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DRhoGEF2 and *Diaphanous* Regulate Contractile Force during Segmental Groove Morphogenesis in the *Drosophila* Embryo

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Morphogenesis of the *Drosophila* embryo is associated with dynamic rearrangement of the actin cytoskeleton mediated by small GTPases of the Rho family. These GTPases act as molecular switches that are activated by guanine nucleotide exchange factors. One of these factors, DRhoGEF2, plays an important role in the constriction of actin filaments during pole cell formation, blastoderm cellularization, and invagination of the germ layers. Here, we show that DRhoGEF2 is equally important during morphogenesis of segmental grooves, which become apparent as tissue infoldings during mid-embryogenesis. Examination of *DRhoGEF2*-mutant embryos indicates a role for DRhoGEF2 in the control of cell shape changes during segmental groove morphogenesis. Overexpression of DRhoGEF2 in the ectoderm recruits myosin II to the cell cortex and induces cell contraction. At groove regression, DRhoGEF2 is enriched in cells posterior to the groove that undergo apical constriction, indicating that groove regression is an active process. We further show that the Formin Diaphanous is required for groove formation and strengthens cell junctions in the epidermis. Morphological analysis suggests that Dia regulates cell shape in a way distinct from DRhoGEF2. We propose that DRhoGEF2 acts through Rho1 to regulate acto-myosin constriction but not Diaphanous-mediated F-actin nucleation during segmental groove morphogenesis.

INTRODUCTION

The body plan of higher animals and insects is segmented to allow controlled patterning and growth in well-defined compartments. Segmentation is associated with the formation of boundaries between segments to prevent distinct populations of cells from intermingling and to allow them to be patterned separately during development. For example, the early *Drosophila* embryo is divided into 14 reiterated developmental units termed parasegments (Martinez-Arias and Lawrence, 1985; Lawrence and Struhl, 1996). Cells within each parasegment are sorted out from neighboring parasegments by the establishment of parasegment boundaries that are visible as transient grooves at the anterior of each stripe of cells expressing the segment polarity gene *engrailed* (*en*) (Vincent and O'Farrell, 1992). Another boundary, the segment boundary, forms posterior to each stripe of *en* expression. Formation of segment boundaries begins shortly after the initiation of germ band retraction, and is associated with distinct morphologic changes in segment border cells. These changes include apical constriction of the most posterior *en*-expressing cells that result in a local folding termed segmental groove. Formation of segmental grooves requires activity of the segment polarity genes *en*, *hedgehog* (*hh*), and *wingless* (*wg*). Embryos mutant for either *en* or *hh* fail to form segment boundaries, and continuous expression of these genes is essential for maintenance of the boundaries. Anterior to *en*-expressing cells, a *wg*-mediated inhibitory signal prevents cells from forming a groove (Larsen *et al*., 2003). However, how these patterning genes direct morphogenesis, and which cytoskeletal regulators control cell shape during this process is currently unknown.

Rho-guanosine triphosphatases (GTPases) play an important role in the control of cell shape changes that are driven by rearrangement of the actin cytoskeleton. In *Drosophila*, *Rho1* has been shown to regulate cell shape and adhesion in many processes, such as cellularization (Padash Barmchi *et al*., 2005), gastrulation (Barrett *et al*., 1997; Häcker and Perrimon, 1998), epidermal development (Bloor and Kiehart, 2002), dorsal closure, segmentation, and head involution (Magie *et al*., 1999) as well as during epithelial folding in wing imaginal discs (Nikolaidou and Barrett, 2004). Activation of Rho1 requires the exchange of guanosine diphosphate for guanosine triphosphate, which is catalyzed by guanine nucleotide exchange factors (GEFs) such as DRhoGEF2.

The role of DRhoGEF2 in epithelial morphogenesis has been studied in most detail in early *Drosophila* embryos. During gastrulation, DRhoGEF2 regulates apical cell constriction that drives the invagination of mesodermal and endodermal primordia (Barrett *et al*., 1997; Häcker and Perrimon, 1998). Evidence identifying factors that connect DRhoGEF2 to the actin cytoskeleton has emerged from genetic studies. In the ventral mesoderm DRhoGEF2 and the Rho-effector Rho-kinase (DRok) are required for apical localization of myosin II, and mutants in both genes result in similar phenotypes (Dawes-Hoang *et al*., 2005). In *Drosophila* Schneider (S2) cells, DRhoGEF2-mediated cell contraction

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can be inhibited by DRok inactivation (Rogers *et al*., 2004). Thus, a Rho-effector pathway including DRok, the regulatory subunit of myosin light chain phosphatase (myosin binding subunit) (Mizuno *et al*., 2002; Tan *et al*., 2003) and myosin II that has been described in other systems (Amano *et al*., 1996) is thought to control acto-myosin contraction in response to DRhoGEF2 activation.

DRhoGEF2 is also required for stabilization and contraction of actomyosin filaments during blastoderm cellularization (Grosshans *et al*., 2005; Padash Barmchi *et al*., 2005), and mutants in DRhoGEF2 and *DRok* have similar defects during this process (Dawes-Hoang *et al*., 2005). DRok may therefore act as a DRhoGEF2-effector in a broad cellular context.

Another Rho1-effector, that plays an important role in early embryos and regulates F-actin nucleation and polymerization together with the actin binding protein Profilin (Wallar and Alberts, 2003) in a pathway parallel to DRok (Watanabe *et al*., 1997) is the Formin homology protein Diaphanous (Dia) (Afshar *et al*., 2000). In mammalian systems, mDia1 and the DRok homologue Rho-kinase (ROCK) have been shown to work concurrently during stress fiber formation. Interestingly, mDia1 and ROCK can induce stress fibers of different thicknesses and densities depending on the balance between their individual activities, indicating that the two Rho-effector pathways may be independently regulated (Watanabe *et al*., 1999).

It has been hypothesized that Rho-effector pathway specificity may be conferred by individual GEFs such as DRhoGEF2. However, whether DRhoGEF2 can activate Dia or signals specifically through DRok is currently unclear.

Here, we show that DRhoGEF2 and Dia regulate cell shape changes during segmental groove morphogenesis. Overexpression of DRhoGEF2 in epidermal cells is sufficient to induce cell contraction and premature formation of segmental grooves. Activation of the Formin Dia results in strengthening of cell junctions, and controls F-actin levels and cell shape in a manner distinct from DRhoGEF2. Our data suggest that DRhoGEF2 may select the outcome of Rho1-activation to regulate cortical myosin II recruitment and constriction of acto-myosin fibers but not cell adhesion or F-actin polymerization.

MATERIALS AND METHODS

Fly Strains

The following mutant alleles were used: DRhoGEF2$^{l(2)04291}$ (Häcker and Perrimon, 1998), *dia*^{5} (Castrillon and Wasserman, 1994), and *sqh-GFP* (Royou *et al*., 2004). The following Gal4 drivers and their responders were used: *prd-Gal4*, *en-Gal4-GFP*, *pnr-gal4* (Bloomington *Drosophila* Stock Center, Department of Biology, Indiana University, Bloomington, IN), *twi-Gal4* (gift from A. Michelson, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA), *wg-Gal4* (gift from N. Perrimon, Department of Genetics, HHMI, Harvard Medical School, Boston, MA), *UAS-DRhoGEF2-RE* (Padash Barmchi *et al*., 2005), and *UAS-dia*^{CA}*-HA* (Somogyi and Rørth, 2004). Germline clones (GLCs) of DRhoGEF2$^{L(2)04291}$ and *dia*^{5} were generated by using the autosomal flippase-mediated recombination dominant-female-sterile technique (Chou and Perrimon, 1996). Females carrying DRhoGEF2$^{L(2)04291}$ GLCs were crossed to DRhoGEF2$^{L(2)04291}$/*Cyo-GFP* males. Embryos maternally and zygotically mutant for DRhoGEF2$^{L(2)0429}$ were identified by the absence of green fluorescent protein (GFP). Females carrying *dia*^{5} germline clones were crossed to *dia*^{5}*/CyO* males and kept at 18°C.

Immunolocalization

For phalloidin stainings, embryos were dechorionated in 50% bleach, fixed in 4 ml of HEPES, 1 mM EGTA, 20 mM $Mgso_4$, 4 or 8% formaldehyde, and 5 ml of heptane for 20 min, and then they were devitellinized by hand, washed in phosphate-buffered saline plus 0.1% Tween 20 (PBT), and incubated in rhodamine-conjugated phalloidin (Invitrogen, Carlsbad, CA) for 1 h, washed three times in PBT, and mounted on a microscope slide. Otherwise embryos were fixed as described above and methanol devitellinized. Primary antibodies used were as follows: rabbit anti-DRhoGEF2 (gift from S. Rogers, Department of Biology, The University of North Carolina at Chapel Hill, Chapel Hill, NC; 1/1000), rabbit anti-Dia (gift from S. Wasserman, Section of Cell and Developmental Biology, University of California at San Diego, La Jolla, CA; 1/2000), rat anti-hemagglutinin (HA) high-affinity (Roche Diagnostics, Indianapolis, IN; 1/100), rabbit anti-Sqh (gift from Tien Hsu, Medical University of South Carolina, Hollings Cancer Center, Charleston, SC; 1/50), mouse anti-Arm (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA; 1/200), rat anti-E-Cad (gift from H. Oda, JT Biohistory Research Hall, Osaka, Japan; 1/250), guinea pig anti-odd (gift from C. Rauskolb, Waksman Institute, Rutgers, the State University of New Jersey, Piscataway, NJ; 1/1000), or mouse anti-En (Developmental Studies Hybridoma Bank; 1/50). The secondary antibodies used were cyanine (Cy)2-goat anti-rabbit immunoglobulin (Ig)G, Rhodamine Red-X goat anti-mouse IgG, Cy2-goat anti-mouse IgG, Cy2-goat anti-rat IgG, Rhodamine Red-X goat anti-rat IgG, Rhodamine Red-X goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Gove, PA), and Alexa 647-goat anti-rabbit IgG (Invitrogen). Images were obtained from a laser scanning confocal microscope (model TCS SP2; Leica, Wetzlar, Germany), and they were processed with Adobe Photoshop software (Mountain View, CA).

Scanning Electron Microscopy

Embryos were collected at timed intervals, dechorionated, fixed in 2.5% glutaraldehyde for 2 h, and devitellinized with methanol. For visualizing filopodia, embryos were devitellinized by hand, using a needle. Embryos were then washed twice in 0.1 M sodium cacodylate, pH 7.4, postfixed in 1% osmiumtetroxide and 0.1 M sodium cacodylate, pH 7.4, for 1 h, and dehydrated in an ethanol series. After exchange of ethanol against carbon dioxide in a critical point dryer, embryos were mounted on stubs, gold-palladium coated in a sputter coater, and photographed in a JEOL LV5600 scanning electron microscope (SEM; JEOL, Tokyo, Japan).

RESULTS

Localization of DRhoGEF2, F-actin, and Myosin II during Segmental Groove Morphogenesis

DRhoGEF2, F-actin, and myosin II play important roles in the generation of acto-myosin–based contractile force, and they are dynamically redistributed during many morphogenetic processes (Nikolaidou and Barrett, 2004; Rogers *et al*., 2004; Dawes-Hoang *et al*., 2005). We investigated the localization of DRhoGEF2 with respect to F-actin and myosin II at the segmental grooves that first become apparent in the embryonic epidermis during stage 12, shortly after the onset of germband retraction. Groove formation occurs first in thoracic segments by dorsal-ventral elongation of the tracheal pits that are in register with segmental grooves, and it gradually proceeds toward more posterior segments in the course of germband retraction during stage 12. Formation of segmental grooves is initiated by apical constriction of a single cell row in each segment corresponding to the posterior-most *en*-expressing cells (Larsen *et al*., 2003). At the onset of groove formation, DRhoGEF2, F-actin, and myosin II are distributed along the apicolateral cortex in all epidermal cells (Figure 1, E–E″). Levels of F-actin and to a much lesser degree DRhoGEF2 were increased in groove founder cells (Figure 1, A–D′, arrowheads) that constrict apically and change shape from cuboidal to wedge shaped. Interestingly, F-actin accumulation was polarized in the plane of the epithelium because F-actin levels were consistently highest at the posterior boundary of groove founder cells (Figure 1, C, C′, D, and D′). A significant enrichment or polarization of myosin II in groove founder cells could not consistently be detected. Apical constriction of groove founder cells during early stage 12 results in the formation of shallow grooves. As the groove deepens, DRhoGEF2, F-actin, and myosin II begin to accumulate basally at the interface between epidermal cells and the underlying mesoderm (Figure 1, F–F″, arrows). Concomitantly, the epidermis is organized into a stratified columnar epithelium with a defined baseline. Grooves have a symmetrical appearance with the apically constricted groove founder cell at the center flanked by three to four cells on either side. No additional cells enter the groove after

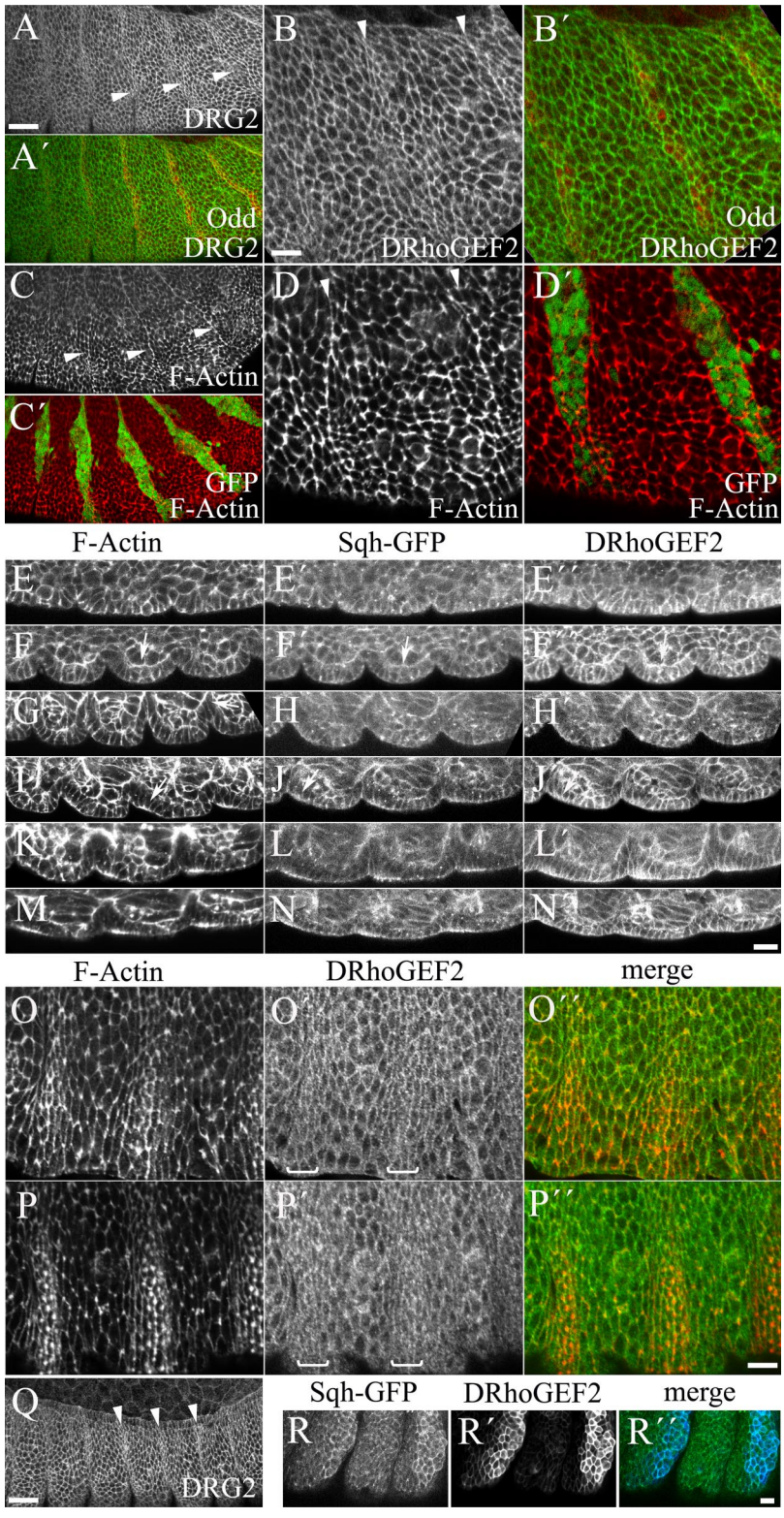

Figure 1. Localization of DRhoGEF2, F-actin, and myosin II during segmental groove morphogenesis. (A and A′) Early stage 12: onset of groove invagination. DRhoGEF2 is enriched in groove founder cells marked by expression of Odd-skipped (Odd). (B and B′) Enlargement of A and A′, respectively. (C and C′) Early stage 12: polarization of F-actin at the posterior boundary of groove founder cells (marked by En-GFP in green) at the onset of groove invagination. (D and D′) Enlargement of C and C′, respectively. (E–E″) Early stage 12: apicolateral distribution of F-actin, myosin II, or DRhoGEF2 at the onset of groove formation. (F–F″) Mid-stage 12: as shallow furrows have formed, F-actin, myosin II, and DRhoGEF2 accumulate basally in epidermal cells (arrows). The epithelium now has a defined baseline. (G, H, and H′) Late stage 12: grooves reach their maximum depth as cells at the groove center elongate along their apical-basal axis (arrow in G). (I, J, and J′) Stage 13: onset of groove regression. F-actin, myosin II, and DRhoGEF2 accumulate at the apical cortex in cells posterior to segment boundaries (arrows). Grooves take on an asymmetrical appearance. (K, L, and L′) Stage 14: F-actin and myosin II form punctate condensations at the apical membrane. DRhoGEF2 remains uniform, and it is found in more apicolateral domains. (M, N, and N′) Stage 15: as grooves complete regression, epidermal cells take on a cuboidal shape. (O–O″) Stage 13: onset of groove regression. F-actin and DRhoGEF2 (brackets) accumulate in constricting cells posterior to grooves. (P–P″) Stage 15: DRhoGEF2 (brackets) does not colocalize with F-Actin punctas. (Q) Stage 14: DRhoGEF2 accumulates posterior to grooves in the lateral epidermis all the way to the dorsal leading edge. (R–R″) *prd-Gal4>UAS-DRhoGEF2-RE; sqh-GFP* embryo at stage 14. DRhoGEF2 overexpression recruits increased levels of myosin II to the cell membrane. E–P″ are ventrolateral views. A–D′ and Q–R″ are lateral views. Anterior is to the left and dorsal is up in all figures. Bars: 25 μm (A, A′, C, C′, and Q) and 10 μm (others).

this stage; however, during the second half of stage 12 the groove continues to deepen as the basal end of cells moves further inside. Simultaneously, groove founder cells and their immediate neighbors elongate approximately twofold along their apical-basal axis (Figure 1, G, H, and H′, arrow).

Shortly after grooves reach their maximum depth at the end of stage 12, DRhoGEF2, F-actin, and myosin II accumulate posterior to the groove founder cell at the apical cortex of four to five cells that constrict their apices, and the grooves begin to regress (Figure 1, I, J, and J′, arrows), first at the ventral side followed by more lateral regions. In parallel to constricting their apices, cells elongate their apical-basal axis (Figure 1, K, L, and L′). This further reduces groove depth posterior to the segment boundary, giving the

groove an asymmetrical morphology (Figure 1, I–L′). In the late phase of groove regression that lasts throughout stage 15, groove cells shorten their apical-basal axis, and they adopt a cuboidal shape (Figure 1, M, N, and N′). In summary, we identify five morphologically distinguishable phases during groove morphogenesis that include apical constriction that results in initial bending of the epithelium during early stage 12, apical-basal elongation of groove founder cells during late stage 12, apical constriction of cells posterior to the groove initiating groove regression during stage 13, apical-basal elongation of cells in the groove, and outward movement of the apical cell surface followed by subsequent apical-basal shortening and outward movement of the basal end of cells.

Groove regression temporally overlaps with the differentiation of denticles, which are F-actin–rich protrusions on the apical surface of cells in the ventral epidermis that together form the denticle belts of the mature larva. The cells that constrict during segmental groove regression coincide largely with cells that form denticles. It has been reported that denticle-forming cells undergo shape changes and accumulate F-actin and myosin II at their apical ends. After initial uniform distribution (Figure 1, I and J, arrows), F-actin and myosin II condense into polarized aggregates (Figure 1, K–N) at the posterior cell boundary that subsequently differentiate into denticles (Price *et al*., 2006; Walters *et al*., 2006). In contrast, DRhoGEF2 remains cortically enriched in more apicolateral regions (Figure 1, L′ and N′), and it is not found in F-actin condensations (Figure 1, O–O″ and P–P″, brackets). In addition, DRhoGEF2 accumulation also occurs in cells of the lateral epidermis that undergo the same cell shape changes as ventral cells, but do not form denticles (Figure 1Q, arrows). These observations are consistent with the view that DRhoGEF2 accumulation may play a role in the regulation of cell shape rather than denticle formation.

Embryos lacking DRhoGEF2 fail to recruit myosin II to the apical membrane of ventral furrow cells during gastrulation (Nikolaidou and Barrett, 2004; Dawes-Hoang *et al*., 2005). To investigate whether DRhoGEF2 is sufficient to recruit myosin II to the cell membrane, we expressed a *UAS-DRhoGEF2-RE* transgene (hereafter referred to as DRhoGEF2) (Padash Barmchi *et al*., 2005) in the epidermis by using *prd-Gal4*. This resulted in cortical accumulation of myosin II in cells of the *prd* domain (Figure 1, R–R″), suggesting that DRhoGEF2 may regulate apical myosin II localization also during segmental groove morphogenesis. An effect of DRhoGEF2 on F-actin accumulation could not be detected (Figure 3).

DRhoGEF2 Is Required for Formation and Regression of Segmental Grooves

DRhoGEF2 is maternally supplied to the embryo, and ubiquitously expressed in the zygote. In embryos that are zygotic mutant for the null allele DRhoGEF2$^{l(2)04291}$, segmental grooves form but occasionally individual grooves fail to regress (Figure 4B), suggesting that DRhoGEF2 may play a role in groove regression. Among embryos derived from DRhoGEF2$^{l(2)04291}$-mutant germline clones, two phenotypically distinct groups can be distinguished that correspond to maternally (DRhoGEF2^{M}) or maternally and zygotically (DRhoGEF2^{MZ}) mutant embryos. Embryos from either group develop with severe defects during early embryogenesis that make analysis of segmental groove formation very difficult. We therefore stained these embryos for En, which highlights the position at which segmental grooves form in the wild type. Our analysis revealed that despite the severe distortion of the epidermis that occurs in the absence of maternal DRhoGEF2, most segmental grooves form (Figure 2A). In contrast, fully developed grooves that are several cell diameters in depth were not observed in DRhoGEF2^{MZ} mutants (Figure 2B). Analysis at the cellular levels showed that DRhoGEF2^{MZ} embryos form shallow indentations in the epidermis that occasionally, but not always, coincide with the posterior-most en-expressing cell. However, cell shapes are randomized and cell size is irregular with many cells abnormally enlarged. Apical constriction of groove founder cells is not observed, and subsequent morphologic changes in epithelial cells such as apical-basal cell elongation do not occur (Figure 2C, arrowheads). F-actin is often distributed irregularly at the cortex of cells throughout the epithelium (Figure 2D, arrowheads). These results show that DRhoGEF2 is required for F-actin organization and the regulation of cell shape changes at the segmental grooves in the embryonic epidermis.

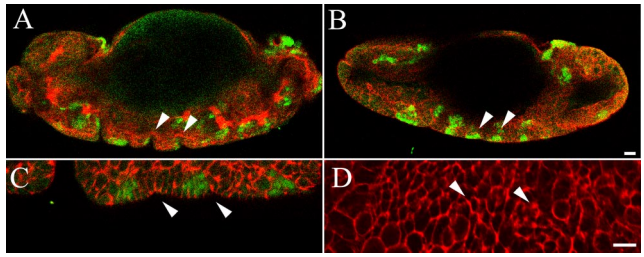

Figure 2. Segmental groove formation requires DRhoGEF2 function. Embryos are stained for F-actin (red) and En (green) to mark segment boundaries. (A) DRhoGEF2^{M}-embryo at stage 13: most segmental grooves form in the correct position posterior to the *en* domains (arrowheads). (B) DRhoGEF2^{MZ} embryo at stage 13: segmental grooves do not form (arrowheads). (C) Ventral epidermis of DRhoGEF2^{MZ} embryo at stage 13: shallow indentations form in the epithelium (arrowheads). However, fully developed grooves do not form. (D) F-actin accumulates in irregular aggregates (arrowheads) in cells throughout the epithelium. Bars, 20 μm (A and B) and 10 μm (C and D).

DRhoGEF2 Regulates Actomyosin Contraction

The irregular shape and size of cells in DRhoGEF2 mutants precluded a detailed analysis of cell behavior in this genetic background. To gain insight into the specific role of DRhoGEF2 in segmental groove morphogenesis, we overexpressed DRhoGEF2 in the epidermis by using *prd-Gal4* because the *prd* domain overlaps with segment boundaries. In the wild type, parasegmental grooves become first apparent in the epidermis at embryonic stage 11. In *prd-Gal4>UAS-DRhoGEF2-RE* embryos, parasegmental grooves form prematurely in *DRhoGEF2*-overexpressing segments. Staining for En showed that parasegmental grooves form anterior to *en*-expressing cells, which is where they also form in the wild type (Figure 3, A and A′). Thus, DRhoGEF2 activation seems to enhance wild-type grooves rather than leading to the formation of ectopic or aberrantly shaped grooves.

Similar to parasegmental grooves, segmental grooves start to form prematurely during stage 12 in DRhoGEF2-overexpressing segments (Figure 3, B and B′). Groove founder cells overexpressing DRhoGEF2 initially constrict apically, similar to their wild-type counterparts, but then contract and assume a rounded shape (Figure 3, C and C′). Apical-basal elongation of groove founder cells does not occur, and this may contribute to a deepening of grooves that is observed in DRhoGEF2-overexpressing segments (Figure 3, C and C′, and D and D′). Contraction is not limited to groove founder

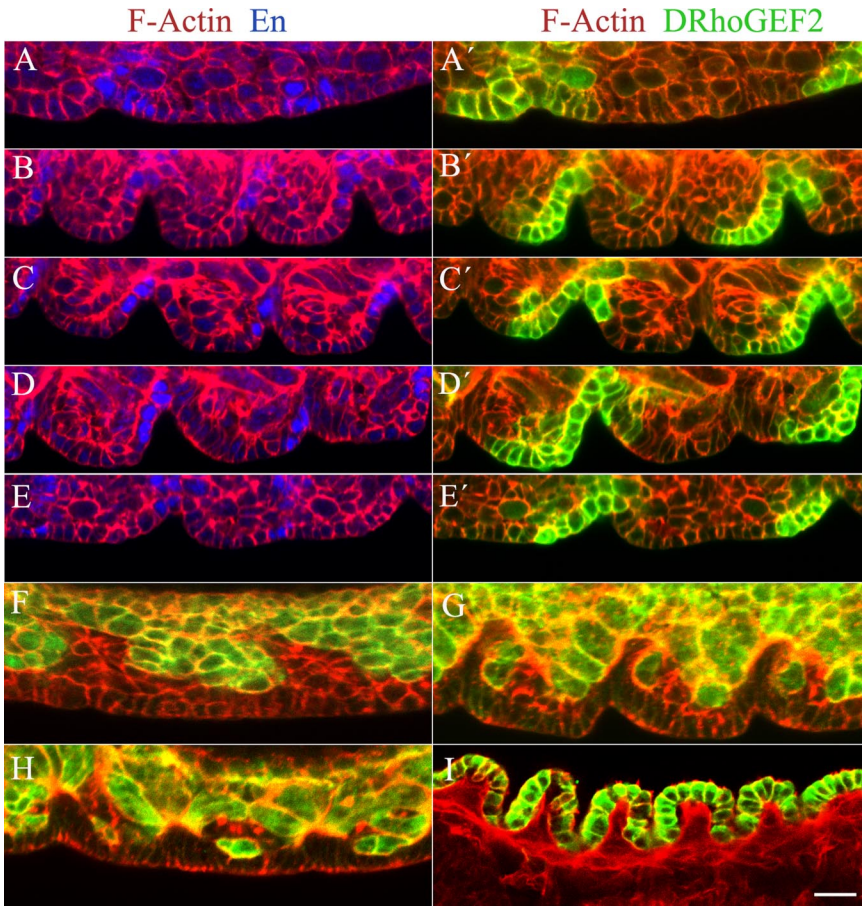

Figure 3. DRhoGEF2 overexpression results in the formation of deep segmental grooves. All embryos express *DRhoGEF2-RE* driven by *prd-Gal4* (A–E′), *twi-Gal4* (F–H), or *pnr-Gal4* (I), and they are stained for DRhoGEF2 (green), F-actin (red), and En (blue). (A and A′) stage 10: parasegmental grooves form prematurely in DRhoGEF2-overexpressing segments anterior to the En domain. (B and B′) Mid-stage 12: Cells contract and segmental grooves have formed prematurely in DRhoGEF2-overexpressing segments posterior to the posteriormost *en*-expressing cell. (C and C′) Late stage 12: groove founder cells overexpressing DRhoGEF2 do not elongate along their apical-basal axis. Segmental grooves are significantly deeper in DRhoGEF2-overexpressing segments. (D and D′) Stage 13: wild-type segments start to regress, whereas grooves persist in DRhoGEF2-overexpressing segments. (E and E′) Stage 14: wild-type grooves have regressed. DRhoGEF2-overexpressing grooves persist often showing reversed asymmetry. (F–H) DRhoGEF2-overexpression in the mesoderm does not influence segmental groove formation. Stages: 11 (F), 13 (G), and 15 (H). (I) Stage 15: DRhoGEF2 overexpression in the dorsal epidermis but not in underlying tissues results in deep segmental grooves. A–D and F–H show ventrolateral views; E and E′ show ventral views. I shows a dorsolateral view. Bar, 10 μm.

cells but is observed in all *DRhoGEF2*-overexpressing cells (Figures 3, D and D′ and E and E′; and 4G, arrowheads) consistent with a previous report showing that DRhoGEF2-overexpression induces contractility in *Drosophila* S2 cells (Rogers *et al*., 2004).

In addition to an approximately twofold increase in groove depth, *DRhoGEF2* overexpression results in delayed groove regression (Figure 3, D and D′ and E and E′), and grooves persist at a time when the wild-type epidermis has returned to a smooth appearance (Figure 4, A and C).

The premature onset of groove invagination and the preservation of normal groove morphology suggest that increased DRhoGEF2-induced contractility at least in part enhances normal aspects of cell behavior and that it can be used to modulate the forces contributing to groove formation. A question that has remained unanswered in the context of groove formation is whether grooves are formed autonomously by shape changes of epidermal cells or whether pulling forces in underlying tissues contribute to furrowing. To address this issue, we expressed DRhoGEF2 exclusively in the mesoderm by using *twi-Gal4* as a driver. Interestingly, no effect on groove formation was observed. Grooves did not form prematurely (Figure 3F), groove depth remained unchanged (Figure 3G), and groove regression occurred normally as in wild type (Figure 3H). In contrast, when *DRhoGEF2* was overexpressed exclusively in the dorsal ectoderm by using *pnr-Gal4*, the *DRhoGEF2*-expressing areas of the epidermis formed deep grooves that persisted throughout dorsal closure of the embryo (Figures 3I and 4, E, F, and F′). These observations suggest that modulation of contractile force in the ectoderm, but not in the underlying mesodermal tissue, can contribute to groove invagination in a cell-autonomous manner.

Role of Diaphanous in Segmental Groove Morphogenesis

To identify other cytoskeletal regulators involved in segmental groove morphogenesis, we investigated Dia, which plays a role in other DRhoGEF2-dependent processes such as pole cell formation and cellularization (Afshar *et al*., 2000). Dia is distributed throughout the epidermis and enriched at the vertices of epithelial cells, were elevated levels of the adherens junction (AJ) protein Armadillo (Arm) and F-actin are also found (Figure 5, A and A′, arrowhead; and C and C′). We found that Dia accumulates apicolaterally in groove founder cells at the onset of groove invagination (Figure 5, B and B′, arrowhead). Similar to F-actin, Dia accumulation is polarized to the posterior boundary of groove founder cells consistent with the view that Dia may control F-actin nucleation in these cells (Figure 5, C and C′).

dia is contributed maternally to the egg and homozygous mutant *dia* embryos show no defects during segmental groove formation. Embryos derived from females that completely lack *dia* function in the germline cannot be analyzed due to severe cytokinesis defects (Castrillon and Wasserman, 1994). We therefore analyzed the strong hypomorphic allele dia^5 in which *dia* protein levels are severely reduced (Figure 5, D and D′). Embryos derived from females homozygous for dia^5 in the germline develop with severe morphologic defects and die at the end of embryogenesis. Staining of these embryos for En and F-actin suggested that segmentation occurs normally, however, cells throughout the epidermis were enlarged, irregular in shape and many

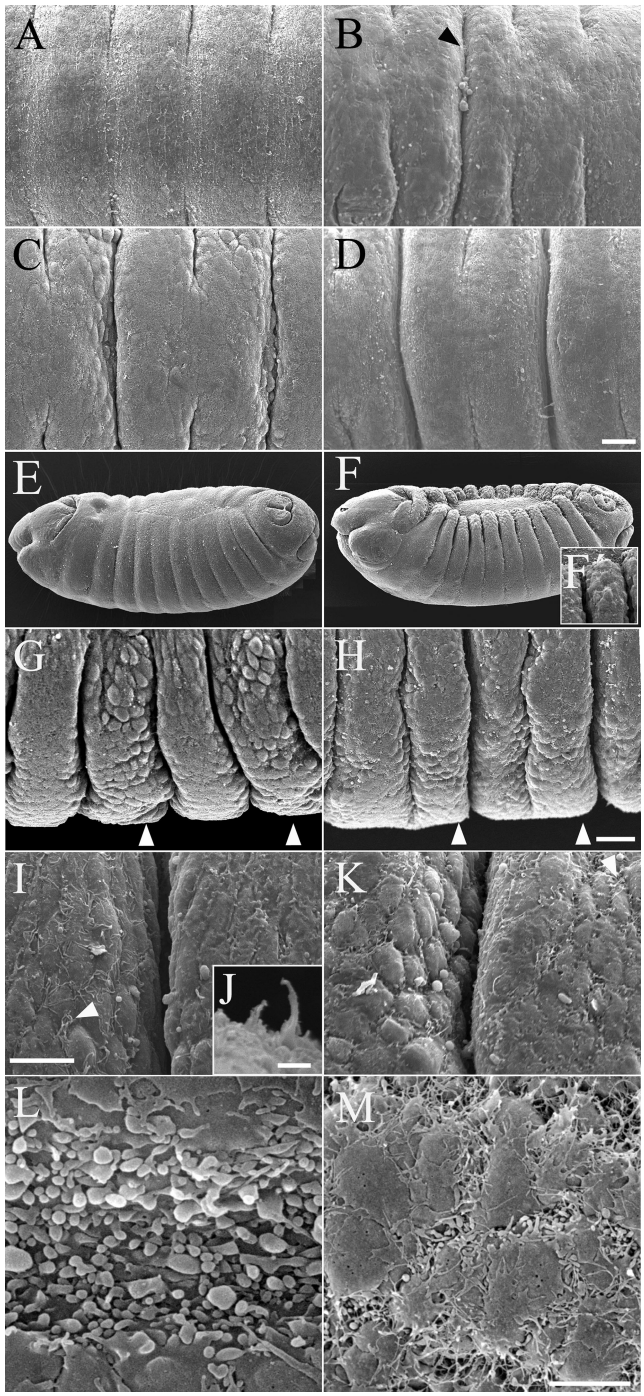

Figure 4. DRhoGEF2 and Dia^{CA} elicit different cell shape changes in epidermal cells. All panels show SEMs. (A) Wild-type embryo, stage 14. Segmental grooves have regressed ventrally. (B) Zygotic *DRhoGEF2*-mutant embryo, stage 14. Occasionally grooves fail to regress (arrowhead). (C) Ventral view of *prd-Gal4>UAS-DRhoGEF2-RE* embryo, Stage 14. Segmental grooves persist in DRhoGEF2-overexpressing segments. (D) Ventral view of *prd-Gal4>UAS-dia*^{CA} embryo, Stage 14. Grooves persist in *dia*^{CA}-expressing segments. (E) Wild-type embryo, Stage 15. Grooves have regressed in the dorsolateral epidermis. (F and F′) *pnr-Gal4>UAS-DRhoGEF2-RE* embryo at the same stage as E. Deep grooves persist in the dorsolateral epidermis. (G) Ventrolateral view of *prd-Gal4>UAS-DRhoGEF2-RE* embryo at stage 13. DRhoGEF2-overexpressing cells (arrowheads) take on a "grape-like" appearance. (H) Ventrolateral view of *prd-Gal4>UAS-dia*^{CA} embryo at Stage 13. *dia*^{CA}-expressing cells (arrowheads) retain a flattened surface (see also I). (I and J) Lateral epidermis of *prd-Gal4>UAS-dia*^{CA} embryos at stage 13. *dia*^{CA}-expressing epidermal cells exhibit extensive filopodia formation (arrowhead in I). (K) Lateral epidermis of *prd-Gal4>UAS-DRhoGEF2*, *UAS-dia*^{CA} embryo at stage 13. Cells in the *prd*-domain take on a rounded appearance. (L) Ventral furrow in the wild type, stage 5. Membrane blebs indicate apical constriction of cells. (M) Ventral furrow in a DRhoGEF2^{MZ} mutant at stage 5. Instead of constricting, cells form large numbers of filopodia-like protrusions. Bars, 10 μm (A–D, G–I, and K), 5 μm (L and M), and 1 μm (J).

cells were multinucleated (Figure 5G, arrowheads). F-actin was localized at the cortex but often distributed in irregular aggregates (Figure 5, D′ and E′). In addition, the homotypic adherens junction protein E-Cadherin (E-Cad) was lost from the cell cortex in some areas (Figure 5, E–E″), suggesting a role for *dia* in the maintenance of adherens junctions. In the majority of embryos, no segmental grooves were observed and a coordinated sequence of specific cell shape changes at the segment boundaries could not be identified (Figure 5, H and H′). In a small subset of embryos (~25%) grooves formed despite the abnormal shape of cells, which is likely due to partial rescue by paternally contributed *dia* (Figure 5, I and I′). We conclude from these observations that *dia* is required for segmental groove formation.

To investigate the function of Dia in groove founder cells and to compare the specific role of Dia to DRhoGEF2, we expressed constitutive active *dia*^{CA} (Somogyi and Rørth, 2004) in the embryonic epidermis. Expression of *dia*^{CA} by using *prd-Gal4* resulted in premature formation of segmental grooves that were significantly deeper than in the wild type (Figure 6A), and they persisted at stages when wild-type grooves had regressed (Figure 4D). Expression of *dia*^{CA} by using *wg-Gal4* was able to induce apical constriction in cells that do not constrict in the wild type (Figure 6B, arrowheads, and C–F′), showing that similar to DRhoGEF2, the ability of *dia*^{CA} to induce cell shape changes is not restricted to cells in the groove.

The morphology of *dia*^{CA}-expressing grooves was significantly different from *DRhoGEF2*-overexpressing grooves (Figure 6A), indicating that Dia activation promotes a different type of cell shape change. In contrast to *DRhoGEF2*-overexpressing cells, *dia*^{CA}-expressing cells did not round up, but they retained a columnar shape (Figures 4H, arrowheads; and 6H). Similar to DRhoGEF2-overexpressing cells, *dia*^{CA}-expressing cells at the center of the groove did not elongate their apical-basal axis. To investigate whether dia stabilizes cell junctions we stained *dia*^{CA}-expressing embryos for E-Cad and the β-catenin homologue Armadillo (Arm). We found that E-Cad and Arm accumulated at the apical membrane of *dia*^{CA}-expressing cells (Figure 6, C–F). No changes in E-Cad or Arm were observed upon *DRhoGEF2*-overexpression. *dia*^{CA}-expression, also caused accumulation of F-actin that was strongest in apicolateral membrane domains (Figure 6H, arrowhead) and colocalized with E-Cad and Arm at AJs (Figure 6, D, D′, F, and F′).

Dia has previously been implicated in maintenance of myosin II at contractile acto-myosin filaments (Dean *et al*., 2005). To investigate whether Dia affects myosin II in the embryonic epidermis, we expressed *dia*^{CA} by using *wg-Gal4* and we found that myosin II levels were increased at the membrane of *dia*^{CA}-expressing cells (Figure 6, G and G′, arrowheads).

Similar to previous reports *dia*^{CA}-expression induced extensive filopodia formation at the surface of cells (Figures 4, I and J, arrowhead; and 6, J and J′, arrowhead). In the large

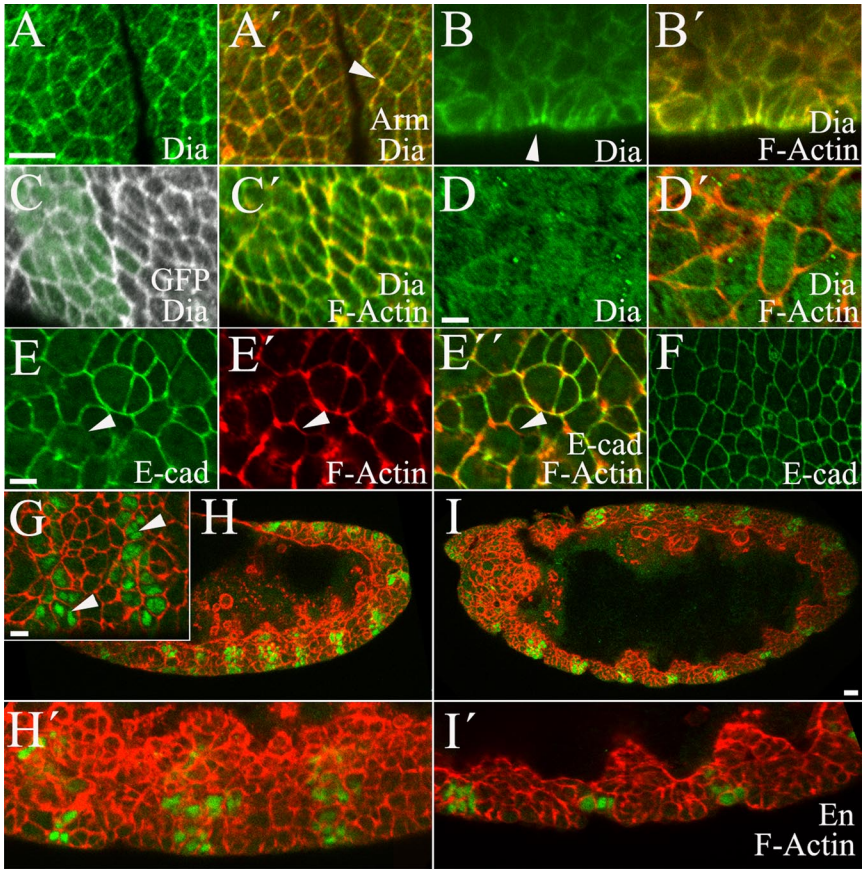

Figure 5. Dia is required for segmental groove morphogenesis. (A–A′) Wild-type, stage 13. Dia, and Arm colocalize at cell vertices (arrowhead). (B–B′) Wild-type, stage 12. Dia and F-actin accumulate apicolaterally in constricting cells (arrowhead). (C and C′) Wild-type, stage 12. Dia (green in C′) and F-actin (red in C′) accumulate at the posterior boundary of groove founder cells (marked by *en-GFP* in green). Dia and F-actin colocalize at cell vertices. (D and D′) Stage 12: levels of *dia* protein are strongly reduced in maternal and zygotic dia^5 mutants. (E and E″) Stage 12: maternal and zygotic dia^5 mutant. E-Cad is lost from the cell cortex in some areas, and F-actin is irregularly distributed in aggregates (arrowheads in E and E′). (F) Stage 12: wild-type embryo stained for E-Cad in green. (G) Stage 12: maternal and zygotic dia^5 mutant. Because of failure of cytokinesis epidermal cells are frequently multinucleated (arrowheads). (H and H′) Stage 13: dia^5 maternal and zygotic mutant. En (green) is distributed in segmentally repeated bands of cells. Segmental grooves do not form. (I and I′) Stage 12: maternally mutant, dia^5 heterozygous embryo. Segmental grooves form at the posterior margin of *en* domains. Bars, 5 μm (A–G) and 20 μm (H and I).

amnioserosa cells, Dia was localized at the apical tip of filopodia (Figure 6I, arrowhead) where it has also been described in other systems (Schirenbeck *et al*., 2005; Williams *et al*., 2007). Filopodia can also be induced by inactivation of *DRok* downstream of Rho^{V14} (Williams *et al*., 2007) consistent with the possibility that a balance of activity between the Dia and Rho-kinase pathways may regulate filopodia formation. We found that DRhoGEF2 mutant cells in the invaginating ventral furrow, and to a lesser degree also cells in the epidermis (data not shown), form large numbers of filopodia-like protrusions (Figure 4M) consistent with the view that DRhoGEF2 may inhibit filopodia, whereas Dia may promote their formation. To test this hypothesis, we coexpressed *DRhoGEF2* and dia^{CA} using *prd-Gal4*. Interestingly, this resulted in the formation of extremely deep segmental grooves (Figure 6, K and K′), indicating an additive effect of Dia and DRhoGEF2. Cells took on a rounded shape characteristic for DRhoGEF2 overexpression (Figure 4K) and at the same time they accumulated high levels of F-actin that was uniformly distributed at the cell cortex (compare Figure 6, J and J′ with L and L′). Formation of filopodia was not observed. Together, our data suggest that activation of DRhoGEF2 or Dia results in distinctly different reorganizations of the actin-based cytoskeleton.

DISCUSSION

Forces Controlling Segmental Groove Morphogenesis

Our analysis of segmental groove morphogenesis revealed that during invagination groove founder cells undergo two distinct phases of shape change. Initial apical constriction is associated with accumulation of F-Actin, DRhoGEF2 and Dia at the site of groove initiation. Together with the loss of apical constriction in *DRhoGEF2* or *dia* mutants and the observation that *DRhoGEF2* or dia^{CA}-overexpressing grooves form earlier, are deeper, and persist longer than wild-type grooves, this indicates that contractile forces in epidermal cells provide a driving force for groove morphogenesis.

Subsequent to apical constriction, groove founder cells and their immediate neighbors elongate their apical-basal axis and extend their basal sides inward. Apical-basal elongation does not occur when *DRhoGEF2* or dia^{CA} are overexpressed, suggesting that activation of these factors may counteract this specific change in cell shape. Inward movement of the epithelial baseline is not affected, and it occurs to a similar depth as in wild type, suggesting that it is not resulting from a pushing force generated by cell elongation. Expression of *DRhoGEF2* in underlying mesodermal cells does not affect groove formation. Thus, cell autonomous bending of the epithelium rather than a pulling force originating in underlying tissues may be the main contributor to groove invagination.

It has been suggested that germband retraction that occurs in parallel to segmental groove formation may contribute to the forces that cause groove founder cells to invaginate (Larsen *et al*., 2003). In DRhoGEF2 mutants, the posterior midgut primordium remains at the posterior pole due to failure of the endoderm to invaginate (Barrett *et al*., 1997; Häcker and Perrimon, 1998). Consequently, the germband is thrown into deep folds that illustrate the pressure exerted on the epithelial sheet. Yet, segmental grooves do not form in these embryos. Furthermore, local expression

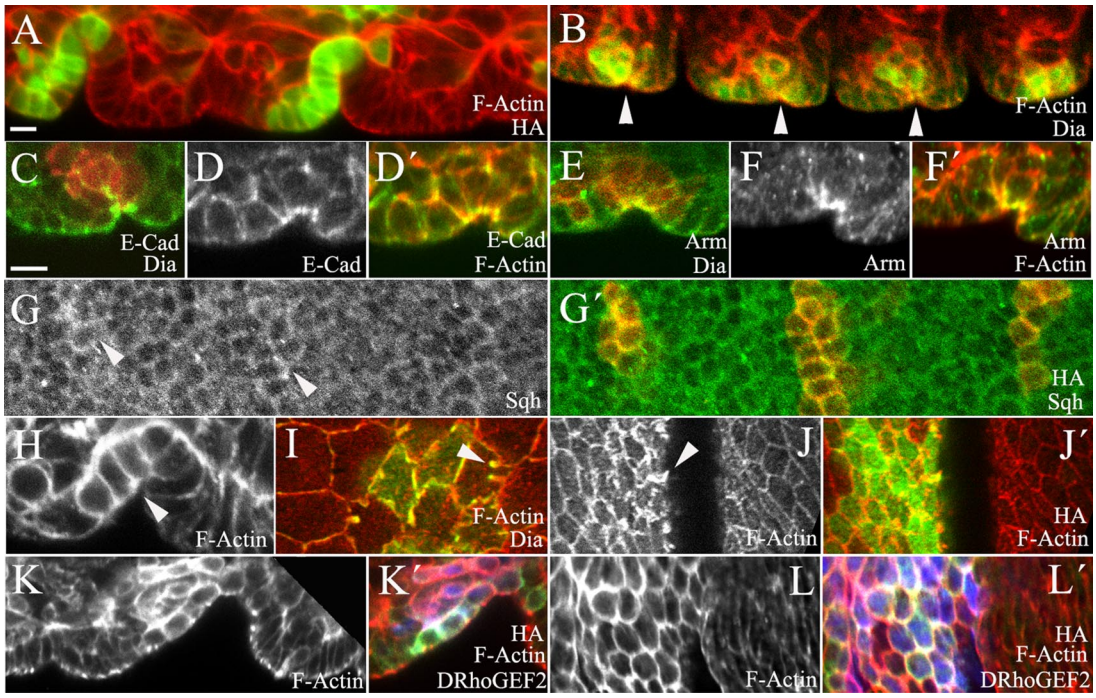

Figure 6. Dia regulates cell shape and adhesion in the epidermis. (A) Late-stage 12: *prd-Gal4>UAS-dia^{CA}*. *dia^{CA}*-expression causes deepening of segmental grooves. Groove morphology is significantly different from *DRhoGEF2*-overexpressing grooves. Cells do not elongate their apical-basal axis. (B–G′) Stage 14: *wg-Gal4>UAS-dia^{CA}*. (B) *dia^{CA}* expression induces ectopic furrowing (arrowheads). (C–F) *dia^{CA}* expression (red in C and E) results in accumulation of E-Cad (green in C and D′), Arm (green in E and F′), and F-actin (red) at AJs. (G and G′) *dia^{CA}*-expressing cells enrich cortical myosin II (arrowheads in G, Sqh in green, HA in red in G′). (H) Stage 12: *en-Gal4>UAS-dia^{CA}*. Cells expressing *dia^{CA}* retain columnar shape. Groove founder cells do not elongate their apical-basal axis. Strong F-actin accumulation is detected apicolaterally (arrowhead) where AJs reside. (I–J′) *prd-Gal4>UAS-dia^{CA}*. (I) Stage 14: Dia (green, F-actin in red) localizes to the apical tips of filopodia (arrowhead) in *dia^{CA}*-expressing amnioserosa cells. (J and J′) Stage 14: *dia^{CA}*-expressing cells (green) in the epidermis accumulate F-actin (red) and form filopodia (arrowhead). (K–L′) Stage 14: *prd-Gal4>UAS-DRhoGEF2>UAS-dia^{CA}* embryos stained for F-actin (red), DRhoGEF2 (green), and HA (blue). (K and K′) Grooves are extremely deep. (L and L′). Cells take on a rounded shape, and they accumulate F-actin uniformly at the cell cortex. Filopodia are not seen. Bars, 5 μm.

of *DRhoGEF2* causes the formation of deep grooves in expressing cells but not in neighboring tissues. These observations do not exclude that global tissue rearrangements support segmental groove invagination, but they suggest that the major contributing force to groove formation is due to autonomous action of epidermal cells.

At the onset of stage 13 when germband retraction has completed, DRhoGEF2, F-actin, and myosin II accumulate apically in four to five cells posterior to the grooves. These cells subsequently constrict apically, which may bend the epithelium in an outward direction. Thus, groove regression may be actively driven by actomyosin-based constriction of epidermal cells. This is supported by previous findings showing that in *zipper* mutants, which partially lack nonmuscle myosin, grooves occasionally do not regress (Larsen *et al*., 2003).

Groove regression coincides with the formation of denticles in ventral epidermal cells posterior to the groove (Price *et al*., 2006; Walters *et al*., 2006). Denticle forming cells undergo cell shape changes that are followed by polarized accumulation of F-actin, myosin II, and Dia into condensations at the posterior cell boundary. Our analysis showed that DRhoGEF2 in contrast to Dia and myosin II, does not polarize to the F-Actin condensations and *DRhoGEF2* mutants are able to form denticles (Padash Barmchi *et al*., 2005). DRhoGEF2 is therefore unlikely to play a role in denticle formation. We propose that the narrowing of cells along the anterior-posterior axis that occurs throughout the ventral and lateral epidermis before hair formation may be due to DRhoGEF2-controlled cell contraction and may contribute to the regression of segmental grooves.

In conclusion, our data suggest that groove invagination and regression are active processes driven by acto-myosin-based contractile filaments in epidermal cells that are regulated by DRhoGEF2 and Dia in a concentration-dependent manner.

DRhoGEF2 Effector Pathways in Segmental Groove Morphogenesis

The small GTPase Rho1 regulates acto-myosin constriction through a pathway including Rho-kinase (Winter *et al*., 2001), the regulatory subunit of myosin light chain phosphatase (Mizuno *et al*., 2002; Tan *et al*., 2003) and myosin II. DRhoGEF2 regulates cell contraction in different developmental contexts and DRhoGEF2-mediated recruitment of myosin II to the apical cortex and cell contraction require Rho-kinase (Rogers *et al*., 2004; Dawes-Hoang *et al*., 2005), suggesting that DRhoGEF2 controls acto-myosin contraction through the Rho-kinase pathway.

Rho1 also regulates F-actin nucleation and polymerization through a pathway including Dia and the actin-binding protein Profilin (Wallar and Alberts, 2003). In cell culture systems, mammalian Dia1 has been shown to act as a positive feedback regulator of Rho-activity through direct interaction with the C terminus of the mammalian DRhoGEF2 homologue LARG (Kitzing *et al*., 2007). However, it is not clear whether Dia can act as an effector of DRhoGEF2 during morphogenesis in vivo. *Drosophila* Dia is required in pro-

cesses such as metaphase furrow invagination, pole cell formation and cellularization (Afshar *et al*., 2000) that are also defective in DRhoGEF2 mutants consistent with the idea that both proteins may function in concert.

Our data suggest that DRhoGEF2 and Dia are required for segmental groove morphogenesis. Interestingly, morphologic changes elicited by Dia^{CA} at the epithelial and cellular level were distinct from those observed with DRhoGEF2. Whereas DRhoGEF2-overexpression caused cells to contract and take on a rounded shape, thereby reducing contact with each other, dia^{CA}-expressing cells remained tightly packed and columnar. In addition, cells showed increased levels of the junctional proteins β-catenin and E-cadherin, suggesting a strengthening of cell–cell contacts. Consistent with this, mammalian Dia1 has been implicated in maintenance and stabilization of adherens junctions. In contrast, Rho-kinase-mediated generation of contractile force leads to the physical disruption of cell–cell contacts and cell rounding reminiscent of DRhoGEF2 overexpression (Sahai and Marshall, 2002). Another distinguishing feature between DRhoGEF2 and *dia* is the accumulation of F-actin in response to dia^{CA} expression that is consistent with the role of Dia in nucleation and elongation of F-actin filaments. F-actin accumulation was not observed in response to *DRhoGEF2*-overexpression consistent with the view that DRhoGEF2 regulates F-actin contraction but not polymerization. We find that DRhoGEF2 and Dia have qualitatively different effects on F-actin remodeling, supporting the view that they may be connected to the actin cytoskeleton by distinct Rho-effector pathways. Despite the differences, overexpression of either *DRhoGEF2* or dia^{CA} in some instances seems to result in increased contractility. In DRhoGEF2, this is likely due to direct regulation of acto-myosin contractility through the Rho-kinase pathway. In Dia^{CA}, the increased levels of actin elicited by Dia activation might indirectly promote the formation of acto-myosin filaments. Alternatively, Dia^{CA} could affect contractility through its effect on myosin.

Expression of either dia^{CA} or *DRhoGEF2* led to increased myosin II levels. In Schneider cells, DRok activity and myosin phosphorylation were found to be required for myosin II recruitment to contractile acto-myosin fibers, whereas Dia was required for maintenance of myosin at contractile rings (Dean *et al*., 2005). Similar to Rho-kinase, DRhoGEF2 is required for apical recruitment of myosin II in the ventral furrow during gastrulation (Dawes-Hoang *et al*., 2005). A role of Dia in this process has not been reported but it is possible that DRhoGEF2 and Dia may regulate different aspects of myosin function also in the *Drosophila* embryo.

In summary, we find that DRhoGEF2 and Dia are required for segmental groove formation and that they may act in concert to regulate a series of specific cell shape changes that lead to groove invagination. However, clear differences in the response of cells to activation of DRhoGEF2 or Dia at the morphologic and molecular level suggest that both genes may regulate the actin cytoskeleton through distinct effector pathways.

Note added in proof. We state that a role for Dia in gastrulation has not been reported. Since submission of this manuscript Homem and Peifer (2008) have reported such a role and also describe a function for Dia in the regulation of myosin and adherens junctions similar to our data.

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