

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

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During heart formation, a network of transcription 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 *Slit*. Placement of *Slit* 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.

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., 2007a; Bryantsev and Cripps, 2009; Medioni et al., 2009; Bodmer 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, FGF) 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 they interact and cross-regulate during heart development has

been studied extensively (for review see Bryantsev and Cripps, 2009; Bodmer et al., 2010), including the global network of target genes controlled by the cardiac master regulator Tinman (Junion et al., 2012; Jin et al., 2013).

In contrast to the regulatory network involved in cardiac specification, the genetic mechanisms controlling the subsequent events in heart morphogenesis are not well understood. Cardiac cells are embedded in a complex environment of ECM and neighboring cells and tissues, with which they communicate by diverse signaling mechanisms. Several studies have shown that signaling by the ECM protein *Slit* and its receptor *Robo* are crucial for heart morphogenesis in *Drosophila* (Qian et al., 2005; MacMullin and Jacobs, 2006; Santiago-Martínez et al., 2006, 2008; Medioni et al., 2008). Mutations in *Slit*–*Robo* have multiple effects on heart morphogenesis, including cardioblast (CB) adhesion, cell shape alterations, and lumen formation. More recently, the *Slit*–*Robo* pathway was also shown to be important for vertebrate cardiogenesis (Medioni et al., 2010; Fish et al., 2011; Mommersteeg et al., 2013). Other important signaling

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Abbreviations used in this paper: AS, amnioserosa; CB, cardioblast; dDAAM, disheveled associated activator of morphogenesis; Dg, Dystroglycan.

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molecules for *Drosophila* heart morphogenesis include Unc5 (Albrecht et al., 2011), integrins (Vanderploeg et al., 2012), Laminin (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; Berzat and Hall, 2010). GTPases control the phosphorylation of myosin regulatory light chains (Rho activation of ROCK and MLCK, and Cdc42 activation of MRCK), as well as actin polymerization through regulation of actin assembly factors like the Arp2/3 activator of WASP or formin proteins (Campellone and Welch, 2010; Hanna and El-Sibai, 2013). For example, the formins Diaphanous and disheveled associated activator of morphogenesis (dDAAM) have been shown to regulate actin cytoskeleton remodeling and actomyosin contractility, e.g., in the context of epithelial morphogenesis and myofilaments formation (Afshar et al., 2000; Matusek et al., 2006; Homem and Peifer, 2008; Molnár et al., 2014).

One method to block the activity of small GTPases is through GDP dissociation inhibitors (GDIs). Cardiac overexpression of Rho-GDI during mouse embryogenesis disrupts heart formation (Wei et al., 2002), indicating that Rho-GTPases in general are crucial for heart morphogenesis. We recently showed that *Cdc42* genetically interacts with the cardiac transcription factor encoded by *tinman/Nkx2-5* to maintain cardiac contractility

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 *Netrin-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, *Cdc42³* (Fehon et al., 1997). Approximately two thirds of *Cdc42³* mutant embryo hearts, stained for Nmr1, 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).

To analyze the heart morphology of *Cdc42³* mutants in detail, we analyzed the localization of Dystroglycan (Dg), which is a basement membrane marker, and of *Slit*, a prominent luminal 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, *Cdc42³* mutant embryos showed misaligned CB nuclei, and Dg and *Slit* no longer delineate a distinct luminal space

(Fig. 1, C' and C''). Although *Cdc42* is known to control cell polarity (Hall, 2005; Tepass, 2012), our data point to a novel role for *Cdc42* in heart development, i.e., facilitating the coordinated alignment of CBs and lumen formation.

To examine a cardiac-specific loss of *Cdc42* function, we overexpressed the dominant-negative allele *Cdc42^{NI7}* (Luo et al., 1994) only in the heart tissue or in all somatic musculature (Fig. 1, D and E). Expression of *Cdc42^{NI7}* with either Gal4 driver resulted in a fully penetrant cardiac phenotype reminiscent of that observed with the zygotic *Cdc42³* loss-of-function allele (Fig. 1, D and E). Expression of dominant-negative Rho GTPases can have nonspecific effects on the activation of other family members (Debreceeni et al., 2004). To investigate this, we overexpressed dominant-negative *Rho1^{NI9}*, *Rac1^{NI7}*, or *RhoL^{N25}* in the cardiac mesoderm or developing heart (Fig. S1, E–J). None of these manipulations affected cardiac morphogenesis, which suggests that the observed cardiac defects are specific to loss of *Cdc42* function.

To determine whether *Cdc42* mutant hearts have defects in cardiac differentiation, we examined early larval hearts from wild-type and zygotic *Cdc42³* mutant flies, or flies with heart-specific expression of *Cdc42^{NI7}*, for changes in late stage cardiac differentiation and ECM structure (collagen IV Pericardin; Fig. S1, B and D). Both *Cdc42³* and *Cdc42^{NI7}* hearts formed dense myofibrillar networks, which is indicative of advanced cardiac differentiation. Adult *tinCΔ4>Cdc42^{NI7}* escaper flies had abnormally beating hearts (as previously reported; Qian et al., 2011). The mesodermal-closure defects in late embryonic/larval stages were accompanied by a lack of ECM near the defective CB alignment (Fig. S1 C). The embryonic luminal defects also persisted into later stages, and the differentiated cardiomyocytes form a layer rather than a tube. Such hearts also showed incomplete sheathing of the cardiomyocytes with Pericardin, which indicates that deposition of ECM depends on cardiomyocyte *Cdc42* activity (Fig. S1, B–D'). In such hearts, the number of pericardial cells (PCs) and Tinman-positive CBs was not changed, which is consistent with the notion that the observed defects are not caused by misspecification by loss of *Cdc42* function.

***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 *Cdc42^{VI2}* 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 *Cdc42^{VI2}* mutant hearts. In contrast to the columnar arrangement of wild-type CBs (Fig. 1 G), *Cdc42^{VI2}* 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 *Cdc42^{NI7}* 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 *Cdc42³* 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 *Cdc42³* background (Table 1). Of the cardiac transcription factors tested, we found that *Cdc42³* strongly interacted with loss-of-function alleles of *tinman* (*tin*), but not with *tail-up* (Islet-1) or *zfh1*. Of note, cardiac-specific *tin^{ABD}*; *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 *robo1* (*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

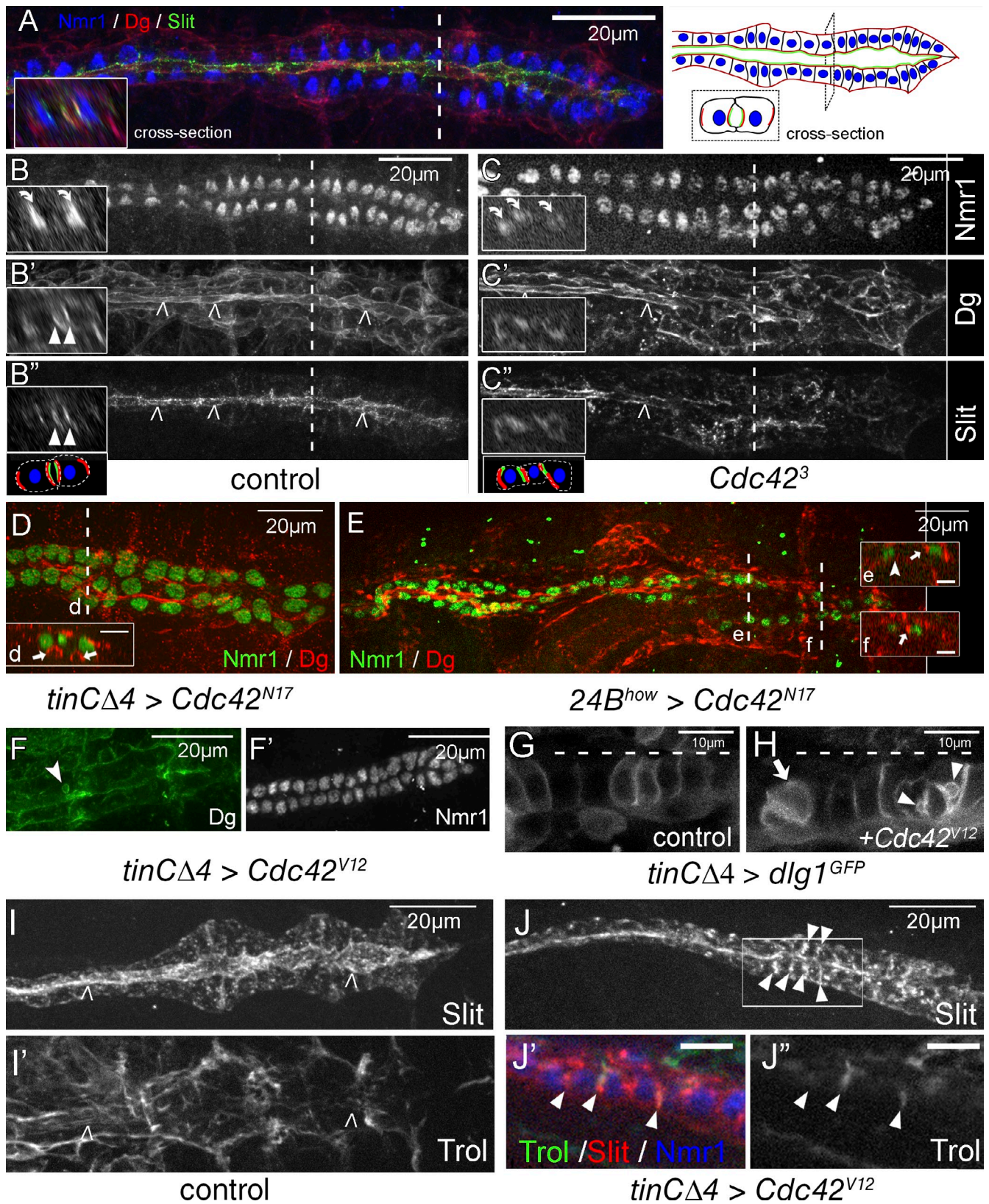


Figure 1. ***Cdc42* is necessary for cardiac alignment and lumen formation.** (A and B) Wild-type *Drosophila* heart at the end of heart morphogenesis, st17. Cardiac nuclei (*Nmr1*, blue) are aligned at the dorsal midline. Basement membrane (*Dg*, red) and heart lumen (*Slit*, green) are properly localized to the respective domains (see schematic in A). (B and C) CB cell shape in wild-type (B–B') and *Cdc42*³ 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 *Cdc42*³ zygotic mutant hearts fail to form a central heart lumen (C' and C'') or are mispositioned (curved

Table 1. Genes tested for genetic interaction with *Cdc42*

<i>Cdc42</i> ³ / <i>FM7</i> x	n	Wild type	DC failure/MC failure	Heart defects	P-value ^a
		%	%	%	
w ¹¹¹⁸	662	60.7	8.5	30.8	
eng ²³	27	66.7	7.4	25.9	0.68
zfh1 ^{75.34}	42	64.3	0.0	35.7	0.74
tup ^{isl-1}	23	47.8	0.0	52.2	0.27
zip ¹	30	30.0	33.3	36.7	0.001
tin ^{EC40}	11	18.2	0.0	81.8	0.009
tin ³⁴⁶	29	6.9	6.9	86.2	<10 ⁻⁸
Abl ²	28	19.9	25	57.1	<10 ⁻⁴
tinCΔ4>dia ^{CA}	19	0	5	14	<10 ⁻⁷
<i>Cdc42</i> ³ ,dDAAM ^{ex68}	22	18.2	81.8		<10 ⁻⁴

Embryos hemizygous for *Cdc42*³ and heterozygous for the indicated alleles were phenotypically analyzed.

^aFisher's exact test was applied to test for significant differences in the expected number of wild-type hearts vs. mutant hearts.

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.

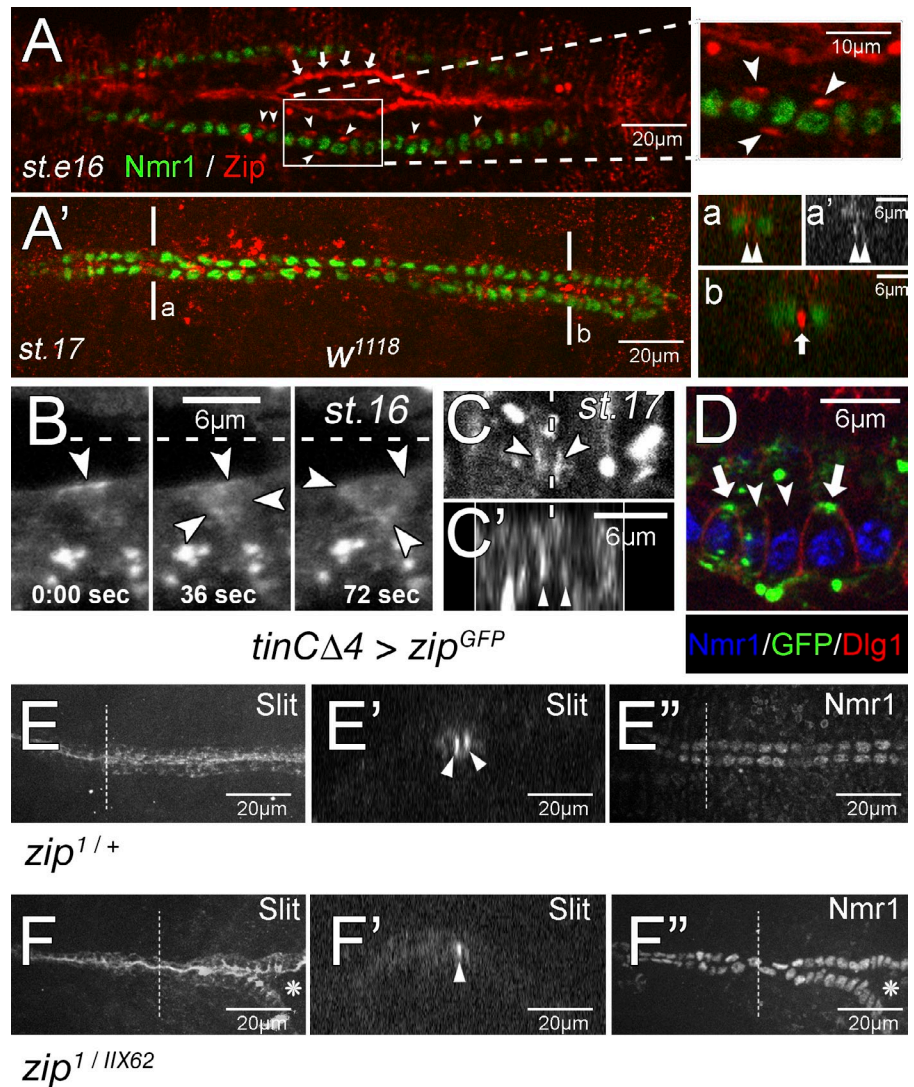
To more closely examine the dynamic pattern of Zipper localization before and during CB alignment, we expressed GFP-tagged Zipper (Franke et al., 2005) in the developing heart and monitored the GFP signal at high resolution in real time.

At stage 15, Zipper^{GFP} 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 Zipper^{GFP} 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 Zip^{GFP} and RFP-tagged actin-linking protein Moesin^{RFP} 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 *zipper* mutant embryos, Slit was localized to the luminal

arrowheads), and Slit protein is located at the CB interface (C'). (D) Cardiac-specific expression of dominant-negative *Cdc42*^{N17} in mid- to late-stage developing hearts (using tinCΔ4-Gal4) causes multiple heart lumina (arrows in d). Dg (red) indicates the ECM. (E) Expression of *Cdc42*^{N17} in the somatic and cardiac mesoderm causes severe alignment defects (e) or collapsed heart lumina at the point of CB alignment (f). Arrows point to the luminal domain marked by Dg. The arrowhead shows lack of Dg at contralateral CB. (F) Expression of activated *Cdc42*^{V12} causes ectopic, ipsilateral “lumen” (enriched for Dg protein; arrowhead in F) but does not interfere with overall CB alignment (F'). (G and H) CB cell shapes are changed upon *Cdc42*^{V12} misexpression. Wild-type CBs are columnar (G), whereas CBs expressing activated *Cdc42*^{V12} are more rounded (H, arrow) and form round structures resembling ectopic lumina (H, arrowheads). Cell outlines are visualized by expression of Dlg1^{GFP}. Broken lines indicate the midline. (I and J) Further characterization of *Cdc42*^{V12}-induced lumina. Slit (I) and Trol (I') are additional markers that localize to the luminal domain in wild-type hearts (open arrowheads). Upon cardiac overexpression of activated *Cdc42*^{V12}, Slit is found between ipsilateral CBs (J, arrowheads), some of which also colocalize Trol (J' and J''). Bars in D and E (insets), J', and J'': 6 μm.

Figure 2. *Drosophila* nonmuscle myosin II, Zipper, shows dynamic localization during heart morphogenesis and is required for lumen formation. (A) Before dorsal closure, Zipper (red) is present at high levels at the leading edge of epidermal cells (arrows). Zipper foci are also present in variable numbers at the leading and trailing edge of CBs (inset, arrowheads; CB nuclei are green). (A') After dorsal closure, high levels of Zipper are at the heart lumen (a, arrowheads) and within (b, arrow) the heart. (B and C) Still frames from a time-lapse movie showing Zipper movement during early and late steps of heart morphogenesis. (B) Before dorsal closure, Zipper accumulates at the leading edge of the cell, disperses through the cytoplasm, and then reaccumulates to initiate another cycle. The broken line indicates the dorsal midline. (C and C') After dorsal closure, Zipper is found dynamically at the heart lumen (arrowheads). (D) Apical constriction of CBs (arrows) coincides with apical concentration of cardiac-specific Zipper^{GFP}. Neighboring CBs do not show accumulation of Zip at that time point (arrowheads). (E) Lumen formation requires Zipper. An early stage 17 embryo heterozygous for the *zip*^{2/llx62} allele has a regular aortic lumen, which is delineated by Slit (arrowheads in center panel; cross-section at the broken line of the side panels). (F) In contrast, the complete *zip*^{2/llx62} null mutant fails to form a lumen, although Slit still accumulates at the contralateral CB interface. Note that there is a cardiac closure defect that is secondary to an epidermal dorsal closure defect (asterisk). Broken lines indicate the position of the cross section.



side and the lumen could easily be seen in both the aorta and the heart proper (Fig. 2 E). In contrast, *zip*^{2/zip^{llx62} mutant embryos showed two types of heart defects: incomplete mesodermal closure at the dorsal midline and a failure to form the heart lumen (Fig. 2 F). 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. 2 F' compared with Fig. 2 E'), 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 Zipper^{GFP} in embryos with cardiac-specific expression of either dominant-negative or constitutively active *Cdc42* (*Cdc42^{N17}* and *Cdc42^{V12}*, respectively; Fig. 3 and Video 5). In contrast to wild-type embryos, *Cdc42^{N17}* expression abolished not only localization of Zipper^{GFP} to the CBs' leading edge but also the dynamic wave-like movement (Fig. 3, A and B). Conversely, upon expression of activated *Cdc42^{V12}*, we still find apically accumulated Zipper that was

more broadly distributed along the leading edge of the CBs (Fig. 3 C). 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.

Actin filament organization is linked to heart lumen formation and slit localization

To gain further insight into the mechanism by which *Cdc42* and Zipper collaborate in lumen formation, we examined their role

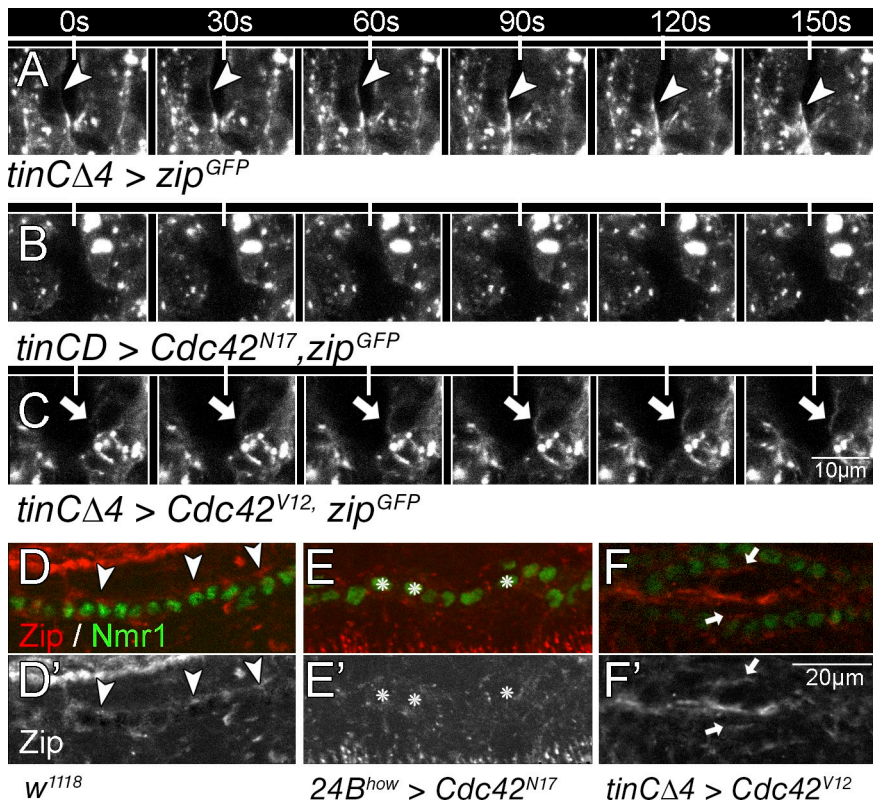


Figure 3. Localization of zipper is dependent on Cdc42 activity. (A–C) Shown are still frames from time-lapse movies of embryos of the indicated genotypes. CB-specific expression of Zipper^{GFP} and Cdc42 overexpression using *tinCΔ4*-Gal4. The midline is indicated by the white vertical lines. Note that overexpressed Zip^{GFP} can form aggregates (intense blobs) that are absent in wild-type tissue. (D–F) Endogenous Zipper localized in fixed heart tissues of the indicated genotypes. (A and D) In wild-type embryos, Zipper concentrates at the CB leading edge in a dynamic, cyclical pattern (arrowheads). It accumulates into a single focus at the leading edge (90–150 s) that dissolves later on. (B and E) Expression of *Cdc42*^{N17} prevents correct localization of Zipper and inhibits foci formation. Asterisks mark positions of selected CB nuclei. (C and F) Upon overexpression of *Cdc42*^{V12}, Zipper still accumulates toward the leading edge but is less concentrated (arrows).

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*^{Ex68} 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*³, *dDAAM*^{Ex68} 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 CdDAAM, 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*^{V12} (Fig. 1, E, H, and J).

When we examined embryos with cardiac-specific overexpression of the activated formin Diaphanous (Dia^{CA}), 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^{CA} 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^{CA} and Zipper^{GFP} showed that Zipper^{GFP} 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^{CA} activity is sufficient to recruit nonmuscle myosin II and to form ectopic heart lumina. Activated Dia^{CA} 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*²/Df(2L)ED1315, *dia*⁵/Df(2L)ED1315, or cardiac *dia*-RNAi) did not produce a cardiac phenotype, which suggests a maternal rescue or functional redundancies among formins.

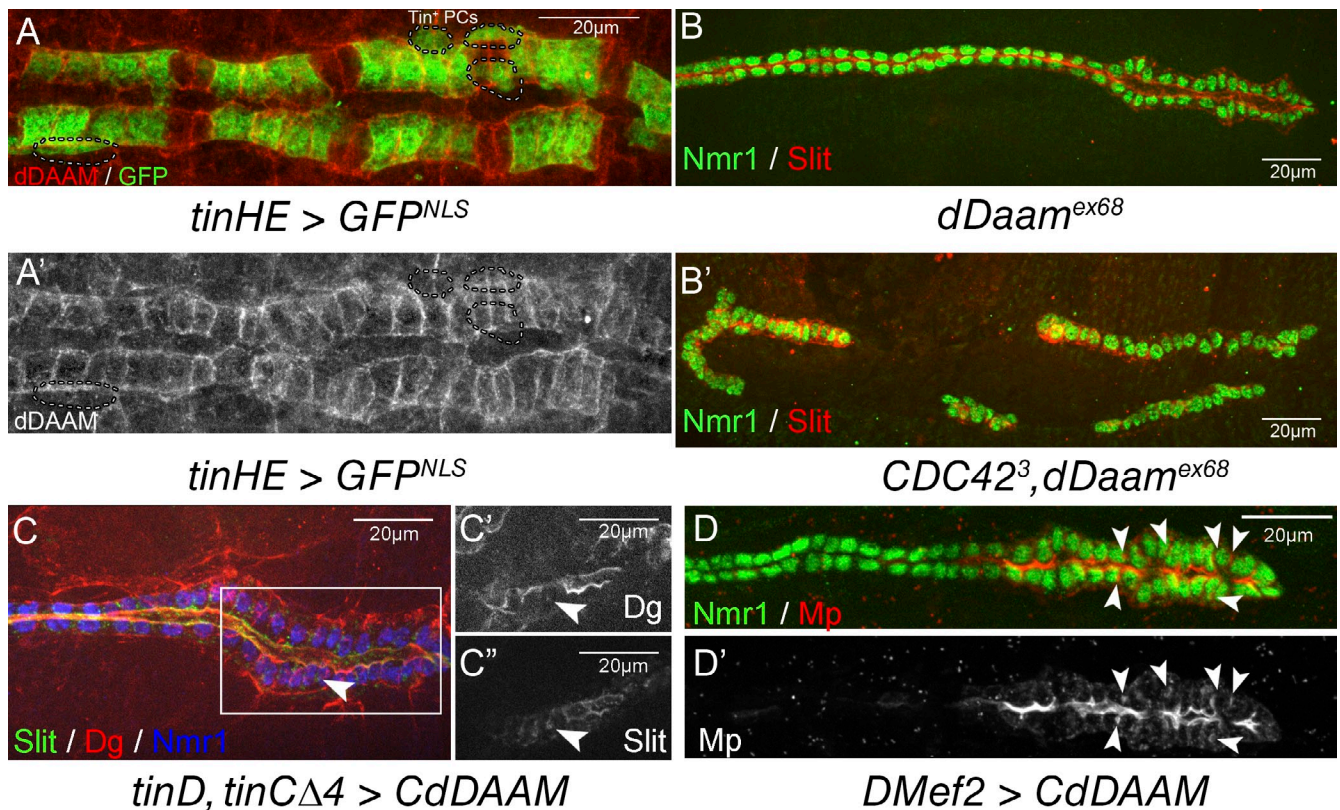


Figure 4. The formin dDAAM is expressed in cardioblasts during heart morphogenesis and genetically interacts with Cdc42. (A) Antibody staining against dDAAM shows that it localizes to the membrane of all CBs including the Tinman-negative ostia cells. Tin⁺ pericardial cells do not express dDAAM (broken lines). Heart cells are visualized with *tin^{HE}-Gal4* driving GFP. (B) *Cdc42* and *dDAAM* genetically interact. The loss-of-function allele *dDAAM1^{Ex68}* does not give rise to embryonic phenotypes (B) due to the maternal contribution of dDAAM. A large proportion (8/18) of *Cdc42³, dDAAM^{Ex68}* double mutants show severe cardiac defects (B') that are not observed in either single mutant, indicating that both genes interact during heart morphogenesis. (C) Strong expression of the C-terminal domains of dDAAM (*CdDAAM*), with *tinD, tinCΔ4-Gal4* Tin inducing ectopic heart lumina (arrowheads) that are positive for Dg (C') and Slit (C''). (D) *tinCΔ4*-driven UAS-*CdDAAM* does not induce ectopic heart lumina (arrowheads) as shown by wild-type Mp localization.

In contrast to *dDaam*, *dia* did not enhance the phenotype of *Cdc42³* mutants, which suggests that *dia* function may not be through Cdc42 in the developing heart. Alternatively, the phenotypes induced by *Dia^{CA}* could be caused by ectopic gain-of-function effects, potentially mimicking activated dDaam.

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

Table 2. Antibody reactivity, dilution, and source

Antibody reactivity and host	Dilution	Source
Nmr1/ H15 (guinea-pig and rabbit)	1:2,000	J. Skeath, Washington University in St. Louis, St. Louis, MO (Leal et al., 2009)
Dg (rabbit)	1:2,000	W.-M. Deng, Florida State University, Tallahassee, FL (Deng et al., 2003)
Zipper (rabbit)	1:1,000	D. Kiehart (Kiehart and Feghali, 1986)
β-Galactosidase (mouse)	1:750	Promega
β-Galactosidase (chicken)	1:500	Abcam
Sex-lethal M18 (mouse)	1:10	DSHB (Bopp et al., 1991)
Slit (mouse)	1:40	DSHB
Discs-large1 (mouse)	1:25	DSHB
Pericardin (mouse)	1:100	DSHB
Trol (rabbit)	1:1,000	S. Baumgartner, Lund University, Lund, Sweden (Schneider et al., 2006)
Multiplexin (rat)	1:100	T. Volk, Weizmann Institute of Science, Israel (Harpaz et al., 2013)
dDaam (rabbit)	1:200	Matusek et al., 2006
GFP (mouse)	1:500	Life Technologies
GFP (chicken)	1:500	Aves Labs

DSHB: antibodies were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biology at The University of Iowa.

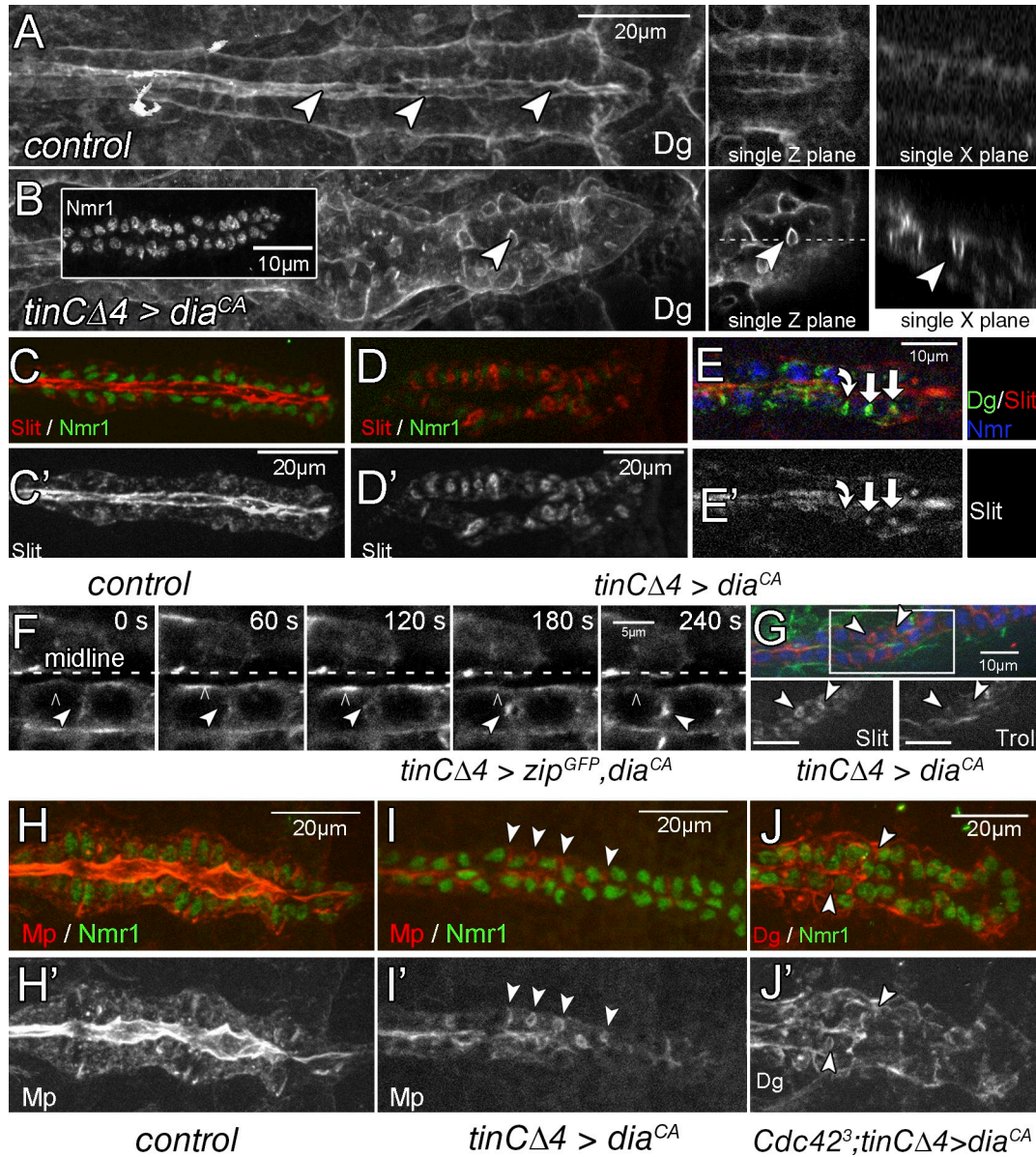


Figure 5. Expression of activated diaphanous induces ipsilateral ectopic lumina. (A) Early stage 17 wild-type embryonic heart stained with anti-Dg (Dg) to label the cell surfaces and anti-Nmr1 to label CB nuclei (inset). A contiguous heart lumen is present (arrowheads in A). (B) Hearts expressing *Dia^{CA}* form ectopic heart lumina (arrowheads) between contralateral CBs. Lumina are completely enclosed, as shown by single sections along the z and x planes of the image stack. The broken line indicates a cross section of the x plane, with intense localization of Dg. (C and D) Slit relocates to the interface of ipsilateral CBs upon expression of *Dia^{CA}* (D), which indicates that the ectopic lumina behave identically to the wild-type heart lumen (C). (E) Ipsilateral heart lumen formation precedes Slit localization. Ectopic lumina are visualized by ipsilateral localization of Dg (E, arrows), some of which do not colocalize with Slit (E', curved arrow). (F) Still frames from time-lapse movies showing ipsilateral Zipper dynamics (arrowheads) and normal apical Zipper (open arrowheads) in CBs overexpressing *Dia^{CA}*. (G) In addition, ectopic lumina are also positive for Slit and the basement membrane marker Trol (arrowheads). (H and I) Relocalization of luminal Multiplexin (Mp) to *Dia^{CA}*-induced ectopic heart lumina. In wild type, Mp is strongly enriched at the lumen domain (H). Upon expression of *Dia^{CA}*, Mp is localized to the ipsilateral ectopic heart lumina (arrowheads in I) showing the luminal character of these structures. (J) Expression of activated *Dia* induces ectopic lumina in *Cdc42* mutant hearts (arrowheads), which suggests that it acts epistatically to *Cdc42*.

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

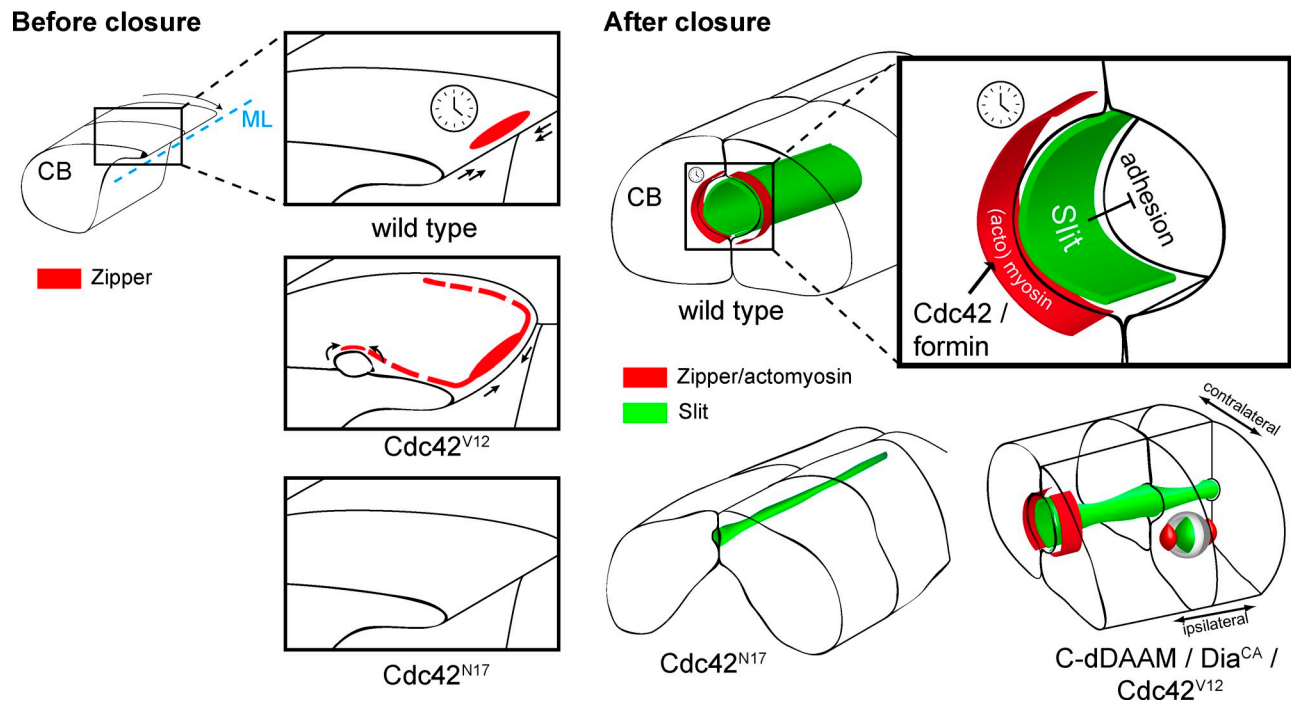


Figure 6. **Model for *Cdc42* and *formin* function in localization of Zipper within cardioblasts during cardiac morphogenesis and lumen formation.** Note that with *Cdc42* loss-of-function and *Cdc42* and *Dia* gain-of-function, cardiac alignment and lumen formation are disrupted. ML, midline; clock icon, changes over time.

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 *Dia^{CA}* or *CdDAAM* favor an intrinsic model. Even though activated formins are more prolonged and continuously localized in the CBs, Zipper still accumulates and fades at ectopic (ipsilateral) points of lumen formation, which indicates temporally regulated signaling events.

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; Heasman 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 (Maillet et al., 2009). In adult *Drosophila* hearts, expression of *Cdc42^{N17}* 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 D/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 Mandal, 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 (Le et al., 2006). The following fly stocks were used: *tinD-Gal4* (Yin et al., 1997), *tinCΔ4-Gal4* (Lo and Frasch, 2001), *tin^{ABD}* (Zaffran et al., 2006), and *tin²⁴⁶* (Azpiazu and Frasch, 1993). All lines were a gift from M. Frasch, University of Erlangen-Nuremberg, Bavaria, Germany. *Robo2^B* (a gift from B. Dickson, Howard Hughes Medical Institute Janelia Farm Research Campus, Ashburn, VA; Rajagopalan et al., 2000), *zfh1^{75.34}* (a gift from R. Lehmann, New York University, New York, NY; Brohier 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), *dDAAM^{Ex68}* and *UAS-CdDAAM* (Matusek et al., 2006), *UAS-dia^{CA}* (a gift from P. Rørth, Institute of Molecular and Cell Biology, Singapore; Somogyi and Rørth, 2004), *Pm/CyO Dfd-YFP*; *CxD/TM6b Dfd-YFP* (a gift from O. Vef, Institut für Genetik, Mainz, Germany), *Cdc42³*, *Abf²*, *UAS-Cdc42^{N17}*, *ena²³*, *UAS-Rho1^{N19}*, *zip¹*, *UAS-Rac1^{N17}*, *zip²*, *UAS-RhoL^{N25}*, *zip^{llx62}*, *UAS-actin5C::GFP*, *robo¹*, *UAS-mCDB8::GFP*, *tup^{isl1}*, *arm::GFP*, *FM7i Dfd-YFP*, *how^{24B}*, *Gal4*, *sna^{ScO}*, *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 (McQuilton et al., 2012).

To test for genetic interaction with *Cdc42³*, YFP and Sxl protein expression was assessed in the embryos derived from the test cross. Absence of the proteins identifies *Cdc42³* 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 min 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 (PBTx), and then placed in ice-cold PBTx (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 PBTx and washed for 1 h in PBTx 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 at a final concentration of 6.6 nM (Life Technologies). Specimens were mounted in ProLong Gold antifade (Life Technologies) and imaged as described in the following paragraphs.

To determine if a gene interacts with *Cdc42*, *Cdc42³/FM7* virgins were crossed to males heterozygous for a loss-of-function allele of the gene to be tested. Fixed embryos were treated with 0.03% H₂O₂ in methanol for

30 min to quench endogenous peroxide before DAB staining. Immunohistochemistry was performed with anti-GFP to detect balancer chromosomes and anti-Sxl to determine the sex (female embryos show uniform brown staining). Embryos hemizygous mutant for *Cdc42³/Y* and heterozygous for the tested allele remain unstained. These embryos were manually removed from the staining solution, and CB nuclei were stained with anti-Nmr1 and an alkaline phosphatase-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 halocarbon 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 μ l of glue was thinly spread on 22 \times 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-GTPase family proteins. Fig. S2 contains the examples and quantification of ectopic heart lumina and filopodia number in different genotypes, including *Cdc42^{N17}*. Fig. S3 shows Slit localization in *tinABD*; *tinF^{C40/346}* mutant hearts, as well as Zipper localization in fixed samples of embryos overexpressing *Dia^{CA}* in the heart, and of *unc5* and *robo*; *robo2* mutants. Fig. S4 shows the presence of AS-derived cells within the embryonic heart lumen, which are also strongly positive for Zip. Video 1 shows filopodia formation in wild-type and *Cdc42^{N17}*-expressing embryos. Video 2 shows the dynamic pattern of GFP-tagged Zipper during heart morphogenesis. Video 3 shows the localization of Moesin relative to Zipper during heart morphogenesis. Video 4 shows Zipper localization at the lumen surface after lumen formation. Video 5 shows the effect of *Cdc42* (dominant negative and constitutively active) on Zipper localization. Video 6 shows ectopic appearance of Zipper at *Dia^{CA}*-induced ectopic heart lumina. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201405075/DC1>.

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