge. We then asked whether any of the known signaling pathways required for heart morphogenesis, such as Slit–Robo and Netrin–Unc5, regulate Zipper localization during heart morphogenesis. In contrast to Cdc42, we found no evidence of changes in Zipper localization (Fig. S2, E and F) in robo, robo2, and unc5 mutants before dorsal closure, despite the heart alignment defects observed at early stages in these mutants (Qian et al., 2005; Albrecht et al., 2011), which indicates that Robo and Unc5 do not control apical Zipper accumulation before CB alignment.

Collectively, these data indicate that Cdc42 affects Zipper localization and cardiac morphogenesis through a pathway distinct from the Slit–Robo or Netrin pathways.
in cytoskeletal organization. Myosin and filamentous actin assemble into contractile bundles, which are in part regulated by myosin light chain (MLC) phosphorylation through the Rho-activated kinases ROCK and MLCK (Vicente-Manzanares et al., 2009). However, manipulation of these kinases in the cardiac mesoderm did not result in any noticeable changes in CB cell shape or alignment (unpublished data). We further considered whether localized assembly of actin filaments might be important for subsequent recruitment of and dynamic changes in Zipper localization. Given that Rho-family GTPases are known regulators of formins, proteins that are central to actin polymerization in many cell types, we asked whether formins are required to direct Zipper localization for lumen formation. Two of the major *Drosophila* formins, dDAAM and Diaphanous (Dia), showed binding of Tinman in ChIP-on-chip experiments (Junion et al., 2012; Jin et al., 2013), which suggests a role for formins during heart morphogenesis.

Antibody staining for dDAAM revealed specific expression in CBs, in particular along the cell membrane (Fig. 4 A), which indicated that formins might indeed play an important role during heart morphogenesis. Although we did not detect any phenotypes in *dDAAM* or *Dia* mutant embryos (Fig. 4 B), either due to strong maternal rescue or possible redundancies with other formin proteins, we found that embryos double mutant for *Cdc42* and *dDAAM* showed severe morphogenesis defects (Fig. 4, B and B'; and Table 2) that are not found in either single mutant alone, which indicates that *Cdc42* and *dDAAM* genetically interact. We then tested if Cdc42, a truncated C-terminal dDAAM that is lacking the diaphanous inhibitory domain (Matusek et al., 2006), was sufficient to induce ectopic heart lumina when overexpressed. Indeed, we found Dg and Slit-positive structures (Fig. 4 C) as well as ectopic Multiplexin protein (Fig. 4 D') upon heart- and mesoderm-specific expression of activated CdDAAM, which were similar to the structures found in hearts expressing *Cdc42* (Fig. 1, E, H, and J).

When we examined embryos with cardiac-specific overexpression of the activated formin Diaphanous (*Dia*), which can induce ectopic Zipper localization in the AS (Homem and Peifer, 2008), we found a strong induction of ectopic lumina (Fig. 5, A and B). Interestingly, Slit localized prominently to these structures (Figs. 5, C–E'), again in support of the notion that they are ectopically localized heart lumina. Zipper protein was also found at these sites (Fig. S3, D and D'), indicating that *Dia* activity was sufficient to ectopically localize both Zipper and Slit. The normal spatiotemporal pattern of Zipper localization was maintained in these ectopic lumina: time-lapse analysis of CBs expressing both *Dia* and Zipper showed that Zipper localized to the newly forming ectopic lumina in a pulsatile and repetitive fashion (Fig. 5 F and Video 6). We also tested additional heart lumen markers such as the basement membrane component Perlecan/Trol and the heart-lumen specific collagen Multiplexin (Harpaz et al., 2013). Both lumen markers are also found at the ectopic heart lumina (Fig. 5, G–I), which is consistent with our findings using activated dDAAM. Based on these observations, we conclude that *Dia* activity is sufficient to recruit nonmuscle myosin II and to form ectopic heart lumina. Activated *Dia* also induced ectopic lumina in a *Cdc42* mutant background (Fig. 5 J), which suggests that formins are acting downstream of *Cdc42*. Similar to *dDaam*, loss of *dia* function (*dia*;*Df(2L)ED1315*, *dia*;*Df(2L)ED1315*, or cardiac *dia-RNAi*) did not produce a cardiac phenotype, which suggests a maternal rescue or functional redundancies among formins.
Collectively, our data show that both formins, when activated, can induce ectopic lumen-like structures. Importantly, one of them, dDAAM, is prominently expressed in the heart and, together with Cdc42, required for heart lumen formation. In summary, we have identified novel functions for the small GTPase in contrast to dDaam, Dia did not enhance the phenotype of Cdc42 mutants, which suggests that Dia function may not be through Cdc42 in the developing heart. Alternatively, the phenotypes induced by DiaCA could be caused by ectopic gain-of-function effects, potentially mimicking activated dDaam.

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Cdc42, and the formins dDAAM/Diaphanous, in promoting lumen formation during heart tube formation by influencing the dynamic relocalization of the nonmuscle myosin II Zipper to the CB leading edge (Fig. 6). Thus, Cdc42 and formin proteins act together to control a cellular mechanism that is sufficient to drive lumen formation and to correctly localize not only Zipper but also the known heart lumen markers Dg, Slit, Trol, and Multiplexin. These data point to critical roles for Cdc42 and formins, dDAAM in particular, acting together in cardiac morphogenesis.

Discussion

Although much is known of how transcription factors and signaling pathways specify and regulate cardiac cell fate, we do not yet have a complete understanding of the downstream effectors that precisely orchestrate heart tube assembly and lumen formation. Here, we present evidence that heart morphogenesis requires the activity of Cdc42, but apparently not Rho1 or Rac1, to regulate the correct positioning and remodeling of CBs during heart formation.
Slit now reduces the adhesiveness of the luminal surface by reducing DE-Cadherin levels (Santiago-Martínez et al., 2008), whereas in parallel, the actomyosin network activity controlled by Cdc42 and formins induces the necessary cell shape changes. Based on our data, we propose that the Slit–Robo pathway is permissive for lumen formation, and that Cdc42 and formins are more directly involved in modulating the actomyosin network in this process (illustrated in Fig. 6). It is interesting to note that polarization of Slit to the luminal domain seems to depend on formin activity: initial lumen formation appears to precede Slit localization, as activated Dia could induce ectopic Dg-delineated lumina that were not Slit positive (Fig. 4, E and E″, curved arrow).

lumen formation. Cdc42 is acting with one or more formins to regulate the assembly of the local actomyosin networks. Accordingly, activation of Cdc42 or Dia is sufficient to reorganize the actomyosin network and initiate lumen formation, which indicates that these proteins are pivotal to the creation of a functional heart tube.

Do Cdc42 and Slit–Robo play distinct roles cardiac morphogenesis?
The Slit–Robo signaling pathway plays a critical role during heart morphogenesis in both Drosophila and vertebrates (Qian et al., 2005; Santiago-Martínez et al., 2006; Medioni et al., 2008; Fish et al., 2011; Mommersteeg et al., 2013). In the developing Drosophila heart, Slit–Robo signaling patterns both the junctional domain of the heart (marked by β-catenin/Armadillo) and the luminal domain (marked by Dg and Slit; Medioni et al., 2008). Slit–Robo mutants show disruption of CB cell–cell contacts, and failure to form heart lumina. Interestingly, slit–robo mutants show no changes in the dynamics of actin (Medioni et al., 2008 and this study) or myosin (this study), which suggests that Slit–Robo signaling may primarily regulate CB cell adhesion in the developing heart. We also found that correct Zipper localization did not depend on Slit–Robo or Unc5/Netrin pathways, which raises the possibility that these pathways may regulate the “adhesive” properties of the cells to allow directed application of force generated by the actomyosin network, in turn resulting in the desired cell shape changes. The CB chain disruption observed in slit–robo mutants (Qian et al., 2005) could be explained by a reduction in the adhesion of CBs before lumen formation, which no longer withstands the force produced by actomyosin at the leading edge. During lumen formation, Slit now reduces the adhesiveness of the luminal surface by reducing DE-Cadherin levels (Santiago-Martínez et al., 2008), whereas in parallel, the actomyosin network activity controlled by Cdc42 and formins induces the necessary cell shape changes. Based on our data, we propose that the Slit–Robo pathway is permissive for lumen formation, and that Cdc42 and formins are more directly involved in modulating the actomyosin network in this process (illustrated in Fig. 6). It is interesting to note that polarization of Slit to the luminal domain seems to depend on formin activity: initial lumen formation appears to precede Slit localization, as activated Dia could induce ectopic Dg-delineated lumina that were not Slit positive (Fig. 4, E and E″, curved arrow).

The actomyosin network and heart morphogenesis
Morphogenesis and the shaping of an organ involves mechanical forces (Patwari and Lee, 2008), as reflected by the critical role of the actomyosin network in many developmental contexts, including cardiogenesis (i.e., nonmuscle myosin II-B is required for cardiogenesis; Tullio et al., 1997). Our experiments with cardiac-specific expression of GFP-tagged nonmuscle myosin showed that the actomyosin network is assembled in a dynamic and localized fashion, and also involves localization of the F-actin tethering protein Moesin at the apical domain. Interestingly, the pattern of dynamic changes in the actomyosin network varies during morphogenesis of different tissues. For example, in Drosophila, dorsal closure of the epidermis is achieved through a pulling force along a bilateral anterior-posterior actin-myosin cable (Young et al., 1993), with little or no apparent change in the actomyosin network. In contrast, pulsation of the
actomyosin network has been observed during egg chamber development (He et al., 2010), gastrulation (Martin et al., 2009), and during dorsal closure (Solon et al., 2009; Blanchard et al., 2010). There are several ways to explain the dynamic, pulsating assembly and disassembly of the actomyosin network, including mechanical force and tension, as well as regulation by signaling pathways. For example, intercalating cells of the elongating Drosophila epithelium are under tension, and Zipper is stabilized within these cells in a nonrandom fashion in response to external force (Fernandez-Gonzalez et al., 2009). Furthermore, ectopically applied pressure can recruit this myosin to the site of indentation, which indicates that these cells are able to sense and respond to mechanical force. During development, CBs make contacts with many cell types that might induce tension, including pericardial cells, AS cells, overlying epidermal cells, and ipsilateral CBs. Of these, only CBs (and some extra-embryonic AS) display pulsating actomyosin assembly behavior. When CBs contract apically during an actomyosin pulse, force could be exerted on the neighboring CBs, resulting in contraction. However, we did not observe actomyosin pulse propagation to neighboring CBs. Therefore, we propose that the actomyosin network responds to a CB-intrinsic oscillator that involves Cdc42. Our results with CBs overexpressing DiaCA or CDAAM favor an intrinsic model. Even though activated formins are more commonly implicated in the regulation of actin dynamics and cell polarity (Etienne-Manneville, 2004; Heasman and Ridley, 2008), its homologue Cdc42. In support of this, Tin has been shown to bind to the only CBs, Cdx and formins regulate Drosophila heart formation

Does Cdc42 play a unique role in cardiac tissue?
The small Rho-like GTPases play important roles in many different cell behaviors. Cdc42 is the family member most commonly implicated in the regulation of actin dynamics and cell polarity (Etienne-Manneville, 2004; Hasman and Ridley, 2008), including in cardiovascular development and function. For example, removal of Cdc42 in developing mouse hearts causes embryonic lethality, whereas in the adult, Cdc42 is required to activate JNK signaling during cardiac hypertrophy (Mailliet et al., 2009). In adult Drosophila hearts, expression of Cdc42N17 also causes cardiac arrhythmias (Qian et al., 2011).

In cultured rat cardiomyocytes, cell shape changes induced by treatment with leukocyte-inhibitory factor (LIF) are specifically inhibited by a dominant-negative form of Cdc42, but not of RhoA or Rac1 (Nagai et al., 2003). Interestingly, activated forms of all three enzymes caused an increase in cell size, but only activated Cdc42 mimicked the LIF-induced phenotype. This correlates with our observation that only dominant-negative Cdc42 delivery to the heart induced cardiac morphogenesis defects. The finding that Cdc42 genetically interacts with tin or its homologue Nkx2.5 in the Drosophila and mouse heart (Qian et al., 2011) further highlights such a cardiac-specific role for Cdc42. In support of this, Tin has been shown to bind to the Cdc42 enhancer during Drosophila heart development (Junion et al., 2012; Jin et al., 2013). In summary, our findings point to a central role for this GTPase during heart development.

In cardiovascular development, Cdc42 is required for the formation of vacuoles and lumina in three-dimensional endothelial cell (EC) cultures (Bayless and Davis, 2002; Koh et al., 2008). In vivo studies have shown that the lumen of the mouse dorsal aorta forms through VE-cadherin—dependent changes in adhesion of aligned ECs and changes in cell shape induced by actomyosin activity (Strilić et al., 2009). The Drosophila heart and mammalian ECs share several commonalities in their ontogenetic origins and their patterns of gene expression (Hartenstein and Manda1, 2006). This study adds their similar use of the actomyosin skeleton for central luminal closure. Our findings also provide an entry point for the genetic and molecular dissection of the interactions among cell adhesion and signaling molecules during heart formation using the Drosophila heart model.

Materials and methods

Drosophila strains and genetics
Fly stocks were maintained at 25°C on standard fly food. Relevant wild-type chromosomes were marked using balancers carrying Dfd-YFP to allow detection in late-stage embryos (Ie et al., 2006). The following fly stocks were used: tinD-Gal4 (Yin et al., 1997), tinCΔ4-Gal4 (Lo and Frasch, 2001), tinN17 (Zaffran et al., 2006), and tinM10 (Aspiziu and Frasch, 1993). All lines were a gift from M. Frasch, University of Erlangen-Nuremberg, Bavaria, Germany. Roba29 (a gift from B. Dickson, Howard Hughes Medical Institute Janelia Farm Research Campus, Ashburn, VA; Rajagopalan et al., 2000), zfh13, [a gift from K. Lehmann, New York University, New York, NY; Brothier et al., 1998], G14-Gal4 (from S. Kramer, Robert Wood Johnson Medical School, Rutgers University, Piscataway, NJ), UAS-dlg1::GFP (a gift from V. Budnik, University of Massachusetts Medical School, Worcester, MA; Koh et al., 1999), unc53 (a gift from G.J. Bashaw, University of Pennsylvania, Philadelphia, PA; Labrador et al., 2005), UAS-zip::GFP (a gift from D. Kiehart, Duke University; Franke et al., 2005), dDAAMN106 and UAS-Cdaam (Matusek et al., 2006), UAS-dia::GFP (a gift from P. Rath, Institute of Molecular and Cell Biology, Singapore; Somogyi and Rørth, 2004), Pin/CyO Dfd-YFP, Cdx/TM6b Dfd-YFP (a gift from O. Vel, Institut für Genetik, Mainz, Germany), Cdc42N17, end23, UAS-Rho1N19, zip1, UAS-Rac1N17, zip2, UAS-Rho1G12V, zip162, UAS-austin5C::GFP, robo1, UAS-mCD8::GFP, tip170, arm::GFP, FM71, Dfd-YFP, howGal4, snail2/Cyo-Dfd-YFP, 332-Gal4, Dr1/TM6b-Dfd-YFP, Df(2R)ED2426, and MTD-Gal4 [all from the Bloomington Drosophila Stock Center]. All alleles are described in the FlyBase database (McGuillen et al., 2012).

To test for genetic interaction with Cdc42N17, YFP and 5xI protein expression was assessed in the embryos derived from the test cross. Absence of the proteins identifies Cdc42N17 male mutant embryos that are also heterozygous for the gene tested.

Immunohistochemistry and imaging
For embryo collections, parental lines were crossed and maintained for 2 d on standard fly food supplemented with dry yeast. Flies were then transferred to cages and allowed to lay eggs on removable cage bottoms containing grape juice agar and rehydrated Baker’s yeast. Collected embryos were dechorionated for 3 min in 3% CloroX and fixed for 22 h in a 1:1 mixture of heptane and 1× PBS, pH 7.4, containing 5% formaldehyde. For immunodetection of Zipper, dechorionated embryos were heat-fixed by immersion for 1 min in boiling PBS containing 0.03% Triton X-100 (PBTrx), and then placed in ice-cold PBTrx (Müller and Wieschaus, 1996). Embryos were devitellinized by briefly vortexing in a 1:1 mixture of methanol and heptane, rinsed with methanol, and stored in methanol at −20°C. For immunostaining, embryos were rehydrated in PBTrx and washed for 1 h in PBTrx before the addition of antibodies.

Fixed embryos were incubated with primary antibodies (Table 2 at 4°C overnight and with secondary antibodies at room temperature for 2 h. Goat secondary antibodies were conjugated to AMCA, Alexa Fluor 488, FITC, Cy3, Alexa Fluor 594, Alexa Fluor 647, or Cy5 and obtained from Jackson ImmunoResearch Laboratories, Inc., and were used at a dilution of 1:500. F-actin was detected by staining with phalloidin–Alexa Fluor 488 (a gift from S. Kramer, Robert Wood Johnson Medical School, Rutgers University, Piscataway, NJ), UAS-dlg1::GFP (a gift from V. Budnik, University of Massachusetts Medical School, Worcester, MA; Koh et al., 1999), unc53 (a gift from G.J. Bashaw, University of Pennsylvania, Philadelphia, PA; Labrador et al., 2005), UAS-zip::GFP (a gift from D. Kiehart, Duke University; Franke et al., 2005), dDAAMN106 and UAS-Cdaam (Matusek et al., 2006), UAS-dia::GFP (a gift from P. Rath, Institute of Molecular and Cell Biology, Singapore; Somogyi and Rørth, 2004), Pin/CyO Dfd-YFP, Cdx/TM6b Dfd-YFP (a gift from O. Vel, Institut für Genetik, Mainz, Germany), Cdc42N17, end23, UAS-Rho1N19, zip1, UAS-Rac1N17, zip2, UAS-Rho1G12V, zip162, UAS-austin5C::GFP, robo1, UAS-mCD8::GFP, tip170, arm::GFP, FM71, Dfd-YFP, howGal4, snail2/Cyo-Dfd-YFP, 332-Gal4, Dr1/TM6b-Dfd-YFP, Df(2R)ED2426, and MTD-Gal4 [all from the Bloomington Drosophila Stock Center]. All alleles are described in the FlyBase database (McGuillen et al., 2012).

To test for genetic interaction with Cdc42N17, YFP and 5xI protein expression was assessed in the embryos derived from the test cross. Absence of the proteins identifies Cdc42N17 male mutant embryos that are also heterozygous for the gene tested.
30 min to quench endogenous peroxide before DAB staining. Immunohis-tochemistry was performed with anti-GFP to detect balancer chromosomes and anti-Sx1 to determine the sex (female embryos show uniform brown staining). Embryos hemizygous mutant for Cdc42/Y and heterozygous for the tested allele remained unstained. These embryos were manually removed from the staining solution, and CB nuclei were stained with anti-Nnr1 and an alkaline phosphate-conjugated secondary antibody. Antibody staining was visualized with WB reagent (Promega).

Late stage 17 embryos and larvae were dissected according to Broadie and Bate [1993], except that the dissections were performed in artificial adult hemolymph (Ocorr et al., 2007b) instead of B&B buffer, and the larvae were cut open along the ventral midline. For time-lapse movies, dechorionated embryos were manually aligned on grape juice agar with their dorsal side facing up. A heptane glue–coated coverslip was carefully placed on top of the embryos, which attach to the coverslip surface. The coverslip with embryos is then placed in a well made from press-to-seal silicone (JTR-SA-0.5; Grace Bio-Labs) filled with holocarbon oil 27 (Sigma-Aldrich), thereby immersing the embryos in the oil. Heptane glue was freshly made by incubation of 10 cm of Scotch tape with 1 ml of n-heptane for 15 min. 20 µl of glue was thinly spread on 22 × 50-mm glass coverslip 30 min before mounting to allow complete evaporation of heptane. Images were acquired using a C-Apochromat 40x/1.2 NA water immersion objective lens on an Imager Z1 equipped with an Apotome (all from Carl Zeiss). Axiocam MRm camera, and Axiovision 4.8.2 software (all from Carl Zeiss). Time-lapse movies were acquired at room temperature using a C-Apochromat 63x/1.2 NA water immersion objective lens on a confocal microscope (LSM710) using Zen 2009 software (all from Carl Zeiss). Images were analyzed using ImageJ software (Schneider et al., 2012) and figures were assembled using Photoshop CS4 (Adobe). Movies were generated using Final Cut Express 4 (Apple).

Online supplemental material
Fig. S1 summarizes embryonic and early larval heart phenotypes found in Cdc42 mutants, and shows a comparison of the cardiogenic effects of different mutant Rho-GTase family proteins. Fig. S2 contains the examples and quantification of ectopic heart lumina and filopodia number in different genotypes, including Cdc42Y/+. Fig. S3 shows Slit localization in tinABD; tinEC40/346; Cdc42N17 mutants, and shows a comparison of the cardiogenic effects of Cdc42 and Rac1 GT-Pases in embryos.
dorsal closure. Cell. 137:1331–1342. http://dx.doi.org/10.1016/j.cell.2009.03.050

Somogyi, K., and P. Rørth. 2004. Evidence for tension-based regulation of Drosophila MAL and SRF during invasive cell migration. Dev. Cell. 7:85–93. http://dx.doi.org/10.1016/j.devcel.2004.05.020

Strilić, B., T. Kucera, J. Eglinger, M.R. Hughes, K.M. McNagny, S. Tsukita, E. Dejana, N. Ferrara, and E. Lammert. 2009. The molecular basis of vascular lumen formation in the developing mouse aorta. Dev. Cell. 17:505–515. http://dx.doi.org/10.1016/j.devcel.2009.08.011

Tepass, U. 2012. The apical polarity protein network in Drosophila epithelial cells: regulation of polarity, junctions, morphogenesis, cell growth, and survival. Annu. Rev. Cell Dev. Biol. 28:655–685. http://dx.doi.org/10.1146/annurev-cellbio-092910-154033

Toyama, Y., X.G. Peralta, A.R. Wells, D.P. Kiehart, and G.S. Edwards. 2008. Apoptotic force and tissue dynamics during Drosophila embryogenesis. Science. 321:1683–1686. http://dx.doi.org/10.1126/science.1157052

Tullio, A.N., D. Accili, V.J. Ferrans, Z.X. Yu, K. Takeda, A. Grinberg, H. Westphal, Y.A. Preston, and R.S. Adelstein. 1997. Nonmuscle myosin II-B is required for normal development of the mouse heart. Proc. Natl. Acad. Sci. USA. 94:12407–12412. http://dx.doi.org/10.1073/pnas.94.23.12407

Vanderploeg, J., L.L. Vazquez Paz, A. MacMullin, and J.R. Jacobs. 2012. Integrons are required for cardioblast polarisation in Drosophila. BMC Dev. Biol. 12:8. http://dx.doi.org/10.1186/1471-213X-12-8

Vicente-Manzanares, M., X. Ma, R.S. Adelstein, and A.R. Horwitz. 2009. Nonmuscle myosin II takes centre stage in cell adhesion and migration. Nat. Rev. Mol. Cell Biol. 10:778–790. http://dx.doi.org/10.1038/nrm2786

Wei, L., K. Imanaka-Yoshida, L. Wang, S. Zhan, M.D. Schneider, F.J. DeMayo, and R.J. Schwartz. 2002. Inhibition of Rho family GTPases by Rho GDP dissociation inhibitor disrupts cardiac morphogenesis and inhibits cardiomyocyte proliferation. Development. 129:1705–1714.

Yarnitzky, T., and T. Volk. 1995. Laminin is required for heart, somatic muscles, and gut development in the Drosophila embryo. Dev. Biol. 169:609–618. http://dx.doi.org/10.1006/dbio.1995.1173

Yin, Z., X.L. Xu, and M. Frasch. 1997. Regulation of the twist target gene tinman by modular cis-regulatory elements during early mesoderm development. Development. 124:4971–4982.

Young, P.E., A.M. Richman, A.S. Ketchum, and D.P. Kiehart. 1993. Morphogenesis in Drosophila requires nonmuscle myosin heavy chain function. Genes Dev. 7:29–41. http://dx.doi.org/10.1101/gad.7.1.29

Zaffran, S., I. Reim, L. Qian, P.C. Lo, R. Bodmer, and M. Frasch. 2006. Cardioblast-intrinsic Tinman activity controls proper diversification and differentiation of myocardial cells in Drosophila. Development. 133:4073–4083. http://dx.doi.org/10.1242/dev.02586