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# *Drosophila* RhoGEF2 Associates with Microtubule Plus Ends in an EB1-Dependent Manner

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## Summary

**Members of the Rho/Rac/Cdc42 superfamily of GTPases [1,2] and their upstream activators, guanine nucleotide exchange factors (GEFs) [3], have emerged as key regulators of actin and microtubule dynamics. In their GTP bound form, these proteins interact with downstream effector molecules that alter actin and microtubule behavior. During *Drosophila* embryogenesis, a Gα subunit (Concertina) and a Rho-type guanine nucleotide exchange factor (DRhoGEF2) have been implicated in the dramatic epithelial-cell shape changes that occur during gastrulation [4–6] and morphogenesis [7]. Using *Drosophila* S2 cells as a model system, we show that DRhoGEF2 induces contractile cell shape changes by stimulating myosin II via the Rho1 pathway. Unexpectedly, we found that DRhoGEF2 travels to the cell cortex on the tips of growing microtubules by interaction with the microtubule plus-end tracking protein EB1. The upstream activator Concertina, in its GTP but not GDP bound form, dissociates DRhoGEF2 from microtubule tips and also causes cellular contraction. We propose that DRhoGEF2 uses microtubule dynamics to search for cortical subdomains of receptor-mediated Gα activation, which in turn causes localized actomyosin contraction associated with morphogenetic movements during development.**

## Results and Discussion

Previously, we characterized the cellular functions of the microtubule plus-end binding protein EB1 in *Drosophila* S2 cells and showed that this protein plays an important role in regulating microtubule dynamics and in the assembly and dynamics of the mitotic spindle [8]. In order to learn more about EB1's functions, we sought to identify EB1 binding partners with affinity purification. We used recombinant *Drosophila* GST-EB1 bound to glutathione Sepharose beads as an affinity chromatography matrix to bind interacting partners from S2 cell extracts. Bound proteins were eluted from the beads and separated by SDS-PAGE (see Figure S1 in the Supplemental Data available with this article online), and excised bands were subjected to tryptic digestion and mass spectrometry fingerprinting. As a negative control, GST alone was attached to beads and used in parallel pull-down experiments with the same extracts. Few proteins (<10) were eluted from the GST column, and these were excluded from analysis.

Twenty "EB1-specific" proteins were identified over the course of five independent pull-down experiments. However, of these, only six candidates were identified in all five trials: CLIP190 (Mr [relative molecular weight] = 190 kDa; the *Drosophila* ortholog of vertebrate CLIP-170, which localizes to the plus ends of microtubules), Orbit/MAST (Mr = 165 kDa; a microtubule plus-end-associated protein that interacts with CLIP-170), nonmuscle myosin II heavy chain (Mr = 230 kDa), the minus-end-directed kinesin, Ncd (Mr = 75 kDa), Shortstop (Mr = 590 kDa; a member of the spectraplakin family of actin/microtubule cross-linking proteins), and DRhoGEF2 (Mr = 280 kDa; a member of the Dbl family of Rho GEFs). In this paper, we focused on DRhoGEF2 for further study; analyses of the other candidate EB1 binding factors will be described elsewhere.

The association of DRhoGEF2 with EB1 in vitro raised the possibility that this protein may localize to the tips of microtubules. To test this idea, we generated polyclonal antibodies against the C-terminal 720 amino acid residues of DRhoGEF2. These antibodies recognized a ~280 kDa polypeptide on immunoblots of S2 cell extracts; this polypeptide was eliminated after DRhoGEF2 RNAi treatment, indicating that the antibodies were reacting with the correct polypeptide (Figure S1B).

By immunofluorescence, anti-DRhoGEF2 antibodies recognized punctate structures distributed throughout the cell (Figure 1A). Superimposed upon this punctate pattern, however, were short (~1 μm) linear tracks that colocalized with the tips of microtubules. Moreover, immunofluorescent staining of DRhoGEF2 in S2 cells expressing low amounts of EB1-EGFP indicated that these two proteins colocalize exactly at microtubule tips (Figure S2). In the perinuclear region of many cells, DRhoGEF2 antibodies also stained larger spots that costained with γ-tubulin, a centrosome marker (Figure S3). Depletion of DRhoGEF2 with RNAi eliminated antibody staining of all of these structures in S2 cells (data not shown). Thus, our immunofluorescence experiments reveal that DRhoGEF2 exists in three pools within S2 cells: punctate throughout the cell, at microtubule tips, and on centrosomes.

We next tagged DRhoGEF2 with green fluorescent protein (GFP) to examine its dynamic behavior through time-lapse imaging with a spinning-disk microscope. As predicted from our immunofluorescence data, "comet-like" structures of DRhoGEF2-GFP moved from the cell center toward the periphery in a manner that was very similar to that observed for EB1-GFP (Movies 1 and 2). In many cells, an intense spot of DRhoGEF2-GFP was observed near the perinuclear region. This spot likely

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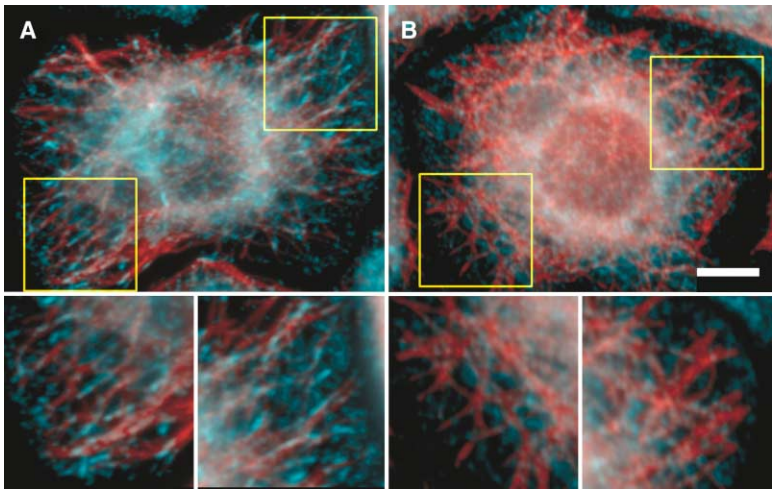


Figure 1. DRhoGEF2 Localizes to Microtubule Tips in an EB1-Dependent Manner

(A) Immunofluorescent localization of DRhoGEF2 (blue) and tubulin (red) in an S2 cell spread upon concanavalin A. DRhoGEF2 antibodies stain punctate cytoplasmic structures and also recognize the tips of microtubules. Boxed regions indicate regions presented at higher magnification below.

(B) Immunofluorescent localization of DRhoGEF2 (blue) and tubulin (red) in a cell depleted of EB1 by a 7 day treatment with dsRNA. DRhoGEF2 is no longer observed at microtubule plus ends. Lower panels show boxed regions at higher magnification. The scale bar represents 5 μm.

corresponded to the centrosome staining because the tips of nucleated microtubules emanated from this point in a radial pattern. Microtubule dynamics are essential for this movement because it could be eliminated with either 10 μM colchicine (data not shown) or 10 μM taxol (Movie 3). Thus, we conclude that DRhoGEF2 associates with the tips of growing microtubules and exhibits plus-end tracking that is qualitatively similar to that described for EB1.

Because DRhoGEF2 was isolated based upon its association, direct or indirect, with EB1, we next depleted EB1 from cells with RNAi and examined whether the association of DRhoGEF2 with microtubule tips was perturbed. In cultures treated with control dsRNA, scoring of fixed cells stained for DRhoGEF2 and microtubules revealed that 94% of the cells (n = 300) had DRhoGEF2 associated with the microtubule tip. In contrast, in S2 cells treated for 7 days with EB1 dsRNA, only 5% of the cells (n = 300) retained DRhoGEF2 at the plus ends (Figure 1B). These results demonstrate that targeting of DRhoGEF2 to growing microtubule plus ends is an EB1-dependent process.

To further understand DRhoGEF2 functions, we examined how overexpression and depletion of the protein affects the morphology of S2 cells. When S2 cells are plated on concanavalin A, they adopt a "fried-egg" appearance with a dome-like central domain defined by the nucleus and perinuclear organelle-rich region and an extended, symmetrical lamella (Figures 2A and 2B; Movie 4). In contrast, we found that overexpressing DRhoGEF2 caused many cells (95%, n = 366) to adopt a smaller, contracted footprint on the substrate and to become significantly taller than control cells. These overexpressing cells formed a skirt of abnormally large membrane ruffles that tapered to the base of a raised, organelle-rich compartment, and the overall morphology resembled a "bonnet" shape (Figures 2C and 2D; Movie 5). This result suggested that DRhoGEF2 could induce contractility, in agreement with the genetic phenotype of DRhoGEF2 mutations. DRhoGEF2 depletion by RNAi did not affect the morphology of S2 cells on con A surfaces (not shown); however, this morphology is the result of lamella extension and not retraction.

Several genetic studies implicate DRhoGEF2 as a positive regulator of Rho1 [5–7]. To test whether Rho activation is involved in generating the unusual phenotype associated with DRhoGEF2 overexpression, we transfected cells with constitutively active Rho1V14 and identified transfected cells with an antibody raised against *Drosophila* Rho1. As predicted, most of the Rho1V14-expressing cells (95%, n = 293) duplicated the morphology produced by DRhoGEF2 overexpression (Figure 2E). In order to next test if inhibition of Rho1 prevented DRhoGEF2-induced shape change, we transfected DRhoGEF2-EGFP into cells that had been treated with Rho RNAi. Depletion of Rho1 by RNAi produced large multinucleate cells that did not contract in response to DRhoGEF2 overexpression (99% of cells, n = 284) (Figure 2F). In contrast, RNAi inhibition of the other six Rho family members did not block DRhoGEF2-induced contraction (data not shown).

Active Rho is known to stimulate nonmuscle myosin II, and Halsell et al. demonstrated a genetic interaction between DRhoGEF2 and myosin II during *Drosophila* morphogenesis [7]. One well-characterized mechanism by which Rho1 activates myosin II is Rho kinase (DROK in *Drosophila*) stimulation, which activates the motor by phosphorylating the myosin light chain and by inactivating myosin light chain phosphatase [9, 10]. In order to determine if DROK is indeed downstream of DRhoGEF2, we depleted DROK with RNAi or inhibited kinase activity with Y-27632, a pharmacological inhibitor, and then examined cell morphology after DRhoGEF2 overexpression. Both treatments significantly reduced the numbers of cells exhibiting the contracted morphology (DROK RNAi to 7% [n = 363] and Y-27632 to 7% [n = 343]; Figure 2G). From these data, we conclude that DRhoGEF2 changes S2 cell morphology through Rho1 and its downstream effector, DROK.

To confirm that myosin II is a downstream effector in the DRhoGEF2 pathway in our system, we compared the behavior of GFP-tagged myosin II in control S2 cells on concanavalin A with that of S2 cells overexpressing DRhoGEF2. To perform this analysis, we generated a stable cell line expressing the myosin II regulatory light chain (RLC), known by *Drosophila* nomenclature as spaghetti squash, under the control of the gene's endogenous promoter. Ectopic expression of RLC-GFP did not

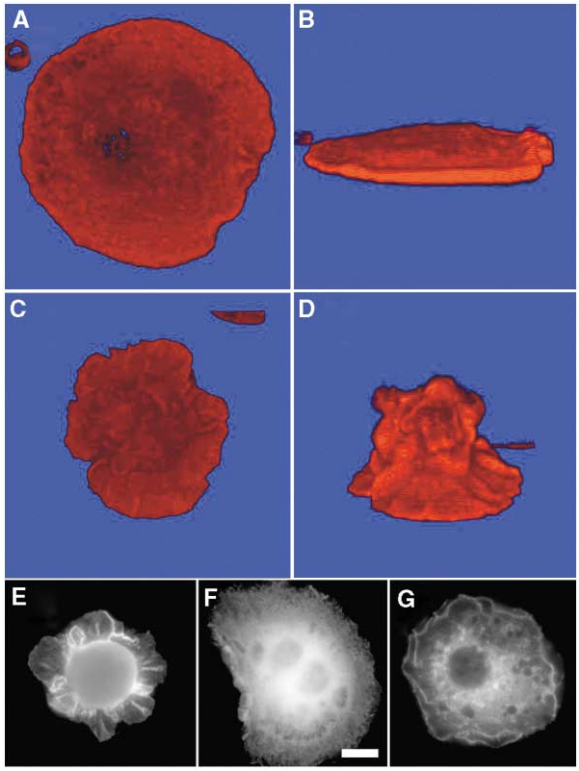


Figure 2. DRhoGEF2-Mediated Cellular Shape Changes Require Rho1

(A and B) Three-dimensional reconstructions of a control S2 cell stained with rhodamine-phalloidin and spread on concanavalin A in the x-y (A) and x-z (B) planes reveal that the cells adopt a discoid shape. Nuclei and organelles reside in the center dome-like region and membrane ruffles are present in the peripheral lamella.

(C and D) In contrast, x-y (C) and x-z (D) projections of a cell overexpressing DRhoGEF2 and identified with our antibodies to the protein show a dramatic change in cell shape. These cells have a smaller footprint on the coverslip, produce massive membrane ruffles that skirt the cell, and push their organelle-rich central cytoplasm axially away from the coverslip.

(E) S2 cells overexpressing constitutively active Rho1V14 and identified by immunofluorescence with an antibody against Rho1 adopt a contracted morphology indistinguishable from that of cells overexpressing DRhoGEF2.

(F) Depletion of Rho1 from S2 cells with RNAi produces large multinucleate cells that do not adopt the contracted morphology upon overexpression of DRhoGEF2-GFP.

(G) Treatment with the Rho kinase inhibitor Y-27632 also prevents shape changes induced by DRhoGEF2-GFP. The scale bar represents 5 μm.

produce observable defects in actin organization or behavior (data not shown and [11]); its distribution exactly coincided with the myosin II distribution determined by immunofluorescence staining of the same cells (data not shown). RLC-GFP typically incorporated into punctae in the cell periphery and into higher-order structures in the central region of the cells (Figure 3A). Time-lapse spinning-disk confocal microscopy revealed that punctae of RLC-GFP formed in the distal cell periphery and then translocated centripetally at a constant rate of 4.0 ± 0.3 μm/min toward the cell center (Movie 6). Such behavior of RLC-GFP is qualitatively very similar to the behavior of fluorescently labeled myosin II in cultured mammalian cells [12, 13].

Upon overexpression of DRhoGEF2, punctae of RLC-GFP were rarely observed. Instead, the majority of RLC-GFP signal was present in circular "purse string" structures surrounding the organelle-dense region at the center of the cell (Figure 3B). Time-lapse observation revealed that peripheral formation of RLC-GFP punctae and retrograde flow were infrequent in DRhoGEF2-overexpressing cells (Movie 7) and that these RLC-GFP-containing purse strings were stable over a span of hours. The location and concentration of the myosin II suggests that actomyosin contraction is responsible for producing the bonnet-shaped appearance of these cells. From these observations, we propose that DRhoGEF2 regulates myosin II dynamics and contractility in S2 cells.

Genetic analyses of epithelial-sheet invagination in the early *Drosophila* embryo suggest that DRhoGEF2 may act downstream of the heterotrimeric Gα protein Concertina (Cta) [4]. To examine directly whether Concertina can activate DRhoGEF2, we transfected cells either with Myc-tagged wild-type Cta or Myc-tagged Cta bearing a constitutively activating point mutation (R277H) that inactivates GTPase activity [14], and we examined the morphology of the transfected cells. Cells expressing Myc-Cta were morphologically indistinguishable from untransfected cells (Figure 3C), and only 3% of cells displayed a mildly contracted phenotype ($n = 392$). In contrast, the majority of cells (90%, $n = 371$) expressing Myc-Cta$^{R277H}$ exhibited the contracted morphology and myosin II purse string reminiscent of DRhoGEF2 overexpression (Figure 3D). Similar results were obtained with three other constitutively activated Concertina constructs (data not shown). However, the shape change was prevented in 88% of these cells ($n = 270$) if they were pretreated for 7 days with dsRNA so that DRhoGEF2 was depleted (Figure 3E). These results suggest that Concertina can act upstream of DRhoGEF2 to regulate S2 cell morphology.

We next wished to determine whether activation of DRhoGEF2 through Concertina affected its association with the microtubule cytoskeleton. Cells expressing Myc-Cta or Myc-Cta$^{R277H}$ were fixed and double stained for the Myc epitope tag and for DRhoGEF2. Overexpression of wild-type Concertina did not affect DRhoGEF2 association with microtubule plus ends or with the centrosome (88% of cells, $n = 125$; Figure 4A). However, constitutively activated Concertina resulted in DRhoGEF2 dissociation from microtubule tips; only 10% ($n = 128$) of the cells showed any colocalization of DRhoGEF2 with microtubule plus ends (Figure 4B). Instead, DRhoGEF2 exhibited a diffuse staining pattern throughout the cell; this pattern likely represents association with the plasma membrane. Targeting of EB1 to the plus ends was not perturbed by Cta$^{R277H}$ (99%, $n = 350$; Figure S4), suggesting that Concertina signaling regulates the interactions between DRhoGEF2 and factors at microtubule tips.

## Conclusions

In our attempt to identify novel cellular factors that interact with EB1, we unexpectedly discovered that DRhoGEF2, a key regulator of morphogenesis in *Drosophila*, associates with the tips of growing microtubules. This

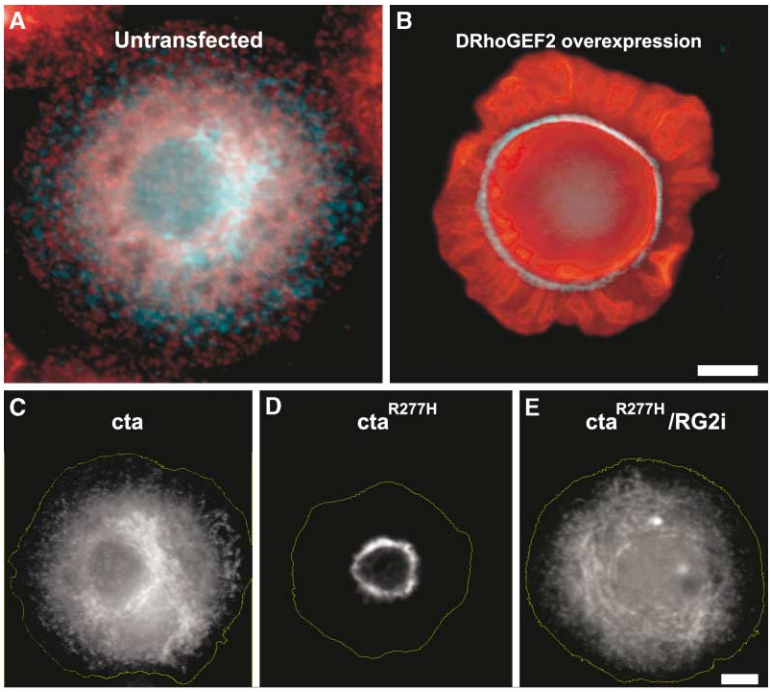


Figure 3. DRhoGEF2 Regulates the Behavior of Nonmuscle Myosin II

(A) An untransfected control S2 cell on concanavalin A. The cell expresses RLC-GFP (blue) and is stained with antibodies to DRhoGEF2 (red). RLC-GFP is present in punctate spots in the cell periphery and circumferential bundles in the cell interior. Note: The fixation technique required to preserve RLC-GFP does not preserve microtubule-tip-associated structures.

(B) An S2 cell both expressing RLC-GFP (blue) and overexpressing DRhoGEF2 (red) and identified by immunofluorescence for the latter. In this cell, RLC-GFP is found predominantly in a circular "purse string" structure surrounding the organelle-rich domain. The scale bar represents 5 μm.

(C–E) Expression of constitutively active Concertina induces cellular contraction in a DRhoGEF2-dependent manner. A cell line expressing RLC-GFP was transfected with Myc-tagged versions of Concertina and identified with an antibody to the epitope tag (not shown). The cell margin is indicated with the yellow lines. (C) Overexpression of wild-type Concertina (Cta) does not affect cell morphology or myosin distribution. (D) However, overexpression of constitutively active Myc-Concertina ($Cta^{R277H}$) causes S2 cells to contract and form a myosin II purse string indistinguishable from transfection with DRhoGEF2 or Rho1V14. (E) Depletion of DRhoGEF2 with RNAi prevents cellular contraction or myosin II redistribution elicited by constitutively active Concertina. The scale bar represents 5 μm.

interesting type of intracellular motility required EB1 in a manner analogous to the EB1-dependent microtubule plus-end tracking of the vertebrate adenomatous polyposis coli (APC) tumor suppressor protein [15]. To our knowledge, this finding represents the first example of a regulator of the actin cytoskeleton that tracks along microtubule plus ends. Moreover, the dissociation of DRhoGEF2 from microtubule tips upon activation of Concertina also represents the first example of a regulated association of a protein with the microtubule plus end.

Our dissection of the DRhoGEF2 pathway at a cellular level is also consistent with genetic studies of *Drosophila* morphogenesis. These studies implicate Concertina in myosin II contractility through the Rho/Rho kinase pathway. The Rho1/Rho kinase/myosin II system is a widely employed module for bundling and contraction of actin filaments; it is involved in the formation of adhesion structures and stress fibers, retraction of the trailing edge in migrating cells, muscular contraction, morphogenetic cell shape changes, and construction of the cleavage furrow at the end of mitosis [9]. Context- and location-specific activation of the Rho1/Rho kinase/myosin II module is likely to reside in the activation of specific RhoGEFs, over 20 of which reside within the *Drosophila* genome [16]. This hypothesis is consistent with observations that inhibition of Rho1 or its downstream effectors causes a dramatic cytokinesis failure in S2 cells and embryos [17–19], but inhibition of DRhoGEF2 does not ([5] and [6]; S.R. and R.V., unpublished data). Instead, DRhoGEF2 has only been implicated in morphogenetic cell shape changes in epithelial cells.

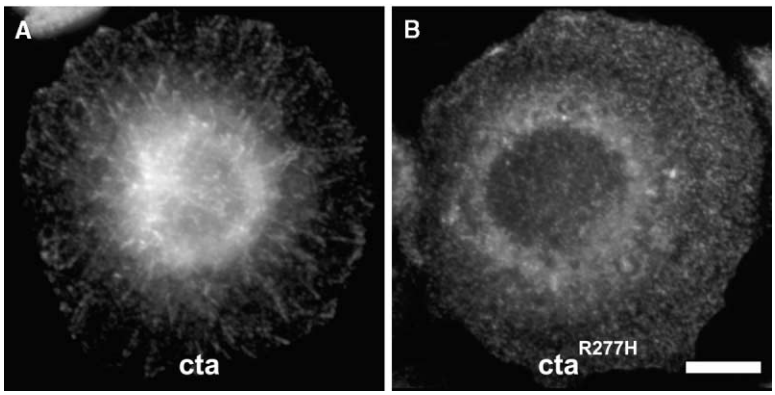


Figure 4. Activation of DRhoGEF2 by Concertina Correlates with Its Dissociation from the Microtubule Tip

S2 cells were transfected with wild-type Myc-Concertina (Cta) or constitutively active Myc-Concertina (Myc-$Cta^{R277H}$). Cells were then stained with antibodies against DRhoGEF2 and transfectants were identified with antibodies to the epitope tag (not shown).

(A) DRhoGEF2 association with microtubule plus ends is unaffected by expression of wild-type Myc-Cta.

(B) However, upon expression of Myc-$Cta^{R277H}$, DRhoGEF2 immunofluorescence no longer exhibits an interaction with microtubule tips and is instead associated with the plasma membrane. The scale bar represents 5 μm.

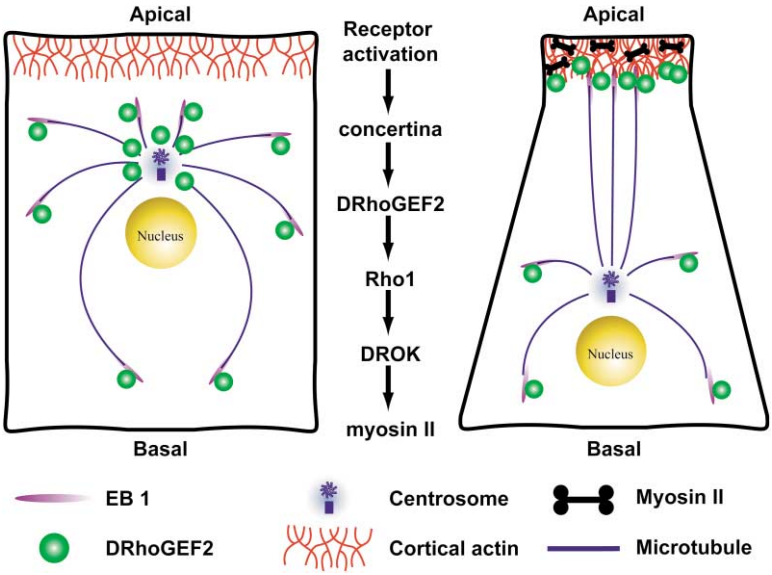


Figure 5. A Speculative Model for the Role of Microtubule Dynamics during DRhoGEF2-Mediated Cellular Shape Change

See text for details.

Thus, we believe that the signaling pathway that we have engineered in S2 cells recapitulates events involved in the cellular shape changes preceding gastrulation in *Drosophila* blastula epithelia cells [20, 21].

However, in *Drosophila* development, this signaling pathway must be activated in a polarized manner by an unidentified receptor and its ligand (possibly the protein encoded by the folded gastrulation gene [22]) so that myosin contraction occurs locally at the apical surface. In such a setting of asymmetric signaling, we propose that the intracellular transport of DRhoGEF2 on microtubule plus ends may play an important role in localized activation of the pathway. We speculate that inactive DRhoGEF2 interacts with the tips of microtubules, whereupon these growing microtubules deliver "packets" of DRhoGEF2 in the vicinity of the actin cortex (Figure 5). If DRhoGEF2 does not receive an activating input, it diffuses back into the cytoplasm to begin the transport cycle anew. However, if DRhoGEF2 is delivered to a subcortical region containing a high concentration of receptor-activated Concertina, DRhoGEF2 can locally activate the Rho1/Rho kinase/myosin II module. Moreover, because DRhoGEF2 possesses potential lipid (pleckstrin homology) and protein-protein (PDZ, RGS, and DH [Dbl homology]) interaction domains, microtubule-delivered DRhoGEF2 may be retained at the cortex if activated by Concertina. Although we propose a microtubule-assisted activation of the Rho pathway during cellular shape changes during morphogenesis (such as in epithelial cells), similar models that account for small GTPase activation during cellular motility have been suggested as well [23, 24].

In principle, interactions between DRhoGEF2 and its cortical activators could occur through diffusion within the cytoplasm. The evolution of this elaborate microtubule polymerization-based transport mechanism undoubtedly reflects some important property of the signaling pathway that we do not yet understand. Perhaps the amount of DRhoGEF2 carried on the tip of a microtubule represents some quanta—a critical concentration of the protein required either to respond to upstream inputs or to locally activate Rho1 in a cortical subdomain. This idea is supported by our observation that, at very low expression levels and without Concertina signaling, DRhoGEF2-GFP efficiently tracks microtubule ends without activating cellular contraction. Alternatively, it is possible that interaction with EB1 or some other protein at the microtubule plus end primes DRhoGEF2 for activation at the cortex. A third possibility is that microtubule dynamic instability is not uniform within a polarized cell but is locally modulated in order to deliver DRhoGEF2 to the cortex in a nonrandom manner. Testing between these hypotheses will require identification of the signaling components (i.e., the ligand-receptor pair) that act upstream of Concertina, reconstitution of the complete pathway in S2 cells, and the selective disruption of the association of DRhoGEF2 with microtubule tips in *Drosophila* embryos.

## Experimental Procedures

### Molecular Biology

DRhoGEF2-EGFP was constructed in three steps. First, making use of a unique EcoRi site (at bp 3129) and engineering a 5′ KpnI site to the first fragment and an XbaI site at the 3′ end of the second, we used PCR to amplify two fragments of the DRhoGEF2 cDNA. The EGFP coding sequence was also amplified with PCR from the EGFP-C1 vector (Clontech, Palo Alto, CA), and the pieces were successively cloned into the pMT-V5-his A vector (Invitrogen, Carlsbad, CA). The Concertina expression constructs were made by PCR with primers containing sequence for the Myc epitope tag and 5′ EcoRI and 3′ NotI restriction sites, and then by cloning into the pMT/V5-His A vector (Concertina and Concertina$^{R277H}$ cDNAs were the gift of Eric Wieschaus, Princeton University). The GAL4 expression plasmid was constructed similarly with yeast genomic DNA as a starting template. pMT-EB1-GFP was constructed by PCR-SOE (splicing by overlap extension) with a cDNA encoding *Drosophila* EB1 and EGFP-C1 as templates.

### Cell Culture and RNA Interference

*Drosophila* Schneider S2 cells were cultured and RNAi was performed according to published methods [8, 25]. Templates for in vitro transcription were generated by PCR with the primers encod-

ing the T7 promoter sequence upstream of the following: EB1 (5′-GAGAATGGCTGTAAACGTCTACTCCACAAATGTG-3′ and 5′-GAG ATGCCCGTGCTGTTGGCACAGGCGTTTA-3′), Rho1 (5′-ATCAAGA ACAACCAGAACATCG-3′ and 5′-TTTGTTTTGTGTTTAGTTCGGC-3′), DROK (5′-GAGAACACTCAAAAGCTGAAAAAG-3′ and 5′-ACAGTTC CTTCTGTAGCTGGTTTT-3′), and DRhoGEF2 (5′-ATGGATCACCCA TCAATCAAAAAACGG-3′ and 5′-TGTCCCGATCCCTATGACCACTA AGGC-3′).

Stable S2 cell lines expressing RLC-GFP were generated by cotransfection with the expression construct and hygromycin resistance plasmid with the Cellfectin transfection reagent and then antibiotic selection as recommended by the manufacturer (Invitrogen, Carlsbad, CA). Transient expression of UAS-Rho1V14 (gift of Liqun Luo, Stanford University) was performed by cotransfection with pMT-GAL4. Gene expression from constructs driven by the metallothionine promoter was induced by addition of 500 μM copper sulfate to the culture medium for 4–6 hr. Y-27632 was purchased from Calbiochem (San Diego, CA).

### Antibodies

To generate antibodies against DRhoGEF2, we prepared a GST fusion protein with the C-terminal 720 amino acids of the protein. Recombinant GST-DRhoGEF2 C terminus was expressed in *E. coli* and purified by glutathione Sepharose affinity chromatography, and antisera was produced in rabbits by Covance (Denver, PA). Polyclonal antibodies were affinity-purified on a GST-DRhoGEF2 C-terminal domain column and the DRhoGEF2 polyclonal antibodies were eluted with low pH. The monoclonal antibody p1D9 anti-Rho1 was obtained from the Developmental Studies Hybridoma Bank (Iowa City, Iowa). Monoclonal antibodies to ∝-tubulin and γ-tubulin were from Sigma (St. Louis, MO). Antibodies to the Myc-epitope tag were obtained from Covance. Polyclonal antibodies raised against *Drosophila* EB1 were previously described [8].

### Immunofluorescence Microscopy

S2 cells were rinsed in HL3 buffer (70 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM trehalose, 115 mM sucrose, and 5 mM HEPES [pH 7.2]) and fixed with either one of two methods: for actin or RLC-GFP staining, S2 cells were fixed for 10 min with 10% paraformaldehyde (EM Sciences, Gibbstown, NJ) in HL3 buffer; for experiments in which we visualized microtubules, EB1, and DRhoGEF2 at microtubule plus ends, cells were fixed for 10 min in a prechilled mixture (to −80°) of 3.2% paraformaldehyde in methanol. This fixation protocol was essential to preserve microtubule tip association. The cells were then washed and permeabilized with 0.1% Triton X-100 in PBS (PBST), blocked in 5% normal goat serum in PBST, and treated with primary antibodies in the same solution for 1 hr. After being washed, cells were stained with secondary antibodies (Cy2 or Cy3 goat anti-rabbit or donkey anti-mouse at a dilution of 1:300) (Jackson Immunoresearch, West Grove, PA). In some experiments, cells were stained with Rhodamine Red-X phalloidin (Molecular Probes, Eugene, OR). After being washed in PBST, the cells were rinsed in distilled water and mounted in fluorescence mounting medium (Dako Cytomation, Carpinteria, CA). Images were acquired with an Orca II cooled CCD camera (Hamamatsu) with a 100×/1.4 N.A objective lens mounted on a Nikon TE300 inverted microscope driven by Simple PCI software (Compix, Cranberry Township, PA). Gray scale images were combined and colorized with Photoshop (Adobe Systems, San Jose, CA).

### Confocal Imaging of S2 Cells

Live-cell microscopy was performed on room temperature S2 cells plated on glass bottom 35 mm microwell dishes (MatTek, Ashland, MA) coated with concanavalin A. Cells expressing GFP-tagged proteins were imaged on a Zeiss Axiovert 200 with a QLC100 spinning disk confocal scan head (Solamere Technology Group, Salt Lake City, UT) equipped with an XR/Mega10 Gen III intensified CCD camera (Stanford Photonics, Palo Alto, CA) and controlled by QED In Vivo software (QED Imaging, Pittsburgh, PA). Individual images were imported into Image J for contrast enhancement, cropping, and analysis. For the production of three-dimensional reconstructions, optical sections were imported into Imaris (Bitplane AG, Zurich, Switzerland), volume rendered, and output as Quicktime movies.

### Supplemental Data

Supplemental Figures and Movies are available with this article online at http://www.current-biology.com/cgi/content/full/14/20/1827/DC1/.

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