Diaphanous regulates myosin and adherens junctions to control cell contractility and protrusive behavior during morphogenesis

Catarina C. F. Homem¹ and Mark Peifer¹,²,*

Formins are key regulators of actin nucleation and elongation. Diaphanous-related formins, the best-known subclass, are activated by Rho and play essential roles in cytokinesis. In cultured cells, Diaphanous-related formins also regulate cell adhesion, polarity and microtubules, suggesting that they may be key regulators of cell shape change and migration during development. However, their essential roles in cytokinesis hamper our ability to test this hypothesis. We used loss- and gain-of-function approaches to examine the role of Diaphanous in Drosophila morphogenesis. We found that Diaphanous has a dynamic expression pattern consistent with a role in regulating cell shape change. We used constitutively active Diaphanous to examine its roles in morphogenesis and its mechanisms of action. This revealed an unexpected role in regulating myosin levels and activity at adherens junctions during cell shape change, suggesting that Diaphanous helps coordinate adhesion and contractility of the underlying actomyosin ring. We tested this hypothesis by reducing Diaphanous function, revealing striking roles in stabilizing adherens junctions and inhibiting cell protrusiveness. These effects also are mediated through coordinated effects on myosin activity and adhesion, suggesting a common mechanism for Diaphanous action during morphogenesis.

KEY WORDS: Myosin, Formins, RhoGEF2, Drosophila

INTRODUCTION
Drosophila embryogenesis provides a superb model of how cell migration and shape changes reshape the body plan during morphogenesis. Small changes in individual cell shapes collectively generate large-scale tissue reorganization. For example, mesoderm invagination requires coordinated apical constriction, germband extension requires cell intercalation, while in dorsal closure epidermal cells elongate in a polarized fashion. One current challenge is to determine how cell fate is translated into cell shape change.

Cell-cell adhesion and the cytoskeleton must be tightly coordinated during morphogenesis. Cell shape changes require remodeling of adherens junctions (AJs) and the actomyosin cytoskeleton. AJs mediate cell-cell adhesion via transmembrane cadherins, with β-catenin (Drosophila Armadillo; Arm) and α-catenin bound to their cytoplasmic tails (Halbleib and Nelson, 2006). α-Catenin also interacts with actin. AJs and actin are intimately interrelated, as disrupting one disrupts the other (e.g. Cox et al., 1996; Quinlan and Hyatt, 1999), but mechanisms regulating this coordination are not well understood.

During morphogenesis, many epithelial cells polarize in the plane of the epithelium, orthogonal to the apical axis. This planar cell polarity involves asymmetric distribution of AJ and cytoskeletal proteins around the apical circumference (Zallen, 2007). For example, during Drosophila germband extension, F-actin enrichment at anterior/posterior (A/P) cell borders is the first break in symmetry. Nonmuscle Myosin 2 (hereafter Myosin) then becomes enriched at A/P cell borders, while Bazooka/PAR-3 and AJ proteins accumulate at reciprocal dorsal/ventral (D/V) cell borders (Bertet et al., 2004; Blankenship et al., 2006; Zallen and Wieschaus, 2004). Planar polarization of AJs, actin and Myosin also occur during dorsal closure, when epidermal cells elongate along the D/V axis and leading-edge cells construct an actomyosin cable along their dorsal borders (Kaltschmidt et al., 2002; Kiehart et al., 2000).

Actin and Myosin regulators, like Rho family GTPases, direct cell movement, shape changes and planar cell polarity. Rho regulates the cytoskeleton and AJs, and Drosophila Rho1 mutants have dorsal closure defects (Magie et al., 1999). Two major Rho effectors are Rho kinases (ROCKs), which are Myosin regulators, and Diaphanous-related formins (DRFs), which are actin regulators. Embryos zygotically lacking Drosophila ROCK are normal, owing to maternal contribution, while removing maternal ROCK blocks oogenesis (Verdier et al., 2006b; Winter et al., 2001). Myosin heavy chain [MHC; encoded by zipper (zip)] is required for proper dorsal closure (Franke et al., 2005). However, zip zygotic mutants retain maternal MHC and thus do not reveal the full spectrum of functions of Myosin.

DRFs, a second class of Rho effectors, nucleate actin filaments and promote filament elongation (Kovar, 2006). DRFs normally are autoinhibited via intramolecular interactions between the N-terminal GTPase-binding domain (GBD) and C-terminal DAD domain. Rho binds the GBD, activating DRFs. DRFs are essential regulators of cytokinesis in yeast, nematodes and flies (Castrillon and Wasserman, 1994; Severson et al., 2002; Swan et al., 1998). Although DRFs exact mechanism of action is not clear, they are crucial in assembling/stabilizing the contractile actomyosin ring. In Drosophila Diaphanous (Dia) is the sole DRF. Dia is essential in conventional cytokinesis and in more specialized events of early embryogenesis, when Dia localizes to tips of syncytial and cellularization furrows, coordinating actin assembly as cells form (Afshar et al., 2000; Grosshans et al., 2005).

Formins also have roles outside of cytokinesis. DRFs can affect transcription by activating MAL/SRF through G-actin depletion, and can stabilize polarized microtubules (Faix and Grosse, 2006). In cultured mammalian cells, both Dia1 and Formin 1 promote AJ

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stability: AJ stabilization by Formin 1 requires its actin polymerization function but is independent of microtubule binding (Kobielak et al., 2004; Sahai and Marshall, 2002; Carramusa et al., 2007).

DRF roles in morphogenesis remain largely unknown. Two challenges impede progress. First, mammals have three DRFs that are at least partially redundant. Mouse DIA mutant mice have hematopoiesis defects, but are otherwise normal (Eisenmann et al., 2007; Peng et al., 2007). Human DIA1 mutations result in deafness (Lynch et al., 1997), but these mutations may be gain of function. Uncovering the full function of mammalian DRFs will require multiple knockouts. The second, more difficult challenge is their essential role in cytokinesis. Although flies and nematodes have only a single DRF, its inactivation disrupts cytokinesis from the onset of development (Afshar et al., 2000; Swan et al., 1998), preventing analysis of morphogenesis.

To address this challenge, we used loss and gain-of-function genetic tools available in *Drosophila* to study the function of Dia during morphogenesis, and to address its mechanisms of action. These studies suggest that Dia plays an important and unexpected role in coordinating actomyosin contractility and adhesion at AJs, and provide evidence that it acts upstream of Myosin as well as actin.

MATERIALS AND METHODS

Genetics

Mutations and Balancer chromosomes are described at FlyBase (flybase.bio.indiana.edu). Fly stocks and sources are in Table 1. Wild-type was y w. Females carrying UAS-transgenes were crossed to males with GAL4-drivers – their expression patterns are described in Table 1. To generate UAS-EGFP::Dia, we began with a derivative of pUASp (Rorth, 1998), modified by T. Murphy for Gateway cloning with an N-terminal EGFP tag (www.ciwemb.edu/labs/murphy/Gateway%20vectors.html). The entire Dia ORF was cloned into this vector, with a short linker as follow: M-EGFP-HRYTSLYKKAGSAAAAPFT-dia (see Fig. S6 in the supplementary material). Flies were generally grown at 25°C. Temperature shifts of dia<sup>−/−</sup>M2 mutants were done as follows. For fixed embryos, embryos were collected for 8 hours at 18°C, aged at 18°C for 4 hours and then shifted to 25°C and aged until the desired stage. For ventral furrow imaging embryos were collected for 8 hours at 18°C and shifted to room temperature for filming. Females with dia mutant germlines were generated by heat-shocking 48-72 hour hsflp<sup>17</sup>; FRT40A dia/FRT40A ovo<sup>D1</sup> larvae for 3 hours at 37°C.

Protein and RNA analysis and immunofluorescence/imaging

Protein samples were prepared by grinding embryos on ice in Laemmli buffer with a plastic pestle and boiling 5 minutes. Immunoblotting was as in Peifer et al. (Peifer et al., 1992). Signal was detected by ECL (Amersham). Immunoblots were quantitated using Image J; we quantitated at least two exposures in each case, with similar results. RNA was prepared and analyzed as described by D'Avino et al. (D'Avino et al., 1995). For anti-myosin, embryos were heat-methanol fixed (Müller and Wieschaus, 1996); otherwise embryos were prepared and imaged as described by Fox and Peifer (Fox and Peifer, 2007). Antibodies, actin and RNA probes are in Table 1. Adobe Photoshop 7.0 was used to adjust input levels to span entire output grayscale, and to adjust brightness and contrast. When protein levels were compared, images were equally adjusted. For fluorescence quantitation, images were acquired from live embryos using a spinning-disc confocal (Perkin-Elmer).

### Table 1. Fly stocks, antibodies and probes

<table>
<thead>
<tr>
<th>Fly stocks</th>
<th>Source</th>
<th>Expression pattern</th>
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<tr>
<td>UAS-GFP-actin</td>
<td>P. Martin (University of Bristol, Bristol, UK)</td>
<td>Expressed in epididermal expressed-striper (posterior cells of each segment) and in scattered amnioserosal cells, presumably from the engrailed-expressing strips. Expression starts at stage 12.</td>
</tr>
<tr>
<td>zip&lt;sup&gt;1&lt;/sup&gt; EnGal4, zip&lt;sup&gt;1&lt;/sup&gt;c381Gal4</td>
<td>D. Kiehart (Duke University, Durham, NC, USA)</td>
<td></td>
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<tr>
<td>Moesin-GFP</td>
<td>D. Kiehart</td>
<td>Expressed in epididermal paired-expressing strips (every other segment) and in small groups of amnioserosal cells, presumably from the paired-expressing strips. Expression starts at stage 12.</td>
</tr>
<tr>
<td>UAS-Dia&lt;sup&gt;CA&lt;/sup&gt;</td>
<td>P. Rorth (EMBL, Heidelberg, Germany)</td>
<td>Expressed in the entire amnioserosa or occasionally in big amnioserosal patches. Expression starts at stage 12.</td>
</tr>
<tr>
<td>UAS-SRF/VP16</td>
<td>M. Krasnow (Stanford, CA, USA)</td>
<td>Expressed ubiquitously in the embryo. Expression starts at stage 12.</td>
</tr>
<tr>
<td>dia&lt;sup&gt;1&lt;/sup&gt; FRT40A</td>
<td>S. Wasserman (UCSD, CA, USA)</td>
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**Antibodies/probes**

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<tr>
<th>Antibodies/probes</th>
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<tr>
<td>Anti-DE-CAD2</td>
<td>1:100</td>
<td>Developmental Studies Hybridoma Bank (DSHB)</td>
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<tr>
<td>Anti-Neurotactin</td>
<td>1:10</td>
<td>DSHB</td>
</tr>
<tr>
<td>Anti-β-P51 integrin</td>
<td>1:3</td>
<td>DSHB</td>
</tr>
<tr>
<td>Anti-Ena</td>
<td>1:200</td>
<td>DSHB</td>
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<tr>
<td>Anti-Dig</td>
<td>1:200</td>
<td>DSHB</td>
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<tr>
<td>Anti-Zipper (Myosin II heavy chain)</td>
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<td>D. Kiehart</td>
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<td>Anti-Phospho-Tyrosine</td>
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<td>Upstate Biotechnology (VA, USA)</td>
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<td>B. Duronio (UNC-CH, NC, USA)</td>
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<td>Anti-MLCK (clone K36)</td>
<td>1:10,000</td>
<td>Sigma (St Louis, MO, USA)</td>
</tr>
<tr>
<td>Anti-Dia</td>
<td>1:5000</td>
<td>S. Wasserman</td>
</tr>
<tr>
<td>Anti-Odd Skipped</td>
<td>1:200</td>
<td>J. Skeath (Washington University of St Louis, MO, USA)</td>
</tr>
<tr>
<td>Anti-Rab5</td>
<td>1:1000</td>
<td>M. Gonzalez-Gaitan (Max Plank Institute, Dresden, Germany)</td>
</tr>
<tr>
<td>Alexa-phallolidin</td>
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<td>Molecular Probes (Carlsbad, CA, USA)</td>
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<tr>
<td>Secondary antibodies: Alexa 488, 568 and 647</td>
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<td>Molecular Probes</td>
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<tr>
<td>MHC antisense RNA</td>
<td>–</td>
<td>LP22476 (BDGP)</td>
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<tr>
<td>RP49 antisense RNA</td>
<td>–</td>
<td>Searles Laboratory (UNC-CH, NC, USA)</td>
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All other fly stocks were from Bloomington Stock Center. Fly stocks and Gal4 drivers are described in more detail at FlyBase (flybase.bio.indiana.edu).
CSU10), a 12-bit camera, and Metamorph. Time-lapse imaging was as described previously (Grevengoed et al., 2001). Images were acquired every 15 seconds. Fluorescence-intensity measurements were as described previously (Harris and Peifer, 2007), using single planes; all fluorescent measurements were conducted on images with a grayscale range of 200-700 (no saturated pixels), using ImageJ.

RESULTS
Dia localizes apically during morphogenesis and overlaps AJs
We hypothesized that Dia helps coordinate adhesion and cytoskeletal regulation at AJs. As a first test, we characterized Dia localization during morphogenesis using anti-Dia antibody (Afshar et al., 2000) and live imaging of EGFP::Dia. UAS-EGFP::Dia driven by e22c-Gal4 can partially rescue dia mutants to adult viability (data not shown), and is expressed at levels roughly similar to those of endogenous Dia (Fig. 1L), suggesting it replicates endogenous Dia localization. During cellularization, Dia colocalizes with actin and Myosin at tips of invaginating furrows (Afshar et al., 2000; Grosshans et al., 2005) (Fig. 1A) but its subsequent localization was unknown. We found that as cellularization ends Dia re-localizes to the apicolateral domain (Fig. 1B,C), overlapping AJs (Fig. 1B), and remains apical during gastrulation (Fig. 1D,E). During germ band extension, when Myosin [visualized using Sqh::GFP (Myosin regulatory light chain)] and AJs take on opposing planar-polarized localizations, Dia is cortical but not notably planar-polarized (Fig. 1F). Both Dia (Fig. 1H,I) and EGFP::Dia (Fig. 1M,N) remain cortically enriched in epidermis and amnioserosa. Consistent with its role in cytokinesis, Dia co-localizes with Myosin in invaginating cytokinetic furrows (Fig. 1G, arrows), but does not remain in midbodies (Fig. 1G, arrowheads). The basal-to-apical re-localization of Dia suggests that it has new functions during morphogenesis, and its overlap with AJs makes it a candidate to regulate actin there.

Dynamic localization of Dia during dorsal closure suggests roles in cell shape change
As a model for morphogenesis, we used dorsal closure. Prior to this stage, the amnioserosa, a squamous epithelium, covers the dorsal surface. During closure amnioserosal cells apically constrict in a radially symmetric fashion, while polarized shape changes cause epidermal cells to nearly double their D/V length, enclosing the embryo. At their leading-edge, epidermal cells construct a contractile actomyosin cable joined cell-cell by AJs. Amnioserosal cell constriction, epidermal cell elongation and actomyosin cable constriction combine to drive dorsal closure (Kiehart et al., 2000). Although most epidermal cells elongate, one row of cells per segment, segmental groove cells, do not elongate like their neighbors, and thus form grooves around the embryo (Larsen et al., 2003).
Dia has a dynamic localization during dorsal closure. In elongating epidermal cells, Dia and EGFP::Dia are cortical (Fig. 1I,J,N arrowheads) and strongly enriched in leading-edge AJs (Fig. 1J,N arrows), where the actomyosin cable is anchored. In segmental groove cells, EGFP::Dia is planar polarized, preferentially accumulating at D/V borders (Fig. 1O1 – because they form a groove these cells are basal to their neighbors, seen in Fig. 1O2). Quantitation of EGFP::Dia at D/V versus A/P borders revealed that this polarization is statistically significant (Fig. 1P). Dia is not planar polarized in other epidermal cells (Fig. 1O2,P). Amnioserosal cells apically constrict during dorsal closure – most do so in concert, but a subset (‘drop-out’ cells) constricts more rapidly than its neighbors (Kiehart et al., 2000). Dia is cortical in all amnioserosal cells (Fig. 1I,K,N), but particularly accumulates in drop-out cells, mirroring Myosin (Fig. 1K arrows). Apical accumulation of Dia in constricting amnioserosal cells and planar polarization in groove cells suggests that it may regulate their cell shape changes during dorsal closure.

**Constitutively active Dia induces precocious amnioserosal cell constriction and stabilizes Myosin and AJs**

As a first test of this hypothesis and to identify mechanisms by which Dia may act during morphogenesis, we explored what activated Dia can do. To do so, we used a constitutively active mutant, DiaCA, that lacks both GBD and DAD domains, preventing intramolecular inhibition (Fig. 2A) (Somogyi and Rorth, 2004).
We first expressed Dia\(^{CA}\) in amnioserosal cells. Their constriction requires an actomyosin contractile ring (diagrammed in Fig. 2I) (Franke et al., 2005). Dia accumulation in wild-type constricting amnioserosal cells (Fig. 1K) suggests that Dia may regulate constriction. We tested this by examining how Dia activation affects amnioserosal cell behavior, using GAL4 drivers to induce HA-tagged-Dia\(^{CA}\) expression in the entire amnioserosa [c381-GAL4 (Table 1); e.g. Fig. 2B,F], in large groups of amnioserosal cells (seen in a small subset of c381-GAL4-driven embryos; e.g. Fig. 2H,J), or in individual cells in a mosaic pattern [enlarged(en)-GAL4 (Table 1); e.g. Fig. 2C.D]. Dia\(^{CA}\) is cortically enriched (Fig. 2B,C), and thus positioned to modulate apical constriction.

Dia\(^{CA}\) strongly induces premature amnioserosal cell apical constriction. Normally, amnioserosal cells are irregular in shape (Fig. 1K; Fig. 2E,G), with most constricting roughly in concert. When we misexpressed Dia\(^{CA}\) in individual amnioserosal cells, they apically constricted prematurely (Fig. 2D, arrowheads); neighboring wild-type cells became elongated (Fig. 2D, arrows). By contrast, when we expressed Dia\(^{CA}\) in all amnioserosal cells, all became apically smaller and uniformly round (compare Fig. 2F with 2G), suggesting that all attempt to constrict simultaneously and the isometric tension rounds the cells.

We next explored mechanisms by which Dia\(^{CA}\) induces apical constriction. Apical constriction is mediated by an actomyosin ring (Fig. 2I) (Franke et al., 2005). Dia stimulates actin polymerization (Wallar and Alberts, 2003). As expected, cortical F-actin is increased in individual Dia\(^{CA}\)-expressing amnioserosal cells (Fig. 2D, arrowheads), and when Dia\(^{CA}\) is expressed in all amnioserosal cells (compare Fig. 2F with 2G). However, Dia\(^{CA}\) also had an unexpected effect on Myosin localization and levels. In wild-type, Myosin accumulates cortically at low levels in most amnioserosal cells (Fig. 2K) and at high levels in drop-out cells undergoing rapid apical constriction (Fig. 1K; Fig. 2K, arrows). Dia\(^{CA}\)-expressing amnioserosal cells had increased cortical Myosin; this was most obvious in embryos expressing Dia\(^{CA}\) in many but not all amnioserosal cells, providing an internal control (Fig. 2J), but was also seen in individual Dia\(^{CA}\)-expressing cells (Fig. 2C, arrowheads). To assess whether this involves more Myosin protein, we compared MHC levels by immunoblotting. Dia\(^{CA}\) expression using the ubiquitous epithelial driver e22c-GAL4 (Table 1) elevated total Myosin ~2.5-fold (Fig. 2L); as only half the embryos receive both driver and UAS-Dia\(^{CA}\), this underestimates the magnitude of the effect. Thus, Dia\(^{CA}\) coordinately elevates both cortical actin and Myosin.

Fig. 3. Dia\(^{CA}\) induces planar-polarized cell shape changes in the epidermis. Drosophila embryos: anterior leftwards, genotypes and antigens indicated. (A-C) Stage 16. Deep segmental grooves (arrows). (D-M) Dorsal closure. (D) Wild-type groove cells (arrows). (E-P) Dia\(^{CA}\)-en-GAL4, DE-cad D/V enrichment and F-actin accumulation (arrows). (G) Myosin in elongating wild-type epidermis. (H-P) Wild-type groove cells, Myosin, DE-cad and EGFP::Dia (e22c-GAL4) D/V enrichment (arrows). (J,J') Dia\(^{CA}\) D/V enrichment. (K-M) Dia\(^{CA}\)-en-GAL4. (K',K") Region indicated by yellow bracket in K". DE-Cad and Myosin D/V enrichment (arrows). (L) Movie frame showing Arm::GFP. (L,M) Dia\(^{CA}\)-expressing cells delimited by double-headed arrows. (N,O) Quantitation of planar polarization in indicated cell types. Error bars indicate s.d. (P) Wild-type elongating and groove cells. Scale bars: 20 μm.
During apical constriction, actomyosin contractile rings are linked to AJs (Fig. 2I) (Dawes-Hoang et al., 2005). AJs and actin have a reciprocal relationship; AJs organize and anchor contractile rings and actin stabilizes AJs. We thus assessed whether DiaCA affects AJs. Mosaic DiaCA expression in the amnioserosa elevated cortical Arm levels (Fig. 2H) and stabilized \(\alpha\)-catenin, as assessed by immunoblotting (Fig. 2L). This suggests that DiaCA stabilizes AJs and underlying actomyosin rings.

To examine how DiaCA affects cell behavior in detail, we imaged Myosin live, using Sqh::GFP. Cell rounding and increased cortical Myosin are clear (compare Fig. 2M with 2N,O). The wild-type network of apical Myosin filaments (Fig. 2P) became reorganized into stress-fiber-like bundles parallel to the epithelium (Fig. 2Q), supporting the hypothesis that these rounded cells are under tension. However, despite this, dorsal closure slowed substantially – in this example it took over twice as long (compare Fig. 2M with 2O). The dorsal opening was abnormal: its normal ‘eye’ shape (Fig. 2K,M) became oval (Fig. 2J,O), suggesting difficulties in epidermal sheets zipping together. However, many features of dorsal closure remained normal, e.g. drop-out cells constricted before their neighbors and accumulated apical Myosin (Fig. 2N, arrowheads). Larval cuticle analysis revealed that most DiaCA-expressing embryos complete closure, but have defects in cell-cell matching as the two sheets meet (Fig. 2R; Fig. 2O5 shows this problem in real time). Together, these data suggest DiaCA accelerates normal amnioserosal apical constriction by stabilizing cortical actin, Myosin and AJs. This is consistent with the hypothesis that during normal dorsal closure, amnioserosal cells regulate Dia activity to coordinate AJs and contractility.

**In epidermal cells DiaCA induces groove cell-like behavior without changing cell fate**

We next examined planar-polarized elongating epidermal cells and segmental groove cells, contrasting the effects of DiaCA to those in radially-symmetric amnioserosal cells. As amnioserosal cells constrict, most epidermal cells elongate in the D/V axis, while segmental groove cells elongate less.

DiaCA expression in segmental stripes induced abnormally deep and persistent segmental grooves [compare Fig. 3A with 3B,C; expression was either in broad stripes in every other segment (paired(prd)-GAL4) or narrow stripes in every segment (en-GAL4)]. Wild-type segmental grooves disappear during dorsal closure (Fig. 3A) but in DiaCA-expressing embryos, grooves persist much longer and become very deep (Fig. 3B,C). Epidermal cells expressing DiaCA are wider in the A/P axis and shorter in the D/V axis than are non-expressing neighbors (Fig. 3E,F), thus forming a shorter belt of cells, explaining the deeper grooves. Interestingly, wild-type segmental groove cells have a similar shape (Fig. 3D), suggesting that DiaCA-expressing epidermal cells mimic groove cell behavior.

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**Fig. 4. Activating Myosin partially mimics DiaCA.**

_Drosophila_ embryos: anterior leftwards, genotypes, GAL4 drivers and antigens indicated. (A-D') Lateral view of stage 13 embryos of indicated genotypes. (E-H') Dorsal view of stage 13 embryos of indicated genotypes. (A-H') Dorsal closure. (I-J') Stage 16. (A,A',E,E') Wild-type. (B,B',D,D') DiaCA or MLCKKCA expression induces deep grooves (arrowheads). (F,F',H-H') DiaCA- or MLCKCA-expressing amnioserosal cells. (C,C') SRF-VP16 expression. Elevated Myosin without deeper grooves (arrowheads). (G-G') SRF-VP16 expressing amnioserosal cells. (I) Wild-type; grooves are no longer present (arrowheads). (K,K') DiaCA expression in zip zygotic mutants still leads to amnioserosal cell rounding. (J-K') DiaCA expression in zig zygotic mutant. Persistent grooves (J', arrowheads). Cortical actin alterations are slightly reduced (see Fig. 3E). Scale bars: 20 \(\mu\)m.
As DiaCA-expressing cells behave like groove cells, we tested whether DiaCA alters epidermal cell fate. Odd-skipped is a groove cell marker (S. Vincent and J. Axelrod, personal communication). Odd-skipped expression is not altered by DiaCA expression; it remains restricted to normal groove cells (a single cell row per segment flanking the DiaCA-expressing cells), and is not misexpressed in cells adopting groove-cell behavior (Fig. 3M). Thus, DiaCA affects cell shape without changing cell fate.

To explore mechanisms driving this cell shape change, we examined the cytoskeleton. Although all wild-type epidermal cells are planar polarized, elongating cells and segmental groove cells differ significantly. In groove cells, Myosin, and, to a lesser extent, actin are enriched at D/V borders (Fig. 3H,P, data not shown), while in elongating cells Myosin is planar-polarized along A/P cell boundaries (Fig. 3G,P) (Walters et al., 2006). AJs are slightly D/V planar-polarized in all epidermal cells, but this is accentuated in groove cells (Fig. 3I; quantitated in 3O). Interestingly, wild-type Dia is D/V planar-polarized in groove cells (Fig. 1O,P; Fig. 3I) but not strikingly polarized in other epidermal cells, consistent with differential Dia function in these two cell types. DiaCA accumulates cortically (Fig. 3F) and at late stages is also D/V planar polarized (Fig. 3J).

Epidermal DiaCA expression dramatically alters the cytoskeleton and AJs. DiaCA triggers elevated cortical F-actin, as expected (Fig. 3E,F). Strikingly, all DiaCA-expressing epidermal cells accumulate cortical Myosin at higher levels than their neighbors, and it is especially enriched at D/V cell borders (Fig. 3K, arrows; quantitation is shown in Fig. 3N). DiaCA also triggers accentuated AJ D/V planar-polarization (Fig. 3K,L; quantitation is shown in Fig. 3O). Thus, DiaCA cells take on both groove cell behavior and planar-polarity properties, with elevated actin and myosin accumulating at stabilized AJs at D/V cell borders.

Thus, DiaCA triggers actin and Myosin accumulation, and stabilizes AJs in both amnioserosa and epidermis. In radially symmetric amnioserosal cells, this triggers premature apical constriction. By contrast, in epidermal cells, actin and Myosin are selectively stabilized at D/V cell boundaries where AJ proteins are already enriched. Perhaps segmental groove cells normally activate Dia, thus regulating their cell shape.

The phenotype of DiaCA cannot be mimicked by increasing Myosin levels

The most surprising effect of DiaCA was its effect on Myosin levels. We thus assessed mechanisms by which this occurs, and whether these are necessary or sufficient to produce the DiaCA phenotype. We tested two hypotheses: (1) Dia increases myosin transcription, possibly through the transcription factor Serum Response Factor (SRF), a Dia target (Tominaga et al., 2000); or (2) Dia activates or stabilizes phosphorylated, active Myosin at AJs.

We hypothesized that SRF might regulate Myosin levels. To test this, we used hyperactive SRF (fused to the strong VP16 activation domain (Guillemin et al., 1996). When SRF-VP16 is expressed in segmental stripes (compare Fig. 4A with 4C) or the amnioserosa
(compare Fig. 4E with 4G), it markedly elevates Myosin, mimicking DiaCA (Fig. 4F). Interestingly, although both SRF-VP16 and DiaCA increase Myosin protein, this does not occur at the transcriptional level (see Fig. S1G,H in the supplementary material).

We next used SRF-VP16 expression to assess whether increased Myosin is sufficient to mimic DiaCA. This is not the case. Whereas DiaCA induces deep segmental grooves when expressed in epidermal stripes (compare Fig. 4A with 4B), SRF-VP16 does not (Fig. 4C; in some embryos there was slight groove deepening; see Fig. S1F in the supplementary material). SRF-VP16 induced Myosin accumulation (Fig. 4C, see Fig. S1A-D in the supplementary material) without changing epidermal cell shape, AJs (see Fig. S1A-D in the supplementary material) or actin (see Fig. S1E,F in the supplementary material). Furthermore, unlike DiaCA (compare Fig. 4E with 4F), SRF-VP16 has no effect on amnioserosal cell shape (compare Fig. 4E with 4G), although Myosin levels are highly elevated. Thus, Myosin accumulation is not sufficient to cause cell shape changes or to affect AJs, suggesting that DiaCA affects not only Myosin levels but also Myosin localization and/or activation.

We also tested whether myosin transcription is necessary for the effects of DiaCA, by expressing DiaCA in MHC zygotically-null mutants (zip; selected by absence of a GFP-tagged Balancer chromosome). zip mutants are normal until late morphogenesis (Franke et al., 2005), owing to maternally contributed Myosin, but cannot upregulate myosin transcription in response to DiaCA as they lack a functional myosin gene. DiaCA expression in zip-null embryos still generated cell shape changes in both epidermis (compare Fig. 4J with 4I; deep grooves) and amnioserosa (Fig. 4K; uniformly rounded cells). Thus, the effects of DiaCA do not require elevated myosin transcription.

**DiaCA is partially mimicked by Myosin activation**

We next explored whether Myosin activation mimicked the DiaCA phenotype. We first tried constitutively active Rho Kinase (Verdier et al., 2006a), but this did not have obvious phenotypic effects (data not shown), perhaps because expression levels were too low. We next induced Myosin activation using constitutively active Myosin light chain kinase [MLCKCA (Kim et al., 2002) derived from chicken MLCK, allowing its specific detection]. MLCKCA expression in the entire amnioserosa led to uniform amnioserosal cell rounding (Fig. 4H), similar to, though weaker than, that caused by DiaCA (Fig. 4F), and expression in individual amnioserosal cells triggered premature apical constriction (Fig. 5A,B, arrows). Furthermore, MLCKCA expression in epidermal stripes triggered deep segmental grooves (Fig. 4D), mimicking DiaCA (Fig. 4B). Thus, MLCKCA and DiaCA have similar morphological consequences, stimulating apical constriction, although the effects of DiaCA are somewhat stronger.

We next examined whether MLCKCA and DiaCA act by similar mechanisms. Some effects were similar, whereas others were distinct. In the amnioserosa, both elevated Myosin (compare Fig. 4F-H with 4E; Fig. 5A) and actin (Fig. 5B), but levels and subcellular localization differed. In epidermal cells, MLCKCA and DiaCA had even more distinct effects. MLCKCA elevated cortical actin, but less so than DiaCA (compare Fig. 4F and Fig. 5E). MLCKCA also elevated cortical Myosin, but this was not planar polarized (compare Fig. 5C,D with Fig. 3K), and the cell shape changes (Fig. 5E) were not as extreme as those induced by DiaCA (Fig. 3K). – MLCKCA-expressing cells may simply apically constrict rather than adopting groove-cell-like shapes. Thus, MLCKCA mimics the morphological phenotypes of DiaCA, but does not replicate its cell biological effects. This is consistent with a model in which Dia triggers Myosin activation at specific sites like AJs, so that global Myosin activation does not precisely mimic its effects.

**Severely reducing Dia function disrupts morphogenesis**

These data are consistent with the hypothesis that regulated Dia activity helps coordinate adhesion with actomyosin assembly/activity at AJs. To test this mechanistic hypothesis, we examined how reducing Dia function affects morphogenesis. To circumvent difficulties caused by the key role of Dia in cellularization, we sought to reduce but not eliminate Dia function.
This was facilitated by our discovery that dia5 is not a null allele, as was thought (Afshar et al., 2000), but produces severely reduced amounts of wild-type Dia, and its phenotype is temperature sensitive (see Fig. S2 in the supplementary material). This allowed us to use temperature shifts to reduce Dia function after cellularization and explore its roles in morphogenesis. We crossed females with dia5 homozygous germlines to males heterozygous for the null allele dia2; half the progeny are zygotically ‘rescued’ (dia5/M) and half are dia5/M/Z mutant (distinguishable by late extended germband using GFP-marked Balancers). As dia5/M/Z mutants retain residual wild-type Dia, we can only draw conclusions from processes that are defective – residual Dia may suffice for other events.

Reduced Dia function has its first consequences at gastrulation, when mesodermal cells along the ventral midline apically constrict in a coordinated way, forming the ventral furrow (Fig. 6A, 10:00, red arrows; see Movie 1 in the supplementary material). Most dia5/M mutants have defects during this process (this is not zygotically rescued). First, a subset of the cells that should apically constrict do not do so (Fig. 6B, 10:00, red arrows; see Movie 2 in the supplementary material), consistent with a role for Dia in apical constriction. However, many cells successfully constrict, revealing a second defect. As wild-type mesodermal cells invaginate, they pull on neighboring cells that stretch toward the midline (Fig. 6A, 13:45, blue arrows). In many dia5/M mutants, these cells become massively multinucleate (e.g. Fig. 6B, blue arrows; see Movie 2 in the supplementary material). In many dia5/M/Z mutants these defects preclude further analysis, but some have less severe disruptions, allowing us to assess later events. These embryos have dramatic alterations in protrusive behavior of amnioserosal cells, which we analyze in detail below.

Fig. 7. Reducing Dia function destabilizes AJs. Drosophila embryos: anterior leftwards, genotypes and antigens indicated. (A,B) Wild type. (C,D,I) Zygotically-rescued dia5/M. (E-H) dia5/M/Z; note less continuous AJs. (I-K) DE-cad. (J-K) dia5/M/Z. (L2,J2 insets) Cross-sections. (L) Immunoblot showing 6-8 hour wild-type and dia5/M/Z embryo extracts. α-tubulin is used as a loading control. (M–N,P,P',R–S) dia5/M/Z. (O,Q,Q') Wild-type. (P,P') Increased apical Rab5-positive vesicles; some colocalize with DE-cad (arrows, lines). (R,S) Abnormal cortical blebbing (arrows). Scale bars: 20 μm.
Dias is required for AJ maintenance

These defects in morphogenesis in embryos with reduced Dia function are consistent with several possible mechanisms of action. Our DiaGain-of-function experiments suggested that Dia helps coordinate actomyosin activity with adhesion at AJs. These data, along with previous work implicating formin as AJ regulators (see Introduction) led us to test the hypothesis that Dia regulates AJ assembly/maintenance.

We compared Arm and DE-cadherin (DE-cad) localization in wild-type (histone-GFP-marked), diaM/Z and zygotically-rescued diaM mutants (identified with a twist-GFP Balancer), all stained and imaged together to allow direct comparison of protein levels. By the end of gastrulation, wild-type AJs are evenly distributed around the apical cortex (Fig. 7A,B). AJs were normal in zygotically rescued diaM mutants (Fig. 7C,D,II,12,). In diaM/Z, AJs formed (Fig. 7E,F) and cortical F-actin and Myosin were not grossly disrupted (see Fig. S3 in the supplementary material). Thus, this degree of reduction in Dia function does not prevent initial AJ assembly.

However, Dia is essential for AJ maintenance. In diaM/Z mutants, Arm (compare Fig. 7E,F with 7A,B) and DE-cad (compare Fig. 7J2,K with 7I2, insets). AJ maintenance was abnormal in embryos with and without ventral furrow disruption (compare Fig. 7G,H with 7E,F), so this is not a secondary consequence of disrupted morphogenesis. To confirm the effect on AJs, we examined DE-cad levels by immunoblotting. DE-cad was noticeably reduced in diaM/Z mutants (hand-selected using a GFP-marked Balancer; Fig. 7L; Arm was slightly reduced and a-catenin unaffected). AJ destabilization is accompanied by Arm and DE-Cad accumulation in cytoplasmic puncta (Fig. 7M,N, arrows). We next stained diaM/Z mutants for Rab5, which marks early endosomes. Rab5 vesicles are normally largely basal to AJs (Fig. 7O). In diaM/Z mutants, Rab5 vesicles accumulate at the apical cortex, partially colocalizing with AJs (Fig. 7P; most internal DE-cad puncta are not Rab5 positive – they may be later endocytic intermediates). Reduction in Dia and consequent reduction in apical actomyosin may expose AJs to endocytic machinery – further work is needed to test this hypothesis. Interestingly, AJ destabilization was accompanied with cortical blebbing on the basolateral cortex just below AJs [Fig. 7R,S, arrows; Discs Large (Dlg) (a basolateral marker)]. Thus, strong reduction of Dia function destabilizes AJs and increases protrusiveness. These data are consistent with the model from our gain-of-function experiments: that Dia promotes mutual stabilization of AJs and junctional actomyosin.

daia and Rho1 exhibit very strong genetic interactions

These data suggest that Dia stabilizes AJs. Drosophila Rho1 plays a similar role (Bloor and Kiehart, 2002; Magie et al., 2002). As DRFs are regulated by Rho binding (Watanabe et al., 1997), this suggested that Rho may work, in part, through Dia.

To test this hypothesis, we examined whether Rho1 and dia genetically interact. We found a very strong genetic interaction. Progeny of heterozygous dia–/+ Rho1 females (subsequently referred to as diaRho1 mutants for simplicity) grow up to be infertile adults, as they lack pole cells, precursors of germ cells, they thus resemble diaM mutants (see Fig. S4H,1 in the supplementary material) (Afshar et al., 2000). diaRho1 mutants also have partially penetrant cellularization defects (see Fig. S4B,E in the supplementary material), similar to but much less severe than those of diaM mutants at 25°C (see Fig. S4C,F in the supplementary material) (Afshar et al., 2000). Thus, reducing both Dia and Rho1 levels by only 50% leads to defects in development, demonstrating that dia and Rho1 exhibit non-allelic non-complementation. This is quite rare and suggests an intimate relationship.

This allowed us to explore how Dia and Rho work together during morphogenesis, by examining what events are compromised when Dia and Rho1 function are reduced. Embryonic cuticles provide an easy means to assess morphogenesis. Zygotic dia–/– mutants are embryonic viable (Castrillon and Wasserman, 1994), dying as late larvae. However, if we reduced maternal Rho1 by 50% in dia–/– mutants (dia–/+Rho1 females crossed to dia–/+ males), nearly all of dia–/– mutant died as embryos (23% of total progeny), with defects in head involution, ventral cuticle or the completion of dorsal closure (Tables 2, 3). Zygotic Rho1 mutants are embryonic lethal with head defects and dorsal cuticle bowing (Magie et al., 1999). Maternal heterozygosity for dia substantially enhanced the phenotype of Rho1 (Tables 2, 3), disrupting the ventral cuticle. This may indicate problems during mesoderm invagination or epithelial integrity, consistent with defects in diaM mutants. Even milder reductions in Rho1 and Dia affect viability; 13% of

Table 3. Embryonic cuticle phenotypes of dia2/Rho172O progeny

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<tbody>
<tr>
<td>Embryonic lethal</td>
<td>1%</td>
<td>23%</td>
<td>22%</td>
<td>42%</td>
</tr>
<tr>
<td>Head defects</td>
<td>n.a.</td>
<td>63%</td>
<td>4%</td>
<td>33%</td>
</tr>
<tr>
<td>Head hole</td>
<td>n.a.</td>
<td>12%</td>
<td>75%</td>
<td>23%</td>
</tr>
<tr>
<td>Dorsal pattern defects</td>
<td>n.a.</td>
<td>7%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Ventral holes</td>
<td>n.a.</td>
<td>9%</td>
<td>0.4%</td>
<td>40%</td>
</tr>
<tr>
<td>Head + dorsal hole</td>
<td>n.a.</td>
<td>0.7%</td>
<td>0%</td>
<td>1.5%</td>
</tr>
<tr>
<td>Fragmentary cuticle/Crumbs</td>
<td>n.a.</td>
<td>0%</td>
<td>0%</td>
<td>2.6%</td>
</tr>
<tr>
<td>n</td>
<td>n.a.</td>
<td>147</td>
<td>272</td>
<td>270</td>
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</tbody>
</table>

*Cuticle preparations were not analyzed for this genotype as 99% of the embryos hatched.

**Table 2. Rho1 and Dia genetically interact**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% Embryonic lethal</th>
<th>n</th>
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<tbody>
<tr>
<td>Rho172O/dia2 × +/-</td>
<td>13%</td>
<td>860</td>
</tr>
<tr>
<td>dia2/+ × dia2/+</td>
<td>1%</td>
<td>352</td>
</tr>
<tr>
<td>Rho172O/dia2 × dia2/+</td>
<td>23%</td>
<td>429</td>
</tr>
<tr>
<td>Rho172O/+ × +/-</td>
<td>5%</td>
<td>305</td>
</tr>
<tr>
<td>Rho172O/+ × Rho172O/+</td>
<td>22%</td>
<td>427</td>
</tr>
<tr>
<td>Rho172O/n × Rho172O/+</td>
<td>42%</td>
<td>444</td>
</tr>
</tbody>
</table>

Croses were carried out at 25°C. dia1/+ = dia1/GYOK Kr-GFP.
progeny of dia\(^2\) +/- Rho1 females die as embryos, largely with defects in head involution (both single mutant heterozygotes are fully viable; less than 7% lethality). These data suggest that Dia and Rho1 work together in morphogenesis, with head involution, dorsal closure and ventral epidermal integrity particularly sensitive to their reduced function.

**Mildly reducing Dia/Rho1 activity triggers dramatic alterations in amnioserosal cell behavior**

We next explored how reducing Dia and Rho1 affects morphogenesis at the cellular level. Although most dia +/- Rho1 mutants hatch, we saw striking alterations in cell behavior. These were most dramatic during germband retraction, when the germband and amnioserosa move in a coordinated fashion (Schock and Perrimon, 2002), with D/V shortening and A/P elongation of amnioserosal cells driving tail retraction. This is facilitated by stable adhesion between the two tissues across a well-defined tissue boundary (Fig. 8A,C, arrows), in which the amnioserosa adheres to and extends over the caudal germband, and extends broad lamellipodia under it (Schock and Perrimon, 2003), while anterior germband cells abut and adhere to lateral amnioserosal cells.

dia/Rho1 mutants exhibit striking differences from wild type. During germband retraction, amnioserosal cells all along the germband of dia/Rho1 embryos form long persistent cell extensions extending over neighboring epidermal cells (Fig. 8B-E). dia\(^5\)M/Z mutants have similar protrusions (Fig. 8F), demonstrating that reduced Dia activity is key. Protrusions are long, thin and stable to harsh fixation (Fig. 8B,E), have Myosin enriched at their tips (Fig. 8E), and contain F-actin (Fig. 8D). Analysis of germband retraction live, using Moesin::GFP (Fig. 8H,I, see Movies 3-5 in the supplementary material; Moesin::GFP binds F-actin) allowed us to examine cell behavior and visualize actin dynamics. This further highlighted altered cell behaviors in dia/Rho1 mutants, revealing protrusions from amnioserosal cells over the epidermis all along the germband. They were especially prominent caudally (Fig. 8H, yellow arrows), but were also observed laterally and anteriorly (Fig. 8H, red arrows). Amnioserosal cells also extended abnormal processes over one another (Fig. 8H, blue arrows), and we saw occasional long protrusions from epidermal cells (Fig. 8L). Alterations in protrusive behavior continued into dorsal closure. Amnioserosal cells normally send out fine filopodia during closure (Fig. 9A, arrows). In dia/Rho1 mutants these were longer and...
sometimes emerged from broad lamella (Fig. 9B, arrows). This was even more exaggerated in dia +/M/Z mutants (Fig. 9C, arrows). dia/Rho1 amnioserosal cells also occasionally produced broad processes over one another (Fig. 9E) or over the epidermis (Fig. 9D). In dia/Rho1, dorsal closure was otherwise normal, with one exception: drop-out cells appeared much earlier in dorsal closure than in wild type (compare Fig. 9F with 9G). However, in dia +/M/Z mutants, dorsal closure was more defective, with cell misalignment as the epidermal sheets met at the dorsal midline (Fig. 9H). Thus, amnioserosal cells are most sensitive to Dia and Rho1 reduction; it disrupts their stable tissue interface with the epidermis, triggering abnormal protrusiveness.

During apical constriction in the Drosophila ventral furrow, Rho is regulated by the guanine nucleotide exchange factor RhoGEF2 (Barrett et al., 1997; Hacker and Perrimon, 1998). We also explored whether dia genetically interacts with RhoGEF2. The progeny of dia +/+ RhoGEF2 females had defective pole cells (see Fig. S4J in the supplementary material), albeit not as defective as those of dia/Rho1, and also had abnormal protrusions of amnioserosal cells over the adjacent epidermis (Fig. 8G), similar to those of dia/Rho1 embryos. This non-allelic non-complementation is consistent with RhoGEF2 working together with Dia and Rho in morphogenesis.

**Dia and Myosin regulation**

To examine mechanisms by which Dia affects protrusiveness, we first examined localization of β-integrin, which is key for germband retraction (Schock and Perrimon, 2003). However, we saw no obvious differences in β-integrin levels or localization in dia/Rho1 mutants in amnioserosa or epidermis (compare Fig. S5A with S5B in the supplementary material; data not shown).

Our gain- and loss-of-function analyses suggested that Dia coordinates adhesion at AJs with actomyosin contractility. We thus tested the hypothesis that abnormal amnioserosal protrusions occur because reduced Dia activity reduces Myosin activity at AJs, destabilizing them and increasing protrusiveness. We activated Myosin by expressing MLCKCA in both the germband and amnioserosa of dia/Rho1 mutants using e22c-GAL4, detecting misexpression with anti-MLCKCA. Interestingly, inducing Myosin phosphorylation rescued the abnormal protrusiveness (compare Fig. 8J with 8K). This is consistent with reduced Dia activity leading to reduced Myosin activity at AJs, destabilizing the normal tissue boundary and triggering protrusive behavior across it.

**DISCUSSION**

Morphogenesis require coordinated adhesion and actomyosin contractility. Here, we implicate Dia as a novel regulator coordinating adhesion and actomyosin contractility during morphogenesis. Our results frame a model suggesting that Dia regulates both actin and Myosin, allowing it to help organize the apical actomyosin network at AJs, thus regulating cell shape change, stabilizing AJs and inhibiting cell protrusion during tissue remodeling.

**Dia helps coordinate adhesion and actomyosin contractility during morphogenesis**

Previous analysis of DRF function in morphogenesis was hampered by mammalian DRF redundancy and the key role of DRFs in cytokinesis. We used the genetic tools available in Drosophila to partially circumvent these difficulties. We knew that Dia regulates cellularization (Afshar et al., 2000; Grosshans et al., 2005). Our analysis revealed new roles during morphogenesis. In embryos with severely reduced Dia function, gastrulation is disrupted. Apical constriction of central furrow cells is delayed or blocked in a subset of cells, suggesting a possible role for Dia in regulated apical constriction. Previous work suggested that an unknown Rho effector regulates actin during this process (Fox and Peifer, 2007); perhaps this is Dia. Adjacent cells, which are stretched during invagination, become massively multinucleate in dia +/+ mutants. This may reflect the fact that the cells of the blastoderm undergo an incomplete form of cytokinesis, remaining connected to the underlying yolk by actin-lined yolk canals. These canals normally close at the onset of gastrulation in ventral furrow cells; this may prevent cell membrane rupture initiated at the yolk canal under stress. Dia may regulate yolk canal closure; it localizes there at the end of cellularization (Afshar et al., 2000; Grosshans et al., 2005) and similar defects are seen in mutants lacking the septin Peanut, a yolk canal component (Adam et al., 2000). Alternately, Dia may stabilize cortical actomyosin or its connections to AJs, with its absence weakening cortical integrity under stress. Dia also plays a key role during germband retraction. The most striking effect of reduced Dia/Rho1 function is altered amnioserosal cell protrusiveness. They normally make stable AJs and form a tight
Roles of Dia in morphogenesis

A mechanistic model of roles of Dia in actin and Myosin regulation

From our data, we developed a mechanistic model for the regulation of actin, Myosin and AJs by Dia during cell shape change (Fig. 10A), in which Dia activation stabilizes actin and active Myosin at AJs. In radially symmetric amnioserosal cells, Dia promotes organization/activation of an apical actomyosin network linked to AJs, inducing precocious apical cell constriction. Activation of endogenous Dia may regulate normal amnioserosal constriction; this now needs to be tested. Dia activation in cells where AJs are planar polarized, such as epidermal cells, promotes actomyosin organization preferentially at cell borders where AJs are enriched. This leads to cell widening or helps cells resist elongation, generating groove-cell-like morphology. Once again, Dia may be normally activated specifically in groove cells to modulate their shape.

How does Dia activation activate Myosin? It does not occur primarily through SRF, which triggers Myosin accumulation but not cell shape changes. Myosin activation via MLCK partially mimicked DiaCA, suggesting that Myosin activation is an important part of the process. However, MLCK did not precisely mimic DiaCA, suggesting that Dia acts preferentially at specific sites such as AJs rather than globally activating Myosin. The dual effects on actin and Myosin suggest the speculative possibility that feedback mechanisms exist to coordinate the Rho-regulated actin and Myosin pathways (Fig. 10B). Recent work revealed that active Dia can activate RhoA by binding the Rho-GEF LARG (Kitzing et al., 2007), and we saw strong genetic interactions between dia and the LARG relative RhoGEF2, making this idea more plausible. Future work is needed to test this hypothesis.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/6/1005/DC1

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