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DRhoGEF2 regulates actin organization and contractility in the Drosophila blastoderm embryo

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Introduction

Animal development is associated with extensive morphological changes of individual cells and entire tissues. Often, these changes require a dynamic rearrangement of the cortical actomyosin-based cytoskeleton. One example is the early phase of embryogenesis in Drosophila melanogaster. Development of the Drosophila zygote begins with 13 synchronous nuclear division cycles that create a syncytial embryo containing ~6,000 nuclei. During mitotic cycles, 10–13 shallow metaphase furrows form transiently between neighboring nuclei at the cell cortex, thus preventing mitotic spindles from colliding. Formation of the furrows is accompanied by relocalization of actin from apical caps into actin rings located at the base of the furrows. Blastoderm cellularization occurs during interphase of cycle 14 when simultaneous cytokinesis transforms the monolayer of syncytial nuclei into a columnar epithelium. This process can be subdivided into a slow and a fast phase, and both phases require distinct cytoskeletal rearrangements. During the early slow phase, cell membranes begin to invaginate radially between the nuclei and form stable infoldings called the furrow canal. The leading edge of the furrow canal is rich in actin and myosin, which form a hexagonal array surrounding the nuclei. As the actomyosin network moves inward, the nuclei, which are spherical at the onset of cellularization, begin to elongate. When the cellularization front has reached the base of the nuclei, the rate of membrane invagination increases by twofold. During this fast phase, actomyosin rings in the furrow canal constrict to close the blastoderm cells basally (for review see Mazumdar and Mazumdar, 2002).

Although some of the genes involved in cytoskeletal rearrangement during cellularization, such as the small GTPase Rho1 (Crawford et al., 1998), the cytoskeletal regulator diaphanous (dia; Afshar et al., 2000) and the zygotic genes nullo, serendipity-α, and bottleneck (bnk; Schejter and Wieschaus, 1993; Postner and Wieschaus, 1994) have been identified, the genetic circuitry regulating the actomyosin contractile apparatus is not well understood.

The small GTPase Rho1 has been identified as a powerful regulator of cytoskeletal reorganization in many contexts. In Drosophila, Rho1 has been demonstrated to play an essential role during oogenesis (Magie et al., 1999), cellularization (Crawford et al., 1998), gastrulation (Barrett et al., 1997; Häcker and Perrimon, 1998), segmentation, dorsal closure (Magie et al., 1999), planar polarity determination (Strutt et al., 1997) and cytokinesis (Prokopenko et al., 1999). In many of these processes, Rho1 controls the spatial and temporal coordination of different aspects of cytoskeletal function such as actin polymerization or contraction of actomyosin fibers in parallel, implying a complex level of regulation of Rho1 activity. Like...
other members of the small GTPase family, Rho1 acts as a molecular switch that is inactive when GDP is bound and activated upon exchange of GDP for GTP by GTP exchange factors (for review see Settleman, 2001).

During cytokinesis, which is mechanistically related to blastoderm cellularization, a linear pathway that includes Dia—the formin homology protein—and profilin—an actin-binding protein that regulates actin polymerization—has been proposed to link RhO1 to the actin cytoskeleton. The RhOGEPF pebble has been shown to activate RhO1 in this context. Pebble is localized to contractile actin rings during cytokinesis in a cell cycle–regulated fashion. The function of pebble is considered essential for the assembly of actin rings during cytokinesis in all cells during Drosophila embryogenesis subsequent to cell cycle 14 (Prokopenko et al., 1999). Recently, pebble has also been shown to regulate the lateral migration of mesodermal cells (Schumacher et al., 2004; Smallhorn et al., 2004). Interestingly, pebble is not required during syncytial nuclear divisions or during blastoderm cellularization, suggesting that a different RhOGEPF may activate RhO1 during this phase of development (Lehner, 1992).

A likely candidate is DRhoGEF2, which is maternally contributed to the embryo and ubiquitously expressed throughout embryogenesis. DRhoGEF2 has previously been shown to regulate the apical constriction of cells during invagination of the mesodermal and endodermal germ layers immediately after cellularization (Barrett et al., 1997; Häcker and Perrimon, 1998). We have used α-DRhoGEF2 antiserum to investigate the role of DRhoGEF2 during early embryogenesis in more detail. Here, we show that DRhoGEF2 colocalizes with actin, myosin II, RhO1, Dia, and Bnk in the furrow canal during cellularization and is required for the recruitment of RhO1 to the cellularization front. DRhoGEF2 regulates actin localization and actin ring constriction during pole cell formation, metaphase furrow formation, and cellularization. Based on our results, we propose that DRhoGEF2 regulates a specific aspect of RhO1-mediated cytoskeletal reorganization throughout Drosophila morphogenesis.

## Results

### DRhoGEF2 is apically enriched in epithelia throughout embryogenesis

DRhoGEF2 transcripts are maternally contributed to the embryo and ubiquitously expressed throughout embryogenesis. However, we observed DRhoGEF2-dependent cytoskeletal reorganization in a tissue-specific fashion during development and suggest that DRhoGEF2 activity may be regulated at the protein level. To investigate the subcellular localization of DRhoGEF2, we stained wild-type embryos with α-DRhoGEF2 antibodies (Rogers et al., 2004). DRhoGEF2 was detected predominantly in epithelia throughout embryogenesis and was enriched at the apical end of cells. In agreement with its previously reported role during gastrulation, DRhoGEF2 levels were elevated at the apical and apico-lateral membrane of cells in the ventral furrow (Fig. 1 A), in the posterior midgut primordium (Fig. 1 B), and in the cephalic furrow (Fig. 1 C). After germ band elongation, DRhoGEF2 was detectable at the apical end of cells in the epidermis (Fig. 1 D). After germ band retraction, DRhoGEF2 levels were elevated in a periodically repeated pattern in the ventral epidermal cells of thoracic and abdominal segments (Fig. 1 E). In the central nervous system, DRhoGEF2 was concentrated in longitudinal and commissural axon fascicles (Fig. 1, E and F). In the lateral epidermis, DRhoGEF2 was highly concentrated at the apical cortex of leading edge cells. Anterior is to the left. B–E show lateral views; A and F show ventral views; and G shows dorsal view. Bars: [A–C] 10 μm; [D–G] 50 μm.

### Figure 1. Expression of DRhoGEF2 during embryonic development. (A–C) DRhoGEF2 protein accumulates apically in cells of the invaginating ventral furrow (A and C, arrows), in cells of the posterior midgut primordium (B, arrow), and in cells in the cephalic fold (C, arrowhead). (D) At late stage 11, DRhoGEF2 is apically localized in all epidermal cells. (E) At late stage 14, DRhoGEF2 is enriched in a periodic pattern in the epidermis (arrows) and in commissural axon tracts (arrowhead). (F) DRhoGEF2 is enriched in longitudinal and commissural axon tracts in the central nervous system. (G) During dorsal closure of the epidermis, DRhoGEF2 is highly enriched at the apical cortex of leading edge cells. Anterior is to the left. B–E show lateral views; A and F show ventral views; and G shows dorsal view. Bars: [A–C] 10 μm; [D–G] 50 μm.

### DRhoGEF2 is localized in actin- and myosin-rich regions during cellularization

During the interphase of syncytial nuclear cycles, DRhoGEF2 was found concentrated in the apical actin caps (Fig. 2 A). At prophase, DRhoGEF2 relocated to the metaphase furrows (Fig. 2 B), where its levels were highest at the center of the broader actin domain (Fig. 2 B, arrow). At the onset of cellularization, the majority of DRhoGEF2
redistributed to the base of the furrow canal (Fig. 2 C). Apical levels were low at this time, but increased again gradually during the slow phase (Fig. 2 D), suggesting that DRhoGEF2 might play a role in the retraction of apical microvilli that occurs during cellularization (Grevengoed et al., 2003). Double labeling experiments showed that DRhoGEF2 colocalized with actin and myosin II at the base of the furrow canal (Fig. 2 and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200407124/DC1). Moderate levels of DRhoGEF2 also remained associated with lateral membranes (Fig. 2, D and E). During basal closure of blastoderm cells, DRhoGEF2 was detectable in a thin stripe at the basal-most boundary of the actin and myosin II domains (Fig. 2 E and Fig. S1 C). The localization of DRhoGEF2 at sites of actin rings suggests that it might play a role in the regulation of actomyosin-based forces during cellularization.

It is noteworthy that DRhoGEF2 was also seen in large particles that are most abundant during the early phase of membrane invagination (Fig. 2, A–C). Similar particles that are transported to the cellularization front in a microtubule-dependent fashion have been reported for myosin II (Royou et al., 2004). Although we were unable to detect myosin II particles, it is possible that the DRhoGEF2 particles might have a similar function in transport of DRhoGEF2 to the apical cortex.

**DRhoGEF2 regulates actomyosin contractility during early embryogenesis**

It has previously been shown that DRhoGEF2 is required for apical constriction of cells in the mesodermal and endodermal primordia during gastrulation (Barrett et al., 1997; Häcker and Perrimon, 1998). DRhoGEF2 protein distribution suggests that DRhoGEF2 may also have an earlier function. To investigate the role of DRhoGEF2 during the syncytial phase of embryogenesis and cellularization, we generated germline clones from a DRhoGEF2 null allele (embryos derived from DRhoGEF2 mutant germline clones are hereafter referred to as DRhoGEF2 mutants; see Materials and methods).

In wild-type embryos, actin cycles between apical actin caps and metaphase furrows during nuclear divisions 10–13 (Fig. 3, A and A’). At the onset of cellularization, actin relocates from the actin caps to the furrow canal where it forms a network of interlinked actin hexagons that surround the nuclei (Fig. 3, C and C’). In the course of the slow phase of membrane invagination, apical actin is almost completely redistributed to the base of the furrow canal (Fig. 3 E). When the cellularization front reaches the base of the nuclei, the actin hexagons detach from each other and begin to contract, thereby expanding the furrow canal (Fig. 3 E’). Contraction of actin rings eventually leads to basal closure of the newly formed blastoderm cells (Fig. 3, G and G’).
In DRhoGEF2 mutants, the depth of metaphase furrows is more variable than in the wild type, and actin is more unevenly distributed (Fig. 3, B and B'). In some areas, metaphase furrows fail to invaginate or break down; this failure can cause adjacent mitotic spindles to collide (Fig. 3 B', arrow) and leads to the elimination of nuclei from the cortex, which results in empty "pseudocells" (Fig. 3 B' and Video 1 [available at http://www.jcb.org/cgi/content/full/jcb.200407124/DC1]; Grevengoed et al., 2003).

At the onset of cellularization, less actin is localized to the furrow canal and apical actin levels remain higher in DRhoGEF2 mutants (Fig. 3 D). Actin rings appear rounded instead of hexagonal, as if they are not under tension. In the wild type, actin hexagons tightly surround the nuclei and slightly squeeze them. In contrast, nuclei in DRhoGEF2 mutants are wider and abnormal, and multinucleated actin rings are frequently observed (Fig. 3 D'). Because abnormal nuclei are removed from the cell surface, we believe that they arise during cellularization rather than being the results of earlier defects during nuclear divisions. When the cellularization front reaches the base of the nuclei, actin rings stay in proximity to each other, and no significant constriction is observed (Fig. 3, F and F'). During the subsequent fast phase, constriction is irregular, and many multinucleated actin rings remain very large (Fig. 3, H and H'). Significant amounts of actin fail to localize to the cellularization front and remain apical (Fig. 3, F and H). We conclude from these results that DRhoGEF2 activity is required for the organization and stabilization of actin rings at invaginating furrows and for the regulation of contractile actomyosin forces during cellularization.

The generation of contractile force requires actin to associate with myosin II, which is encoded by the spaghetti squash (sqh) gene. To assess whether the defects in actomyosin contraction may be caused by mislocalization of myosin II, we expressed an sqh-GFP fusion protein (Ryou et al., 2004) in DRhoGEF2 mutants, but no significant changes in localization or levels of myosin II were observed (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200407124/DC1). Next, we followed the time course of cellularization by time-lapse confocal microscopy (Video 2). Interestingly, a decrease in the rate of membrane invagination was not observed in DRhoGEF2 mutants. Often, it seemed that the rate was slightly increased instead; this observation suggests that actomyosin contractility might not play a role or play only a very minor role in membrane invagination. In addition, these experiments revealed that the radial movement of nuclei was irregular in DRhoGEF2 mutants. In the wild type, nuclei move inward in parallel to each other during the slow phase. In DRhoGEF2 mutants, coordination of this movement is disrupted and some nuclei remain apical, suggesting that actomyosin-based forces may play a role in nuclear alignment.

**DRhoGEF2 is required for Rho1 localization**

DRhoGEF2 has previously been implicated in the regulation of Rho1 activity (Barrett et al., 1997; Häcker and Perrimon, 1998). To investigate whether Rho1 may be a target for DRhoGEF2 during cellularization, we determined the localization of Rho1 with respect to DRhoGEF2 and found that Rho1 colocalizes precisely with DRhoGEF2 at the base of the furrow canal (Fig. 4, A–C). To define the domain of DRhoGEF2 and Rho1 localization more precisely, we double-labeled embryos with β-Heavy spectrin and Rho1. It has been shown that β-Heavy spectrin is present in the furrow canal in a domain apically adjacent to myosin II (Thomas and Kiehart, 1994). We found that Rho1 and, by inference, DRhoGEF2 are localized basal to β-Heavy spectrin at the basal-most boundary of the myosin II domain (Fig. 4, D and E).
Rho1 is essential for cytoskeletal regulation during oogenesis in Drosophila, and female germline clones mutant for Rho1 null alleles do not survive (Magie et al., 1999), precluding a genetic analysis of Rho1 function in the embryo. To overcome this problem, we have generated a piggyBac insertion in the 5′-untranslated region of Rho1. Embryos, derived from germline clones mutant for this allele, Rho1L3, develop with variable defects that range from failure to establish organized actin structures in the most severe cases (Fig. 5 A and Fig. S3 A [available at http://www.jcb.org/cgi/content/full/jcb.200407124/DC1]) to irregular and often only partial membrane invagination in the least severe cases (Fig. 5 B and Fig. S3 B). Although low levels of Rho1 must be present in these embryos, the Rho1 that localized to the furrow canal was consistently below the detection threshold of α-Rho1 antibodies (Fig. 5 B). If Rho1 were required to recruit DRhoGEF2, one might expect that this reduction in Rho1 levels would result in a reduction of DRhoGEF2 at the furrow canal. However, this was not observed (Fig. 5 B). Although we cannot exclude that the reduced amount of Rho1 might be able to recruit similar levels of DRhoGEF2 to the furrow canal as in the wild type, we believe it is more likely that DRhoGEF2 is localized independent of Rho1. Conversely, when DRhoGEF2 mutants were stained with α-Rho1 antibodies, localization of Rho1 was disrupted (Fig. 5 C), suggesting that DRhoGEF2 may recruit Rho1 to the furrow canal.

It has been proposed that the formin homology protein diaphanous (Dia) is a Rho1 effector during the assembly of actin rings. Dia is required for the localization of several actin-binding proteins such as anillin (Field and Alberts, 1995) and peanut (Pnut; Cooper and Kiehart, 1996) at the furrow canal (Afshar et al., 2000). To determine whether Dia may be involved in DRhoGEF2 localization, we generated germline clones of the null allele dia5. Although maternally mutant dia5 embryos have severe cellularization defects, DRhoGEF2 concentration at the furrow canal was unaffected (Fig. 5 D). Conversely, localization of Dia (Fig. 5 C) and the Drosophila septin family protein Pnut (not depicted) was unaffected in DRhoGEF2 mutants. We conclude that DRhoGEF2 may be localized to the cellularization front independently of Dia and Rho1.

DRhoGEF2 acts in concert with bnk during cellularization

The actin-binding protein Bnk plays an important role during cellularization (Schejter and Wieschaus, 1993). It has been pro-

Figure 4. Localization of DRhoGEF2 with respect to Rho1 and β-Heavy spectrin. (A–C) DRhoGEF2 (green) and Rho1 (red) colocalize precisely at the furrow canal during membrane invagination. (D and E) β-Heavy spectrin (green) and Rho1 (red) are localized in adjacent nonoverlapping membrane subdomains [compare arrowheads]. (Right) Merge of the left two panels. All panels have the same magnification. Bar, 10 μm.

Figure 5. Localization of DRhoGEF2 in Rho1L3, dia5 and, bnk mutants. (A) Rho1L3 embryo stained for actin (red) and DRhoGEF2 (green). Example of severe phenotype. Although actin structures are severely disrupted, DRhoGEF2 is concentrated at actin-rich structures [compare arrows]. (B) Rho1L3 embryo stained for Rho1 (red) and DRhoGEF2 (green). Example of less severe phenotype (possibly caused by paternal rescue; Fig. S3). DRhoGEF2 localization is unaffected although Rho1 is reduced to background levels [compare arrows]. (C) DRhoGEF2D205G206 embryo stained for Rho1 (red) and Dia (green). Concentration of Rho1 at the furrow canal is abrogated [compare arrows]. Localization of Dia at the furrow canal is not affected. (D) dia5 embryo stained for actin (red) and DRhoGEF2 (green). DRhoGEF2 concentration at the furrow canal (arrow) is unaffected. (E) Δf[3R]f[e embryo [bnk mutant] stained for actin (red) and DRhoGEF2 (green). Actin rings contract prematurely pinching the nuclei. DRhoGEF2 distribution is not affected by the absence of bnk function. DNA is stained in blue. (Right) Merge of left two panels. Bars, 10 μm.
posed that Bnk links the actomyosin hexagons surrounding individual nuclei and thereby creates a network that is kept under tension by the contractile force of actomyosin fibers (Theurkauf, 1994). Bnk is tightly associated with the lateral edges of adjacent actin hexagons, but is absent from the vertices (Fig. 6 A’). To investigate the functional relationship between bnk and DRhotech2, we stained wild-type and DRhotech2 mutant embryos for Bnk.

bnk is a zygotically expressed gene and bnk protein is first detectable shortly after the onset of zygotic transcription when it is present at the apical surface during interphase and at metaphase furrows during syncytial divisions (Schejter and Wieschaus, 1993). At the onset of cellularization, Bnk redistributes to the base of the furrow canal (Fig. 6, A and A’), where it is detectable throughout the slow phase of membrane invagination (Fig. 6, C and C’). Bnk is rapidly degraded during the fast phase.

In DRhotech2 mutants, a significant change in the overall levels of Bnk was not observed. However, levels of Bnk concentrated at the base of the furrow canal were decreased, as seen from the less intense staining and the more restricted distribution in the grazing confocal sections (Fig. 6, B and B’). At the time when the furrow canal had reached the base of the nuclei, Bnk was still present in the wild type (Fig. 6, C and C’), whereas no localized Bnk was detectable in DRhotech2 mutants, (Fig. 6, D and D’). Conversely, DRhotech2 protein levels or localization was unaffected in bnk mutants (Fig. 5 E). The premature disappearance of Bnk in DRhotech2 mutants is not caused by a decreased rate of membrane invagination (see DRhotech2 regulates actomyosin...).}

This led us to speculate that DRhotech2 might play a role in Bnk stabilization. To test this hypothesis, we generated the DRhotech2 expression construct UAS-DRhotech2-RE. When UAS-DRhotech2-RE was expressed ubiquitously using the maleGal-VP16 driver (Häcker and Perrimon, 1998) in DRhotech2 mutants, the ventral open cuticle defect, was rescued (Fig. 7 B), suggesting that UAS-DRhotech2-RE can provide D Rhotech2 function. When we expressed UAS-DRhotech2-RE using prd-Gal4, Bnk accumulated in a pair rule pattern in the epidermis (Fig. 7 C). This accumulation was not caused by D Rhotech2-mediated transcriptional activation, because no increase in bnk mRNAs was observed (unpublished data). We conclude from these results that D Rhotech2 and Bnk may be recruited to actin-rich regions independent of each other, and that D Rhotech2 may play a role in the stabilization of Bnk.

The development of b nk mutants during syncytial nuclear divisions is indistinguishable from the wild type. However, shortly after the onset of membrane invagination, actin hexagons transform into rings, which constrict prematurely and cause the “bottleneck” phenotype (Fig. 5 E; Schejter and Wieschaus, 1993). To determine whether the premature constriction of actin rings requires D Rhotech2 function, we generated D Rhotech2-bnk double mutants. During syncytial divisions,
the development of these embryos resembled DRhoGEF2 single mutants. Metaphase furrow formation was variable and failed in some areas (Fig. 6, E and E’), which led to the loss of nuclei (Fig. 6 E) and the formation of empty pseudocells (Fig. 6 E’). During cellularization, the actin network of DRhoGEF2-bnk double mutants deteriorated progressively. Breaks in actin rings were observed more frequently than in the single mutants (Fig. 6, F and F’), and toward the end of the slow phase, the actin network was severely fragmented. Only isolated rings were found, but these did not constrict prematurely as observed in bnk single mutants (Fig. 6, G and G’). At later stages, as determined by the increased depth at which actin is found, the actin network was completely disintegrated. The majority of actin did not associate with the invagination front but remained localized at the cortex. We conclude that bnk and DRhoGEF2 may act together to stabilize and organize actin filaments during cellularization and that the premature constriction of actin rings in bnk mutants requires DRhoGEF2 function.

**DRhoGEF2 is required for pole cell formation**

The first individual cells that form in the *Drosophila* embryo are the germline precursors. After cortical migration of syncytial nuclei, actin caps form dome-like protrusions called cytoplasmic buds around interphase nuclei. During nuclear cycle 10, cytoplasmic buds at the posterior pole grow extensively, and constriction of an actin ring at the base of each bud results in the formation of a set of pole cells (Swanson and Poody, 1980; Foe and Alberts, 1983). DRhoGEF2 is detectable at the membrane furrow between polar cytoplasmic buds and at the base of the forming pole cells (Fig. 8 A), in an area where actin rings are located. DRhoGEF2 colocalizes in these areas with the contractile ring marker Pnut (Fig. 8 A; Neufeld and Rubin, 1994), and we suggest that DRhoGEF2 may play a role in pole cell formation.

In DRhoGEF2 mutant embryos, polar cytoplasmic buds form in a manner very similar to that of the wild type. Although actin is more unevenly distributed, a set of pole cells forms at the posterior pole (Fig. 9 A). The concentration of Pnut and myosin II at the base between the forming pole cells suggests that contractile rings have been assembled (Fig. 8, B and C; and Video 1). In the wild type, the pole cells pinch off from the somatic syncytium immediately after nuclear division cycle 10. The pole cells of DRhoGEF2 mutants show severe defects in actin and myosin II organization at this stage and fail to form independent cortical actin structures that are separated from the somatic nuclear layer (Fig. 9 B and Video 1). The defects are particularly severe during the metaphase of nuclear divisions (Fig. 9 C) and lead to the elimination of nuclei from the cortex, which accumulate in the yolk at the posterior pole (Fig. 9 D and Video 1). In contrast to wild-type pole cells, which sit on top of the syncytium, the pole cells of DRhoGEF2 mutants remain embedded in the somatic nuclear layer. Cellularization occurs independent of these defects, and the inward movement of the cellularization front obliterates the majority of pole cells (Fig. 9, E–G). We conclude from these observations that DRhoGEF2 function is required for actin distribution and for reorganization of the actin cytoskeleton of the pole cells during somatic syncytial divisions and cellularization.

**Discussion**

The role of DRhoGEF2 during development

Guanine nucleotide exchange factors regulate the activity of the small GTPase Rho1, which is thought to act as a molecular switch in a broad spectrum of morphogenetic processes that require a complex reorganization of the actin cytoskeleton. However, the manner in which different aspects of Rho1 function are regulated by RhoGEFs is not well understood. We found that DRhoGEF2 protein is broadly distributed in epithelia during oogenesis (unpublished data) and embryonic development and concentrated at the apical surface of cells, suggesting that it may regulate Rho1 throughout morphogenesis. The defects of DRhoGEF2 mutants are less severe than those of Rho1 mutants, suggesting that DRhoGEF2 regulates specific aspects of Rho1 function.

DRhoGEF2 has previously been shown to regulate cell shape changes during gastrulation. A recent paper implicates...
DRhoGEF2 in epithelial folding during imaginal disc development, a process that depends on cell shape changes that are similar to those driving invagination of the germ layers (Nikolaidou and Barrett, 2004). In this paper, we show that DRhoGEF2 regulates cytoskeletal reorganization and function during pole cell formation and blastoderm cellularization. All of these processes require the contraction of actomyosin rings. We propose that DRhoGEF2 regulates Rho1 activity during cell shape changes requiring actomyosin contractility. Our results support the hypothesis that individual RhoGEFs may regulate specific aspects of Rho1 function during development (Van Aelst and D’Souza-Schorey, 1997).

Interestingly, DRhoGEF2 has been found to be nonessential during cytokinesis (Nikolaidou and Barrett, 2004; unpublished data), which also involves the contraction of contractile actin rings. The function of Rho1 during cytokinesis is regulated by the RhoGEF pebble that initiates actin ring assembly (Prokopenko et al., 1999). In pebble mutants, cytokinesis is blocked at mitotic cycle 14 and subsequent mitoses occur without cytokinesis, creating polyploid, multinucleated cells. Although large multinucleated cells are also observed in DRhoGEF2 mutants at the extended germ band stage (not depicted) it is not clear whether these cells are caused by a block in cytokinesis or are caused by earlier defects during cellularization. In contrast to pebble, DRhoGEF2 may not be required for the assembly of actin rings, but may play a nonessential role in the separation of daughter cells. This is reminiscent of our observations during cellularization. Although the function of actin rings appears compromised throughout cellularization, our data suggest that some contractile activity remains that leads to the basal closure of blastoderm cells and is responsible for the cellularized appearance of DRhoGEF2 mutants at the onset of gastrulation.

At the retracted germ band stage, DRhoGEF2 is enriched at the apical cortex of cells in the leading edge of the lateral epidermis, which is consistent with the view that it may regulate Rho1 during dorsal closure. Rho1 function is essential for dorsal closure, and the cuticles of zygotic Rho1 mutants show dorsal holes (Harden et al., 1999; Magie et al., 1999). In DRhoGEF2 mutants, we observed that the lateral epithelial sheets closed the embryo dorsally. This does not exclude the possibility that constriction of actin cables may contribute to dorsal closure and that DRhoGEF2 may play a role in this process. Overall, our data suggest that DRhoGEF2 function may not be essential for the generation of contractile force, but rather regulate the temporal and spatial coordination of actomyosin contractility.

The role of DRhoGEF2 during early embryogenesis

During syncytial nuclear divisions and cellularization, DRhoGEF2 is localized specifically at the invaginating furrows. In DRhoGEF2 mutants, actin is irregularly distributed and metaphase furrow formation is less uniform than in the wild type. The defects in furrow formation lead to mitotic defects and the subsequent elimination of abnormal nuclei from the cortex so that, at the onset of cellularization, ~20% of the nuclei have been lost. These phenotypes are reminiscent of the defects seen in mutants of the nonreceptor tyrosine kinase Abl (Abl; Grevengoed et al., 2003). The abnormalities in actin distribution observed in abl mutants are likely caused by the mislocalization of Dia, which leads to ectopic actin polymerization at the apical end of cells. Changes in Dia distribution were not observed in DRhoGEF2 mutants, suggesting that DRhoGEF2 may regulate actin distribution by a different mechanism (see next section). Perturbations in actin distribution are observed throughout early development in DRhoGEF2.
mutants. During cellularization, significant amounts of actin fail to redistribute to the base of the furrow canal. These observations show that one of the roles of DrhoGef2 is to regulate furrow assembly. The defects in actin distribution also affect the polar cells, which fail to reorganize their cortical actin cytoskeleton and remain embedded in the somatic nuclear layer rather than sitting on top of it. Consequently, they are obliterated during invagination of the cellularization front.

We propose that DrhoGef2 may have a function in the assembly of actin cables by regulating the association of actin with other proteins such as myosin II. The mislocalization of actin observed in DrhoGef2 mutants may be caused by failure of actin to associate with myosin. Interestingly, although myosin II is present at the metaphase furrows, it plays no essential role in their formation (Royou et al., 2004), and this suggests that the function of DrhoGef2 in furrow assembly may be independent of actomyosin contractility.

Our phenotypic analysis suggests that DrhoGef2 regulates actomyosin contractility during cellularization. Previously, the actin-binding protein Bnk has been implicated in the regulation of contractile forces (Schejter and Wieschaus, 1993). In bnk mutants, actin hexagons detach from each other and constrict prematurely. Based on this phenotype, Schejter and Wieschaus (1993) proposed a model and suggested that, during the slow phase, cortical actin hexagons are linked to each other through Bnk, and that actomyosin constriction causes the network to contract as a whole, thereby pulling the membrane front inwards. Once the cellularization front has reached the base of the nuclei and Bnk is degraded, actin hexagons detach from each other and contract as individual rings, thereby closing the blastoderm cells basally. We propose that DrhoGef2-mediated activation of Rho1 may regulate the force that keeps actin hexagons under tension. Bnk counteracts constriction during the slow phase by linking individual actin rings to each other. Degradation of Bnk during the fast phase releases individual actin rings, and the DrhoGef2-mediated contractile force now contributes to basal closure. Therefore, DrhoGef2 and bnk act in concert to coordinately actin ring contraction during cellularization. In DrhoGef2-bnk double mutants, the actin network disintegrates progressively, suggesting that DrhoGef2 and bnk may play an additional role in the assembly or stabilization of actomyosin filaments.

It has been proposed that actin network contraction contributes to the inward movement of the furrow canal. Although our data suggest that network tension is severely reduced in DrhoGef2 mutants, we find that the rate of membrane invagination is unaffected. This is consistent with reports on the role of myosin II during cellularization, suggesting that network tension may not contribute to membrane invagination (Royou et al., 2004). In the wild type, actin rings squeeze the nuclei slightly and push them basal-wards as the actin network moves over them. This may contribute to the parallel alignment of astral microtubules surrounding the nuclei and to nuclear elongation (Bate and Martinez Arias, 1993). In DrhoGef2 mutants, nuclei are wider than in the wild type and irregularly aligned. We propose that network tension may create an ordered hexagonal array of actin rings that contributes to a parallel alignment of nuclei during cellularization. The force moving the actin network inward may be created by plus end–directed tracking of actin on astral microtubules and by membrane insertion as previously suggested (Bate and Martinez Arias, 1993). Our observations suggest that actomyosin contractility plays a role in the spatial coordination of cytoskeletal function during cellularization.

The molecular pathway transducing DrhoGef2 activity during blastoderm cellularization

Two effector pathways have been implicated in the transduction of Rho1 activation to the actin cytoskeleton. During cytokinesis, which is mechanistically related to cellularization, a linear pathway including profilin and Dia have been proposed to link Rho1 to the contractile actomyosin ring (Prokopenko et al., 1999). The maternally supplied Dia plays a role in a spectrum of cytoskeletal functions during early embryogenesis that also require DrhoGef2 function, such as metaphase furrow formation, pole cell formation, and cellularization. Dia is localized at the cellularization front and is necessary for the recruitment of cytoskeletal components such as the actin-binding protein anillin and the septin homologue Pnut. The phenotypes of dia mutants suggest that dia is necessary for the assembly of contractile actin rings at sites of membrane invagination (Afshar et al., 2000).

The similarities between dia and DrhoGef2 mutants might suggest dia as a downstream effector of DrhoGef2. However, the defects of dia mutants are morphologically different from those of DrhoGef2 mutants. In dia mutants, metaphase furrows do not form and contractile rings at the base of polar cytoplasmic buds fail to assemble. During cellularization, actin fails to condense into individual rings, and the network disintegrates during the second phase of cellularization. In DrhoGef2 mutants actin rings form and remain largely intact but fail to constrict. In addition, the temporal and spatial localization of Dia and Pnut to the cellularization front was unaffected in DrhoGef2 mutants and dia was not required for the localization of DrhoGef2. These findings do not exclude that DrhoGef2 activity may in part be mediated by dia, however, they suggest that some dia-dependent aspects of Rho1 function are still active in DrhoGef2 mutants and that another pathway may be involved in transduction of the DrhoGef2 signal.

A well-characterized pathway regulating actomyosin contractility in mammalian cells (Fukata et al., 2001) and in Caenorhabditis elegans (Wissmann et al., 1997) links Rho1 to actin via Rho kinase (Winter et al., 2001), the regulatory subunit of myosin light chain phosphatase (MBS; Mizuno et al., 2002; Tan et al., 2003) and myosin II. Rho kinase-mediated phosphorylation inhibits the activity of MBS and induces a conformational change in myosin II allowing it to form filaments that promote sliding of antiparallel actin filaments. Our data are consistent with a model in which DrhoGef2 regulates the association of actin with myosin II, thereby stabilizing actomyosin cables. We propose that failure to activate the Rho kinase pathway may compromise the recruitment of actin into
contractile cables. This may destabilize actin cables and lead to the mislocalization of actin and to the defects in actomyosin contractility observed in DRhoGEF2 mutants. The Drosophila homologue of Rho kinase, Drok, and myosin II have recently been identified as downstream effectors of DRhoGEF2 during the regulation of actomyosin contractility in Schneider (S2) cells (Rogers et al., 2004). In addition, myosin II is required for basal closure of blastoderm cells (Royou et al., 2004) and the myosin II heavy chain encoded by zipper (zip) interacts genetically with DRhoGEF2 (Halsell et al., 2000). These data support the model that DRhoGEF2 may regulate actomyosin contractility through the Rho kinase pathway. Mutants in Drok and Drosophila myosin light chain phosphatase have been identified, however, their role during early embryogenesis has not been reported. Interestingly, inhibition of Drok activity by injection of the specific Rho kinase inhibitor Y-27632 into embryos before cellularization disrupts the localization of myosin II (Royou et al., 2004). Similar observations have been made in Drok mutant cell clones in imaginal discs (Winter et al., 2001). By contrast, DRhoGEF2 mutants reveal no significant changes in the localization of myosin II during cellularization. It is possible that differences in myosin II localization between DRhoGEF2 and Drok mutants are due to different mechanisms of action at the molecular level. In mammalian cells myosin II phosphorylation is required for the generation of contractile force but not for its localization (Fumoto et al., 2003). Further investigations will be necessary to resolve how the DRhoGEF2 signal is transduced to the cytoskeleton.

The localization of DRhoGEF2 during cellularization

Little is known about the events that regulate the specific subcellular localization and activation of DRhoGEF2. It has recently been shown that DRhoGEF2 particles are transported from the cytoplasm to the cell periphery by tracking microtubule plus ends in Drosophila S2 cells (Rogers et al., 2004). We have observed DRhoGEF2 particles during syncytial development that may be involved in a similar process in the embryo. We speculate that DRhoGEF2 may be delivered to specific membrane subdomains at the cellularization front by microtubules. The G-protein α-subunit encoding gene concertina (cta) has been shown to regulate the dissociation of DRhoGEF2 from microtubules. cta has previously been implicated in the activation of DRhoGEF2 during gastrulation (Barrett et al., 1997), but is not required during cellularization (Parks and Wieschaus, 1991). It has been suggested that the force moving the actin network inward may be generated by plus end-directed crawling of actin on astral microtubules (Bate and Martinez Arias, 1993). We speculate that DRhoGEF2 may regulate actin ring constriction during cellularization while associated with the tip of astral microtubules by recruiting Rho1 to the site of actin rings. DRhoGEF2 is concentrated in actin-rich regions throughout development and the human orthologue of DRhoGEF2, PDZ-RhoGEF, has been shown to bind to actin directly (Banerjee and Wedegaertner, 2004). Although the domain structure of DRhoGEF2 and PDZ-RhoGEF is very similar, the actin-binding region of PDZ-RhoGEF is not conserved in DRhoGEF2. Nevertheless, the localization of DRhoGEF2 is consistent with the view that it may associate with actin, however, further experiments are needed to corroborate this theory.

Materials and methods

Fly strains and genetics

Germline clones of DRhoGEF2(l(2)04249), Drok, DRhoGEF2(l(2)04218), dia, and Rho1 were generated using the autosomal FLP-FRT technique (Chau and Perrimon, 1996). DRhoGEF2(l(2)04249) and DRhoGEF2(l(2)04218) are considered null alleles (Barrett et al., 1997; Häcker and Perrimon, 1998) and showed very similar phenotypes. All data shown regarding DRhoGEF2 mutants were obtained from the analysis of embryos derived from females carrying DRhoGEF2(l(2)04249) germline clones crossed with heterozygous males. Parental rescue was not observed. In DRhoGEF2(l(2)04249) null mutants, no DRhoGEF2 was detectable. In embryos derived from DRhoGEF2(l(2)04218) germline clones wildtype DRhoGEF2 levels and distribution were observed and phenotypes were less severe, suggesting that DRhoGEF2(l(2)04218) is a hypomorphic allele. Rescue of DRhoGEF2(l(2)04249) mutants was performed by crossing DRhoGEF2(l(2)04249)/P{FRT[w[hs]]}3G13 P{w[1-18R]; m4-Gal-VP16/TM3, Sb females carrying germline clones to UAS-DRhoGEF2-RE males. DRhoGEF2(l(2)04249) cnk double mutants were made by crossing DRhoGEF2(l(2)04249) D{3[R]f[1]11; cnk/TM6b, Tb, ca males. Double mutant embryos were identified by α-Bnk antibody staining. DRhoGEF2 mutants expressing sqh-GFP (Royou et al., 2004) were made by crossing DRhoGEF2(l(2)04249)/sqh-GFP to females carrying germline clones with sqh-GFP males. The wild-type controls used were sqh-GFP/TM3, Sb females crossed with sqh-GFP males. Rho1 was made by mobilizing pBac[3xPH3; P{P{neoFRT}[40A, P{FRT[w[h]]}G13 as a target chromosome and the pBac insert in the stock was determined as described previously (Häcker et al., 2003). The Rho1 insertion is in the 5′ untranslated region of Rho1 at nt16014 in GenBank scaffold AE003808.3. The bnl mutant used was D{3[R]f[1]11; bnl/TM6b) (Schejter and Wieschaus, 1993). Gal4 drivers were used m4-Gal-VP16 (gift of D. St. Johnston, University of Cambridge, Cambridge, UK) and pre-Gal4 (Yoffe et al., 1995). UAS-DRhoGEF2-RE was generated by P element–mediated germline transformation of pUAST-DRhoGEF2-RE (performed at the EMBL Drosophila injection service).

Molecular biology

We isolated several partial cDNAs, representing different parts of DRhoGEF2 transcripts, by screening a random primed Drosophila embryonic cDNA library (CLONTECH Laboratories, Inc.). pUAST-DRhoGEF2-RE was generated by joining a partial cDNA encompassing the region from a unique central EcoRI site to the 3′-end of DRhoGEF2 with a cDNA representing the transcript DRhoGEF2-RE (FlyBase) from the same EcoRI site to the 5′-end and cloning of the resulting DNA fragment into the XbaI site of pUAST (Brand and Perrimon, 1993).

Immunohistochemistry

For phalloidin stainings, embryos were dechorionated, fixed by shaking in 4 ml HEM (100 mM Hepes, 20 mM MgSO₄, and 1 mM EGTA), 4% formamide for 20 min, dehydrillized by hand using a needle, washed in PBT (PBS containing 0.1% Tween 20), incubated in rhodamine-conjugated phalloidin (Molecular Probes) for 1 h, washed three times in PBT, and mounted on a microscope slide. To visualize DRhoGEF2, Rho1, Dia, Pnut, β-Heavy spectrin, or Bnk embryos were fixed as just described, dehydrillized by adding 10 ml methanol and stained using rabbit α-DRhoGEF2 (Rogers et al., 2004), mouse α-Rho1 (Magie et al., 2002), mouse α-Pnut (obtained from the Developmental Studies Hybridoma Bank and developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa), rabbit α-Dia (Ashfar et al., 2000) (gift of S. Wasserman, University of California San Diego, CA), rabbit α-β-Heavy spectrin (gift of G. Thomas, Pennsylvania State University, University Park, PA; Thomas and Kiehart, 1994) or rat α-Bnk (Schejter and Wieschaus, 1993) (gift of E. Wieschaus, Princeton University, Princeton, NJ) antibodies, respectively. DNA was stained using TO-PRO (Molecular Probes). Primary antibodies were detected with Cy2-, rhodamine RedX- or FITC-conjugated goat antisera (all obtained from Jackson ImmunoResearch Laboratories). Images were collected on a laser scanning confocal microscope (model TCS SP2, Leica) and imported directly into Adobe Photoshop software or assembled into movies using NIH image, ImageJ 1.62 software.
Online supplemental material

Time-lapse videos supplement Fig. 3 (Videos 1 and 2) and Fig. 8 and Fig. 9 (Videos 1 and 3). Videos play at 10 frames/s. Fig. S1 shows the distribution of DRhoGEF2 and myosin II during cellularization. Fig. S2 shows the distribution of myosin II in DRhoGEF2 mutants. Fig. S3 provides an overview of the phenotype of embryos derived from Rho13 germine clones.

Online supplemental material is available http://www.jcb.org/cgi/content/full/jcb.200407124/DC1.

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