Available online at www.sciencedirect.com

ScienceDirect

Developmental Biology 297 (2006) 417–432

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Drosophila Rho-kinase (DRok) is required for tissue morphogenesis in diverse compartments of the egg chamber during oogenesis

Valerie Verdier [a], James E. Johndrow [b], Martha Betson, Guang-Chao Chen [a], David A. Hughes [c], Susan M. Parkhurst [b], Jeffrey Settleman [a,*]

[a] *Massachusetts General Hospital Cancer Center and Harvard Medical School, 149 13th Street, Charlestown, MA 02129, USA*

[b] *Division of Basic Sciences, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, A1-162, PO Box 19024, Seattle, WA 98109-1024, USA*

[c] *The Faculty of Life Sciences, The University of Manchester, Sackville Street, Manchester, UK*

Received for publication 21 October 2005; revised 11 May 2006; accepted 15 May 2006
Available online 19 May 2006

Abstract

The Rho-kinases are widely utilized downstream targets of the activated Rho GTPase that have been directly implicated in many aspects of Rho-dependent effects on F-actin assembly, acto-myosin contractility, and microtubule stability, and consequently play an essential role in regulating cell shape, migration, polarity, and division. We have determined that the single closely related *Drosophila* Rho-kinase ortholog, DRok, is required for several aspects of oogenesis, including maintaining the integrity of the oocyte cortex, actin-mediated tethering of nurse cell nuclei, "dumping" of nurse cell contents into the oocyte, establishment of oocyte polarity, and the trafficking of oocyte yolk granules. These defects are associated with abnormalities in DRok-dependent actin dynamics and appear to be mediated by multiple downstream effectors of activated DRok that have previously been implicated in oogenesis. DRok regulates at least one of these targets, the membrane cytoskeletal cross-linker DMoesin, via a direct phosphorylation that is required to promote localization of DMoesin to the oocyte cortex. The collective oogenesis defects associated with DRok deficiency reveal its essential role in multiple aspects of proper oocyte formation and suggest that DRok defines a novel class of oogenesis determinants that function as key regulators of several distinct actin-dependent processes required for proper tissue morphogenesis.

Keywords: Oogenesis; Rho-kinase; Actin; Moesin; Cytoskeleton; *Drosophila*

Introduction

The molecular pathways underlying the complex morphogenesis of tissues in developing multicellular organisms are beginning to be elucidated. At the cellular level, it is clear that numerous highly coordinated shape changes and movements are among the key regulatory processes required for proper tissue development, and these depend on dynamic cytoskeletal rearrangements that are stringently regulated by the activities of the Ras-like GTPases of the Rho subfamily (Hall, 1998; Nobes and Hall, 1995b; Ridley, 1996). These evolutionarily conserved proteins, which include the prototypical family members, Rho, Rac and Cdc42, have been implicated in a variety of cellular processes associated with cytoskeletal rearrangements, including cell shape change, cell migration, cell adhesion (Nobes and Hall, 1995a, 1999; Raftopoulou and Hall, 2004), gene transcription (Hill et al., 1995; Sahai et al., 1998) and protein trafficking (Qualmann and Mellor, 2003; Symons and Rusk, 2003), among others.

The Rho proteins function as molecular switches, cycling between an inactive GDP-bound state and an active GTP-bound state (Symons and Settleman, 2000). In their active form, Rho GTPases transduce signals by binding various downstream effector targets to trigger cellular responses to extracellular stimuli (Burridge and Wennerberg, 2004; Van Aelst and D'Souza-Schorey, 1997). Among the key identified Rho effectors is Rho-kinase, a serine/threonine kinase that has been found to mediate Rho-directed cellular responses through direct phosphorylation of various protein substrates that participate in diverse biological processes, including smooth muscle contraction, cytokinesis, axon outgrowth, cell migration, and cell adhesion (Riento and Ridley, 2003). Extensive

* Corresponding author. Fax: +1 617 726 7808.
E-mail address: settleman@helix.mgh.harvard.edu (J. Settleman).

0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.05.016

studies of Rho-kinase function in mammalian smooth muscle as well as nonmuscle cells have implicated Rho-kinase in acto-myosin contractility: Upon stimulus-induced activation, GTP-bound Rho binds to and activates Rho-kinase which in turn phosphorylates myosin regulatory light chain and the myosin-binding subunit of myosin phosphatase. This dual phosphorylation results in a conformational change that allows myosin II to form filaments and increases its actin-activated ATPase activity, leading to the formation of actin stress fibers and focal adhesions in nonmuscle cells (Kawano et al., 1999; Kimura et al., 1996), to smooth muscle contraction (Uehata et al., 1997), and to neurite retraction (Amano et al., 1998; Hirose et al., 1998).

Rho-kinase has also been shown to mediate Rho-induced effects on the actin cytoskeleton through phosphorylation of other actin-binding proteins, such as adducin, to regulate the organization of the subcortical spectrin-F-actin meshwork in MDCK epithelial cells (Fukata et al., 1999b), and the ERM (Ezrin–Radixin–Moesin) family proteins, to ensure the anchoring of F-actin to the plasma membrane and subsequent microvilli-like structure formation (Fukata et al., 1999a; Oshiro et al., 1998). In addition, Rho-kinase directly phosphorylates LIM kinase, which regulates the turnover of actin filaments through an inactivating phosphorylation of the actin-capping protein, cofilin (Maekawa et al., 1999).

In light of its prominent role in mediating Rho-dependent cytoskeletal dynamics, Rho-kinase function has also been studied in the context of tissue morphogenesis in several multicellular model organisms where it has been implicated in various developmental processes, including organogenesis in higher vertebrates such as chicken and mouse (Wei et al., 2001), embryo elongation and cytokinesis in *Caenorhabditis elegans* (Piekny and Mains, 2002; Piekny et al., 2000; Wissmann et al., 1997), and gastrulation in zebrafish (Lai et al., 2005). Rho-kinase has also been shown to function downstream of the Wnt/planar cell polarity pathway to ensure convergent extension cell movements during vertebrate gastrulation in the *Xenopus* embryo (Kim and Han, 2005). In *Drosophila*, analysis of somatic clones of $Drok^2$, a loss-of-function mutation of the single closely related Rho-kinase ortholog, *Drok* (Mizuno et al., 1999), revealed a role for DRok in the evolutionarily conserved Frizzled-Dishevelled pathway that controls planar cell polarity. Thus, $Drok^2$ mutant clones exhibit tissue polarity defects resulting in an abnormal number of wing hairs and improper orientation of photoreceptor clusters in the eye (Winter et al., 2001). In this developmental context, DRok's ability to regulate acto-myosin contractility appears to account largely for its biological function.

Zygotic $Drok^2$ mutant animals die just prior to the third instar larval stage (Winter et al., 2001), and maternal contribution of *Drok* mRNA or DRok protein to the egg may preclude the identification of roles for DRok in earlier fly development. Therefore, to further explore DRok biological functions in early *Drosophila* development, we generated germline clones (GLCs) of DRok, using a null allele. We observed that DRok is required for proper oogenesis, and that DRok deficiency results in loss of integrity of the oocyte cortex and in defective cytoplasmic transport, two developmental processes that depend on actin cytoskeletal organization (Polesello et al., 2002; Theurkauf and Hazelrigg, 1998). Many of the previously reported Rho-kinase-mediated developmental functions have been found to involve Rho-kinase signaling to nonmuscle myosin light chain and consequent acto-myosin contractility. Here, we show that DRok is likely to control the oocyte cortex integrity through its regulation of the phosphorylation and localization of the cytoskeletal-membrane anchoring protein, DMoesin, at the oocyte cortex. We also demonstrate that DRok is involved in the establishment of egg polarity and the trafficking of yolk granules. We conclude that *Drosophila* oogenesis provides a complex developmental setting in which DRok functions as a spatio-temporal regulator of tissue morphogenesis in diverse compartments of the egg chamber through the engagement of multiple downstream effectors.

Materials and methods

Drosophila strains

Drosophila stocks were maintained at 25°C. To generate germline clones of $Drok^2$, the following stocks were used: the *y, w,* rok^2 *P[neoFRT19A]/Fm7i Act-GFP* mutant line, kindly provided by L. Luo, from which the mutation was segregated from the *FRT19A* site and consequently recombined with *P[neoFRT18E]* to generate the *y, w,* rok^2 *P[neoFR18E]/Fm7i, Act-GFP* line; $ovoD^2$*P[neoFRT18E]/Fm6;P{ hs-Flp}38* flies. Third instar larval progeny of the genotype *y, w,* rok^2 *P[neoFR18E]/*$ovoD^2$*P[neoFRT18E];P{ hs-Flp}38/+* were incubated at 37°C for 2 h each day for 3 days (Chou and Perrimon, 1996). Females with $Drok^2$ homozygous GLCs (non-*FM6* females) were allowed to lay eggs and tested for sterility. Germline clones of $Drok^1$ (Bloomington Stock Center) were generated similarly. Other stocks utilized include *w1118* and *w1118*; *P[nosVP16-GAL4] P[UAS-*α*Tub84B-GFP]* (Bloomington Stock Center).

RNA in situ hybridization

In situ mRNA hybridization to adult ovaries of the wild-type genotype was performed using a full-length *Drok* cDNA. *oskar* and *bicoid* mRNA in situ hybridizations to adult ovaries of the wild-type and $Drok^2$ germline clone genotypes were carried out using PCR fragments from wild-type fly genomic DNA, corresponding to amplified sequences from *oskar* or *bicoid* mRNA regions. 5′-ACGT**TCTAGA**CAAAAATGCCAGTACCCATCA-3′ and 5′-CATG**GGATCC**CCCTTTCGTTGATTAGACAGGA-3′, and 5′-ACGT**TCTAGA**CACCACTTTTACCAGCTCTCAA-3′ and 5′-CATG**GGATCC**CTGTAGCGTCGTCTTCTTGCT-3′ were used as left and right primers for *oskar* and *bicoid*, respectively, with the introduction of the *Xba*1 and *Bam*H1 restriction sites (in bold type) for subsequent subcloning into the *Xba*1 and *Bam*H1 linearized pBSK vector. Ovaries from 2- to 4-day old well-fed female flies were dissected in EBR (13 mM NaCl, 0.47 mM KCl, 0.19 mM CaCl2, 1 mM HEPES, pH 6.9), fixed in 1.6 ml of fixing solution (0.1 M HEPES, pH 6.9, 2 mM MgSO4, 1 mM EGTA), 0.4 ml of 20% paraformaldehyde and 8 ml of Heptane, for 29′ with vigorous shaking. The following steps were performed according to a standard protocol for in situ hybridization, with the indicated modifications (O'Neill and Bier, 1994). Proteinase K treatment was performed for 10 min. Hybridization with a digoxygenin-labeled RNA probe, prepared as suggested by the manufacturer (Boehringer Mannheim), was performed at 50°C overnight and followed by washes in hybridization solution, 1:1 mixture of hybridization solution and PBT (PBS, 0.1% Tween-20), and PBT at 50°C. The stained ovaries were mounted in 50% glycerol in PBS.

Immunohistochemistry

Ovaries were dissected and fixed as described (Cant et al., 1994). The following antibodies were used: rabbit anti-Phospho-ERM (1:50, Cell Signaling

Technology), mouse anti-Hu-li-tai-shao-RC (1:10, anti-Hts-RC), kindly provided by Lynn Cooley, anti-Gurken (1D12, 1:10, Developmental Studies Hybridoma Bank), mouse anti-β tubulin (1:500, mouse ascites E7, Developmental Studies Hybridoma Bank). Immunostaining using rabbit anti-Moesin antiserum (1:5000, D. Kiehart, Duke University) was performed as described. The secondary antibodies Cy3-conjugated or Cy2-conjugated goat anti-rabbit (Jackson Labs), Cy3-conjugated or Alexa 488-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories), were used at a dilution of 1:200. F-actin was visualized after staining with TRITC-Phalloidin (Sigma, 1:200). Nuclei were stained with Alexa 488-conjugated Wheat Germ Agglutinin (WGA) (1:1000, Molecular Probes). Lectin staining was performed according to Jankovics et al. (2002). Ovaries were mounted in anti-fade solution (50% glycerol containing 0.05% propyl gallate), and fluorescent images were recorded using a Zeiss LSM510 confocal microscope.

In vitro kinase assays

Reactions of 30 μl containing 50 mU of purified recombinant mRok (Upstate), 2 μg of GST fusion protein, 0.5 μl of 32P-ATP (10 mM HEPES [pH 7.5], 150 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT and 25 mM β-glycerol phosphate) were incubated for 20 min at 30°C and the reaction products were subjected to SDS-PAGE electrophoresis. Gels were stained with Coomassie solution to reveal protein content, dried, and subjected to autoradiography.

Timelapse confocal imaging

Female flies were fattened for 2 days on yeast–cornmeal–molasses with supplemental dried active baker's yeast at 25°C. Females were injected in the abdomen with 0.4% trypan blue in normal saline. Two hours after injection, ovaries were dissected into ovarioles in halocarbon 700 oil on glass-bottom culture dishes (Bioptechs). For injection of C3 transferase, wild-type egg chambers were prepared as above and were injected with C3 transferase (approximately 100 nM) following dissection into halocarbon oil using a microinjection apparatus. As controls, egg chambers were injected with GST protein alone (in the same buffer) or buffer alone. In both cases, there was no effect on ovaries (no premature swirling) — even with higher concentrations of GST (up to 500 nM) injected compared to GST-C3. Timelapse movies were recorded by taking images of a single 1- to 2-μm central section of the oocyte every 10 s on a Zeiss LSM 510 META confocal microscope. Excitation was

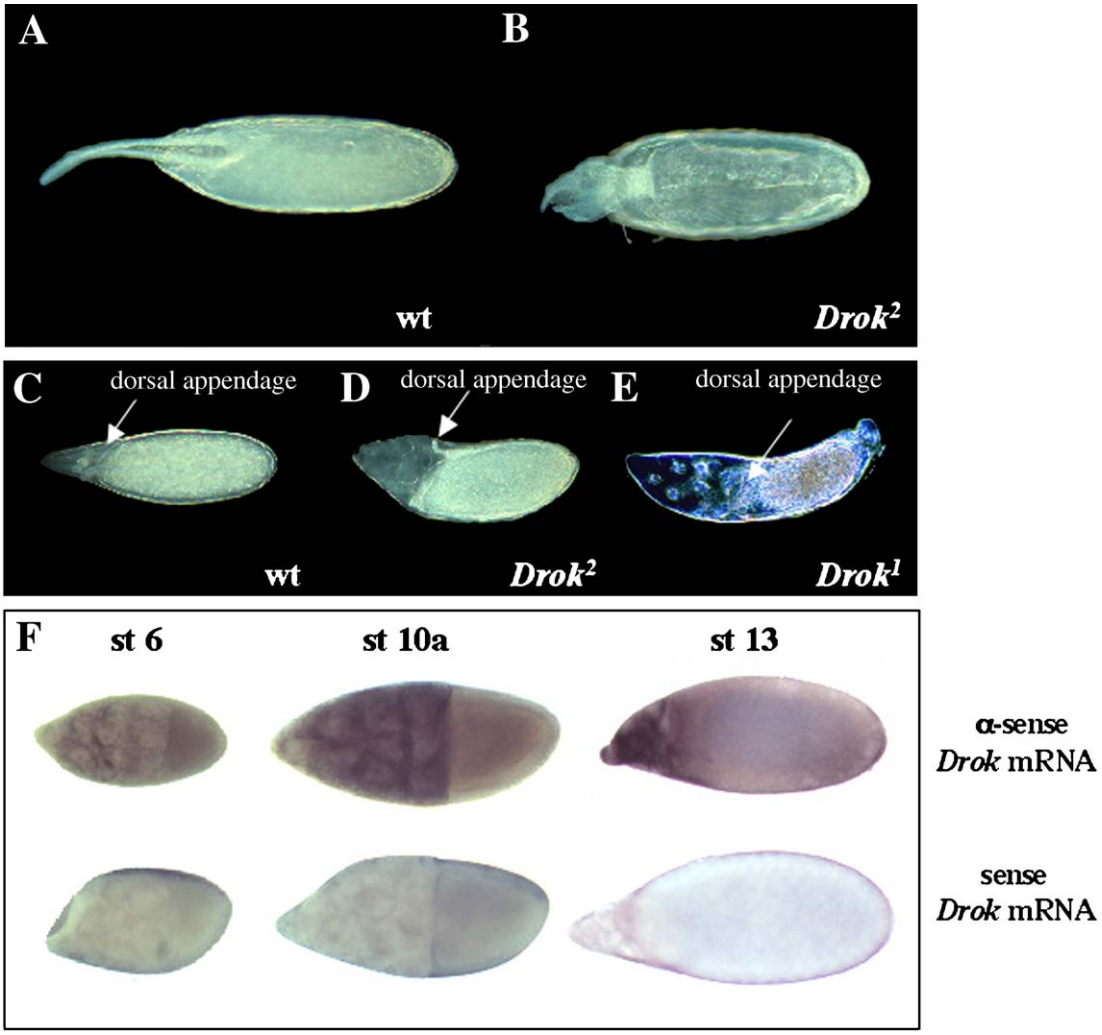


Fig. 1. *Drok*^{2} and *Drok*^{1} germline clones produce abnormal eggs with dorsal appendage defects. (A, B) Phase contrast images of dorsal views of a wild-type stage 14 egg chamber (A) and a *Drok*^{2} germline mutant stage 14 egg chamber (B). *Drok*^{2} germline clones produce eggs with chorion patterning defects such as fused and flat dorsal appendages. (C-E) Phase contrast images of a wild type (C), a *Drok*^{2} mutant (D), and a *Drok*^{1} mutant (E) stage 13 egg chambers. *Drok*^{2} and *Drok*^{1} mutant egg chambers exhibit dumpless-like nurse cells, resulting in eggs smaller than their wild-type counterparts. The arrows point to the newly forming dorsal appendages, which marks the developmental stage of these eggs. (F) In situ hybridization to *Drok* mRNA in wild-type stage 6 (st 6), stage 10a (st 10a) and stage 13 (st 13) egg chambers. *Drok* is mainly expressed in nurse cells, along with a diffuse expression in the oocyte throughout oogenesis. Sense mRNA was used as a negative control. Anterior is to the left and dorsal is to the top.

achieved with a 543-nm laser and detection was through a 560-long pass filter. Projected images represent six consecutive time points.

Immunofluorescence imaging of microtubules

Females were fattened as for timelapse and dissected into Robb's medium (55 mM KOAc, 40 mM NaOAc, 100 mM sucrose, 10 mM glucose, 1.2 mM $MgCl_2$, 1.0 mM $CaCl_2$, 100 mM HEPES, pH 7.4) at room temperature for a maximum of 8 min, and were fixed and stained immediately thereafter according to the method of Cha et al. (2001), using a FITC-labeled anti-α-tubulin monoclonal antibody DM1A (Sigma). Visualization was by confocal microscopy using a Zeiss LSM 510 META with excitation at 488 nm and collecting emission using a BP-505-560 filter. A Plan-Apochromat 40× 1.3 Oil objective was used in all cases for imaging.

Recombinant protein production

The Rho-inhibitory C3 transferase coding sequence, in a pGEX vector (GE/Amersham), was transformed into Rosetta-Gami-B BL21(DE3) pLysS (Novagen). Cultures were inoculated and grown overnight at 37°C, diluted tenfold, and grown to OD_{600} = 1.0. Expression was induced with 50 μM IPTG, cultures were transferred to 18°C and grown overnight (at least 18 h). Cells were lysed by sonication in lysis buffer (50 mM Tris pH 7.6, 150 mM NaCl, 5% glycerol, 5 mM DTT) with Complete protease inhibitor tablets (Roche). Triton X-100 was added to 1% and lysates were incubated at 4°C for 30 min. Lysates were centrifuged at 10,000 rpm in a JA-17 or SS-34 rotor, and the supernatants were coupled to Glutathione-sepharose 4B (GE/Amersham) by 90-min nutation at 4°C. The matrix was washed three times with lysis buffer (without protease inhibitors) and eluted two times 1 h with elution buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5% glycerol, 5 mM DTT, 20 mM reduced glutathione). Elutions were pooled, dialyzed into injection buffer (5 mM Kcl, 0.1 mM $NaPO_4$ pH 6.8), and stored at 4°C.

GST-DMoe and GST-DMoeT559A C-terminal protein fusions were generated by PCR and cloned into the pGEX20 vector. The oligonucleotides 5′-ACGT**GGATCC**AAGAATATGGAGGCCGTCGAG-3′ and 5′-CATG**TCTAGA**TTACATGTTCTCAAACTGATCGACGC-3′ were used as left and right primers containing *Bam*HI and *Xba*I restriction sites (in bold type), respectively, to amplify the DMoe C-terminal fragment containing the putative Rho-kinase phosphorylation site. Primers 5′-CGTGACAAGTACAAGGCGCTCCGCGAGATTCGT-3′ and 5′-CTTACGAATCTCGCGGAGCGCCTTGTACTTGTC-3′ were used to generate the threonine to alanine mutation at position 559 in the same DMoe fragment. GST fusion proteins were produced as described (Jiang et al., 2005). In vitro kinase assays were performed with purified rat active mRok (Upstate) and either GST, GST fused to the DMoe C-terminal fragment, or to the DMoe C-terminal fragment with threonine 559 mutated to alanine, eliminating the putative Rho-kinase phosphorylation site.

Results

DRok is required for normal oogenesis

The developmental function of DRok has, thus far, only been examined in the context of tissue polarity and axon outgrowth using zygotic loss-of-function mutants (Ng and Luo, 2004; Winter et al., 2001). Therefore, to examine a potential requirement for DRok in early *Drosophila* development, we generated germline clones (GLCs) of a *Drok* null allele. This strategy relies upon the Flp-FRT recombination system to eliminate maternal contribution of *Drok* mRNA and thereby provides an opportunity to examine potential roles for DRok in developing eggs or early embryos. Using this strategy, we observed that females carrying $Drok^2$ GLCs are sterile and lay a relatively small number of eggs, all of which are abnormally shaped. Unlike wild-type eggs, in which the dorsal appendages adopt a tubular morphology (Fig. 1A), the DRok-deficient eggs, which invariably fail to hatch, exhibit fused flat dorsal appendages (100% of the clones) (Fig. 1B and Table 1). In addition, whereas in wild-type egg chambers at stage 13 nurse cells have transferred most of their cytoplasm to the oocyte (Fig. 1C), in $Drok^2$ mutant egg chambers, nurse cells largely retain their cytoplasm, and about half of the mutant oocytes are reduced in size (Fig. 1D and Table 1). Similar phenotypes were found in $Drok^1$ germline clones, where $Drok^1$ is an independently derived loss-of-function allele of *Drok*, suggesting that the observed defects reflect the genetic disruption of *Drok* and are not due to a nonspecific secondary mutation (Fig. 1E and Table 1). These findings indicate that DRok is essential for normal oogenesis.

We used in situ hybridization to confirm that *Drok* mRNA is normally present in developing egg chambers. *Drok* mRNA is expressed somewhat uniformly throughout the cytoplasm of nurse cells (Fig. 1F) and diffuse mRNA expression is detected in the oocyte, throughout oogenesis, suggesting that DRok is in fact maternally provided to the oocyte.

Table 1
$Drok^2$ and $Drok^1$ GLCs exhibit multiple oogenesis defects

Genotype	Oocytes with fused dorsal appendages (%)	Egg chambers with dumpless Nurse cells (%)	Small eggs (%)	Oocytes with actin clumps in ooplasm (%)	Oocytes with altered plasma membrane (%)
$Drok^2/Drok^2$	100 (N = 152)	97 (N = 127)	50 (N = 152)	95 (N = 180)	82 (N = 180)
$Drok^1/Drok^1$	100 (N = 30)	96 (N = 45)	58 (N = 40)	75 (N = 92)	62 (N = 92)
wt	0 (N = 100)	0 (N = 100)	3 (N = 100)	0 (N = 100)	0 (N = 100)

Genotype	Egg chambers with defective NC actin cytoskeleton (%)	Oocytes with reduced/diffused Phospho-DMoesin at the cortex (%)	Oocytes with Phospho-DMoesin clumps in ooplasm (%)	Egg chambers with misshapen ring canals (%)
$Drok^2/Drok^2$	86 (N = 64)	70 (N = 133)	22 (N = 133)	93 (N = 77)
$Drok^1/Drok^1$	72 (N = 92)	NA	NA	NA
wt	0 (N = 50)	8 (N = 120)	0 (N = 120)	0 (N = 55)

N equals the total number of egg chambers.
NC = Nurse Cell.
NA = not available.

DRok is required for establishing normal oocyte polarity

Our observation that stages 13–14 *Drok*^{2} mutant oocytes harbor incompletely formed or fused dorsal appendages, a defect that is often associated with abnormal egg polarity (Neuman-Silberberg and Schupbach, 1994, 1996), prompted us to investigate the polarity of *Drok*^{2} mutant oocytes. By examining *oskar* mRNA localization as a marker of antero-posterior polarity (Kim-Ha et al., 1991) and the localization of *bicoid* mRNA as a marker of the anterior pole (van Eeden and St Johnston, 1999), we observed that, whereas *oskar* mRNA is restricted to the posterior tip of wild-type oocytes (Fig. 2A), its localization is altered in *Drok*^{2} mutant oocytes, where *oskar* mRNA is detected throughout the ooplasm (Fig. 2B). The loss of posterior localization of *oskar* mRNA in *Drok*^{2} GLCs is also observed in early oocytes (stage 8/9) (data not shown). We note that, as has been reported in GLCs of *Dmoesin* (*Dmoe* GLCs), a putative effector target of DRok (Polesello et al., 2002), a small fraction of correctly positioned *oskar* mRNA can be detected in *Drok*^{2} GLCs (Fig. 2B), indicating that the absence of either DRok or DMoesin does not entirely abolish the localization of *oskar* mRNA. On the other hand, similar to Dmoe GLCs, *bicoid* mRNA localizes properly at the anterior margin of wild-type and *Drok*^{2} mutant oocytes (Figs. 2C, D). Together, these results indicate that DRok, like DMoesin, is not required for the formation of the anterior pole, but is required for formation of the posterior pole.

We also investigated dorso-ventral polarity of oocytes by examining the subcellular localization of the Gurken product, a TGFα-like ligand for the *Drosophila* EGFR. The antero-dorsal

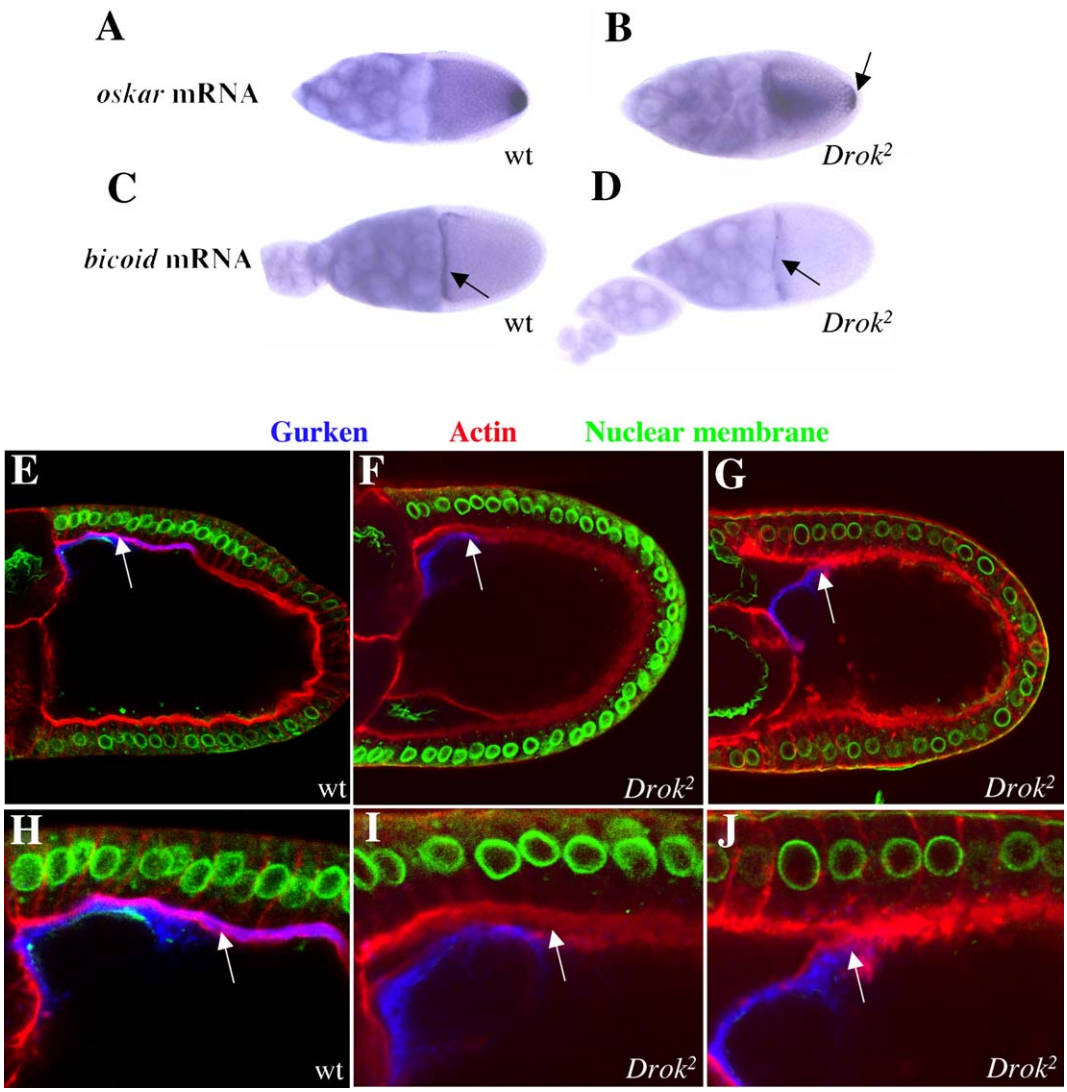


Fig. 2. *Drok*^{2} GLCs exhibit polarity defects. (A–D) In situ hybridizations to *oskar* mRNA (A, B) or *bicoid* mRNA (C, D) in wild-type (A,C) or *Drok*^{2} GLC mutant (B, D) egg chambers. *oskar* mRNA is normally restricted to the posterior side of wild-type oocytes (A) but this localization is disrupted in *Drok*^{2} mutant oocytes (B). The arrow denotes some *oskar* mRNA still anchored at the posterior tip of mutant oocytes. *bicoid* mRNA anterior margin distribution is unaltered in *Drok*^{2} mutant oocytes (C, D). (E–G) Triple anti-Gurken, TRITC-phalloidin and nuclear staining of a wild-type (E and close-up H) or *Drok*^{2} mutant (F and close-up I, G and close-up J) oocytes. In both the wild-type and mutant oocytes, Gurken accumulates in close proximity to the oocyte nucleus and distributes normally to the antero-dorsal region of the oocyte. However, unlike in wild-type oocytes, Gurken is not being secreted in the extracellular space between the oocyte membrane and the neighboring follicle cell apical membranes, in *Drok*^{2} mutant oocytes (arrows in H, I and J).

localization of *gurken* mRNA and the localized activity of its product are responsible for the formation of the dorso-ventral axis (Neuman-Silberberg and Schupbach, 1993, 1994, 1996). We determined that the Gurken protein is present near the oocyte nucleus and is correctly positioned to the antero-dorsal region of both wild-type and *Drok*^{2} mutant oocytes (Figs. 2E, F), even in *Drok*^{2} mutant oocytes with a severely affected morphology (Fig. 2G). This indicates that DRok is not required for proper localization of Gurken in the establishment of dorso-ventral polarity.

Gurken regulates the activity of the EGFR, which is expressed in the surrounding follicle cells, and during oogenesis, intercellular communication events, which involve the Gurken/EGFR pathway, take place between the germline and the follicle cells (Nilson and Schupbach, 1999). Previous studies indicate that Gurken is cleaved at the oocyte membrane, and its soluble extracellular domain is then secreted for subsequent activation of the EGFR on the plasma membrane of neighboring follicle cells (Ghiglione et al., 2002). Therefore, we examined Gurken secretion in *Drok*^{2} GLCs. In wild-type oocytes, we observed that Gurken is secreted into the intercellular space between the oocyte plasma membrane and the adjacent follicle cells, which are revealed by F-actin staining (Figs. 2E, H). However, Gurken fails to be secreted from the majority of *Drok*^{2} GLC oocytes (80% of the *Drok*^{2} GLCs) and remains within the oocyte, either adjacent to the plasma membrane or apparently "stuck" within the plasma membrane (Figs. 2F, G, I, J). Taken together, these results suggest that DRok is not required for the proper localization of Gurken but is necessary for the proper distribution of Gurken between the oocyte and adjacent follicle cell plasma membranes. This potentially reflects a role for DRok in the normal processing of Gurken and its routing through the secretory pathway, or its role in maintaining oocyte plasma membrane integrity, as described below.

DRho1 and DRok are required for yolk granule distribution

A polarized microtubule cytoskeleton is required for the localization of polarity determinants within the oocyte, and Rho GTPase signaling has been directly implicated in microtubule dynamics in several experimental systems (Theurkauf et al., 1992). Therefore, we determined whether microtubules are affected in *Drok*^{2} oocytes. Anti-α-tubulin staining of wild-type oocytes reveals a subcortical array of microtubules at stage 8 (Fig. 3A) followed by a gradient of bundled microtubules from anterior to posterior from stage 10a (Fig. 3B) and a more lateral bundling of microtubules from stage 11 (Fig. 3C). This microtubule cytoskeleton arrangement appears to be normal in *Drok*^{2} mutant oocytes where microtubules seem to be well organized (Figs. 3D–F).

Both actin and microtubule cytoskeletons have been functionally linked in the establishment of oocyte polarity during *Drosophila* oogenesis (Theurkauf et al., 1992). The fact that disruption of the actin cytoskeleton either with cytochalasin D or with mutants in genes such as *chickadee*, which encodes the actin-binding protein Profilin, results in microtubule-based premature streaming in the oocyte (Manseau et al., 1996) prompted us to examine microtubule cytoskeleton dynamics in *Drok*^{2} mutant oocytes. This was assessed by comparing the movement of autofluorescent yolk granules during ooplasmic streaming in wild-type and *Drok*^{2} mutant oocytes. Ooplasmic streaming is a process by which microtubules move within the oocyte to mix the oocyte cytoplasm with the cytoplasm being rapidly added from the nurse cells, during the microfilament-dependent dumping from the nurse cells into the oocyte, starting at stage 10b (Gutzeit, 1986). While we readily observe the movement of yolk granules throughout the ooplasm of wild-type oocytes starting at stage 10b (Fig. 3J and Supplementary Material, movie 1), there is no detectable movement of yolk granules at similar stages in *Drok*^{2} GLC oocytes (Fig. 3K and Supplementary Material, movie 2). In fact, unlike in wild-type oocytes, most of the yolk granules in *Drok*^{2} GLCs accumulate and remain at the oocyte cortex beginning as early as stage 8 as seen by the more intense red staining along the oocyte cortex (Figs. 3G, H, Supplementary Material, movies 3, 4). This prevented the visualization of microtubule movement and ooplasmic streaming in later stages (Fig. 3K). There is also some cortical accumulation of yolk granules in wild-type stage 10b oocytes, but this is far less than that seen in *Drok*^{2} mutant oocytes. Interestingly, the observed accumulation of yolk granules at the oocyte cortex has not been previously described in other *Drosophila* mutants that exhibit oogenesis defects.

To determine whether this role of DRok in the oocyte reflects a downstream activity of the Rho GTPase, we injected wild-type egg chambers with the Rho-specific bacterial inhibitory toxin, C3. Significantly, the C3-injected egg chambers exhibit the same yolk phenotype as *Drok*^{2} mutant oocytes (Fig. 3I). Together, these results suggest that a DRho1-DRok pathway is required prior to the onset of ooplasmic streaming to regulate the trafficking of the yolk granules within the ooplasm, and that such trafficking is independent of the microtubule cytoskeleton.

Subcortical F-actin organization and plasma membrane integrity are disrupted in DRok-deficient oocytes

Since Rho-Rho-kinase signaling plays an important role in regulating F-actin assembly in numerous experimental models, we examined the actin cytoskeleton in *Drok*^{2} mutant eggs. Dissection of *Drok*^{2} GLC egg chambers followed by phalloidin staining to detect F-actin revealed that subcortical F-actin is disorganized in most mutant oocytes (Table 1) and is characterized by the presence of actin clumps within the oocyte and along the length of the oocyte cortex (Figs. 4B, C). Moreover, the cortical F-actin in *Drok*^{2} mutant oocytes exhibits a somewhat diffuse organization along the oocyte membrane (Fig. 4B, arrowhead). This is in striking contrast to the "tight" subcortical F-actin distribution seen in a wild-type oocyte (Fig. 4A) and suggests that DRok contributes to the maintenance of cortical actin integrity in *Drosophila* oocytes. Significantly, genetic disruption of the DRho1 GTPase in oocytes results in similar effects on F-actin organization along the cortex (Magie et al., 1999), consistent with a role for DRho1-DRok signaling in the regulation of cortical F-actin at the oocyte membrane.

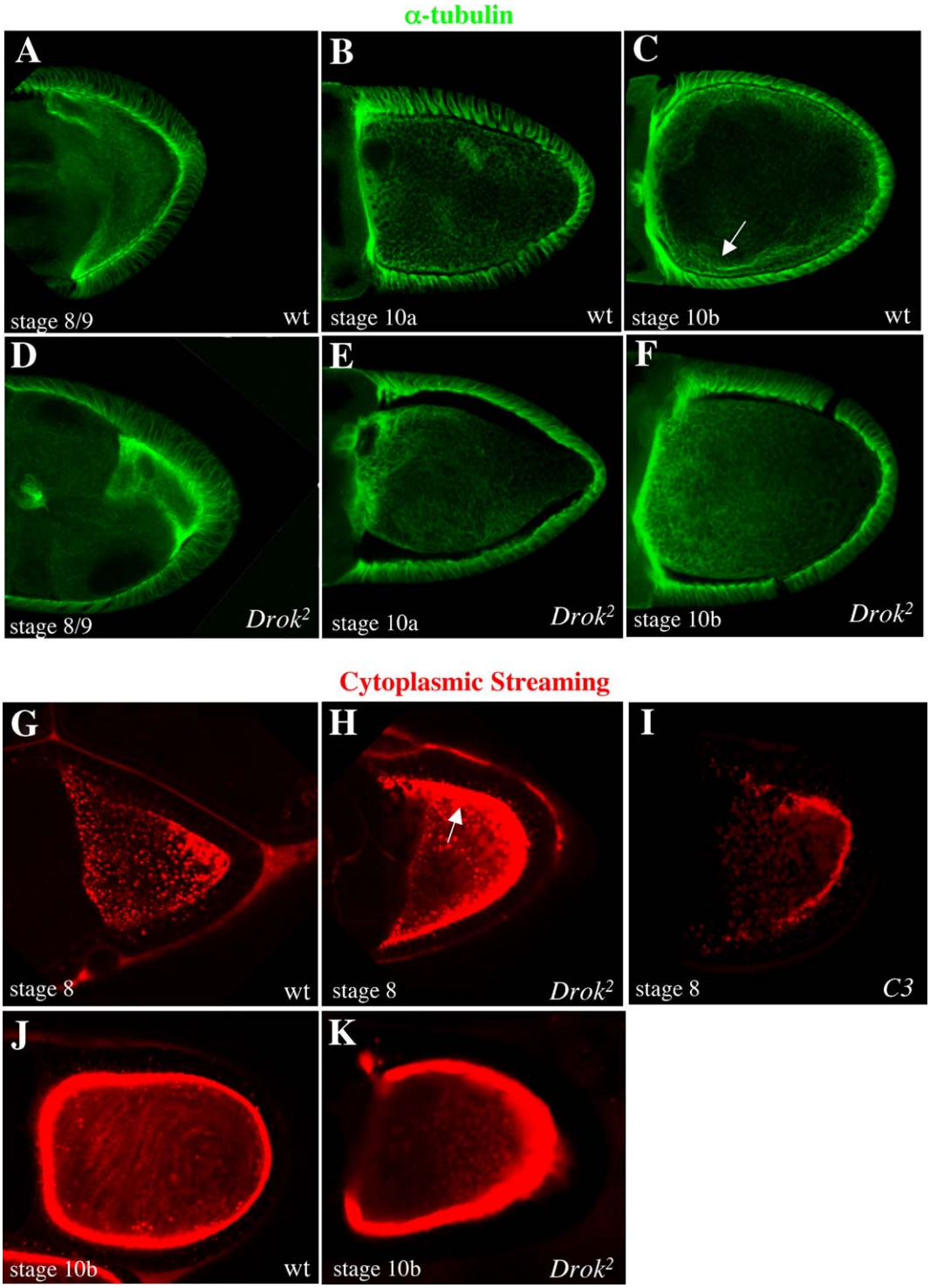


Fig. 3. DRok does not perturb the bundling of microtubules but is involved in yolk granule trafficking. (A–F) Visualization of wild-type (A–C) and *Drok*^{2} mutant oocytes (D–F), all expressing the Tubulin-GFP fusion protein to reveal microtubules. As expected in wild-type oocytes, early stages (stage 8/9) are marked by an array of subcortical microtubules around the oocyte (A). Starting from stage 10a, microtubules are bundling from the anterior margin of the oocyte, and this creates an anterior to posterior gradient of microtubules in the oocyte (B). Later stages are characterized by anterior and more lateral bundling of microtubules that extend more in the ooplasm, as they are ready for cytoplasmic streaming (C). This microtubule distribution is not perturbed in *Drok*^{2} mutant oocytes and microtubules bundle quite normally from early to late stages (D–F). (G–I) Visualization of auto-fluorescent yolk granules by time-lapse microscopy in wild-type (G, J), *Drok*^{2} GLCs (H, K) and C3-treated wild-type egg chambers (I). While yolk granules are uniformly present throughout stage 8 wild-type oocytes (G), they aggregate and accumulate at the cortex of same stage *Drok*^{2} mutant oocytes (H). This phenotype has also been detected in C3-treated oocytes (I). While cytoplasmic streaming can be observed in stage 10b wild-type oocytes as swirling arrays corresponding to temporal projections of yolk granules movements (J), visualization of streaming is not possible in stage 10b *Drok*^{2} mutant oocytes as most yolk granules have accumulated at the cortex (K).

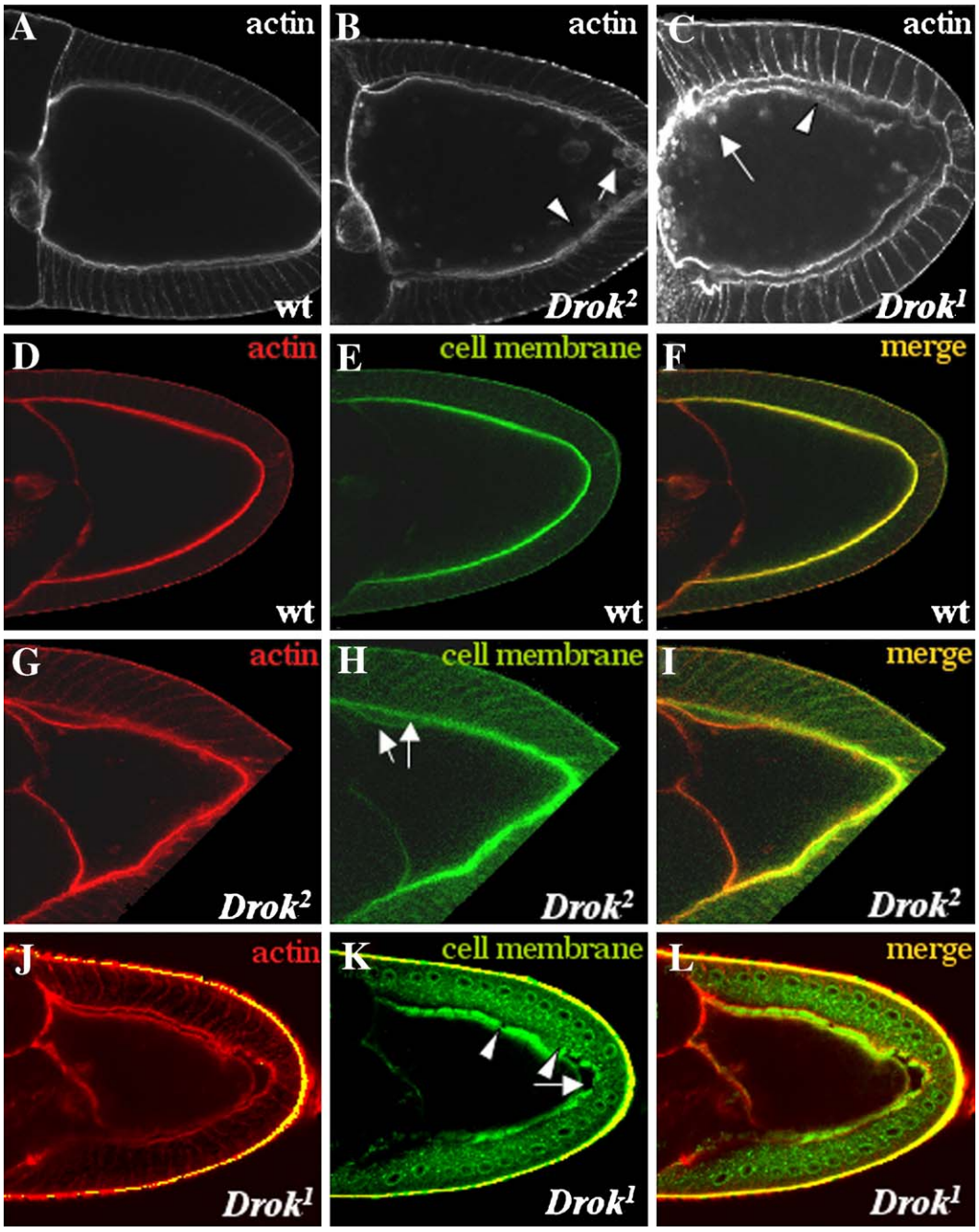


Fig. 4. Subcortical F-actin organization and plasma membrane integrity are disrupted in DRok-deficient oocytes. (A–C) Phalloidin staining of a wild type (A), a *Drok*^{2} mutant (B) and a *Drok*^{1} mutant (C) stage 10a oocytes. *Drok*^{2} and *Drok*^{1} mutants display disorganized F-actin at the cortex of the oocyte (B and C, arrowheads pointing to discontinuous F-actin staining), along with the presence of actin clumps near the cortex or within the ooplasm (B, C, arrows). (D–L) Visualization of the F-actin network (D, G and J), using phalloidin, and cell membranes (E, H and K), using a fluorescein-conjugated lectin. (F, I and L) are the merged images of actin and membrane stainings. In the wild-type stage 9 oocyte, the lectin co-localizes with F-actin and outlines juxtaposed membranes between the oocyte and the surrounding follicle cells (F). Mutant stage 9 or 10 oocytes display co-localization of F-actin and lectin (I) but exhibit an abnormal lectin distribution, associated with an apparent detachment of the oocyte membrane from the follicle cell layer (H, arrows), as well as a defect in the oocyte shape itself. This lectin defect was also observed in *Drok*^{1} mutant oocytes (K, arrow). Anterior is to the left and dorsal is to the top.

In addition to the actin defects described above, we frequently observe a significant deformation along localized regions of the cortical membrane in *Drok*^{2} GLC oocytes, resulting in protrusions into the oocyte cytoplasm in a majority of stage 9 and later stage oocytes (Table 1). To determine whether these deformations result from an abnormally shaped oocyte or reflect a detachment of subcortical actin from an otherwise normal cell membrane, we used a cell membrane-specific marker (fluorescently labeled lectin) that co-localizes with F-actin and outlines juxtaposed membranes between the oocyte and the surrounding follicle cell layer in a wild-type egg chamber (Figs. 4D–F). In *Drok*^{2} GLCs, the lectin marker co-

localizes normally with F-actin, revealing an abnormal oocyte cell shape and a significant detachment of the oocyte membrane from the apical membrane of follicle cells (Figs. 4G–I). The expressivity of that phenotype ranged from mild (stage 9 egg chambers) to very severe (stage 10 and 11 egg chambers) (data not shown), suggesting that the defect progresses during oocyte development. In severe cases, the oocyte membrane seems to lose its integrity, as indicated by the appearance of diffuse lectin staining between the oocyte and the follicle cell layer (data not shown). Moreover, strong phenotypes are associated with discontinuous lectin staining of the oocyte membrane (Fig. 4K, arrowheads in *Drok*^{1} mutant oocytes). These results suggest that DRok is required for the integrity of the oocyte plasma membrane and is consequently required to maintain the proper shape of the oocyte. Similarly, *Drok*^{1} germline clones are characterized by a disorganized subcortical F-actin network associated with the presence of actin clumps and a reduction in plasma membrane integrity (Figs. 4C, J–L and Table 1), confirming that *Drok*^{2} germline clone-generated defects are due specifically to the genetic disruption of the *Drok* gene. These results suggest that DRok is required for the integrity of the oocyte plasma membrane and is consequently required to maintain the proper shape of the oocyte.

Drok^{2} *mutant nurse cells exhibit a "dumpless-like" phenotype*

During oogenesis, the 15 polyploid nurse cells of the egg chamber continually provide the oocyte with nutrients, proteins, and maternal RNAs to support its growth through actin-rich structures called rings canals. At stage 11 of oogenesis, the entire cytoplasmic content of the nurse cells is rapidly transferred to the oocyte within 30 min, in a process known as "dumping" (see Spradling, 1993 for a review of oogenesis). Nurse cell dumping was previously shown to depend upon an intact actin cytoskeleton (Cant et al., 1994; Verheyen and Cooley, 1994; Xue and Cooley, 1993). In wild-type stage 13 egg chambers, the nurse cells retain a cluster of nuclei after they have dumped all of their cytoplasm into the oocyte. In contrast, in similarly staged *Drok*^{2} (or *Drok*^{1}) GLC egg chambers, the nurse cells have retained most of their cytoplasm (Figs. 5A–C). This dumpless-like nurse cell phenotype was observed in most late stage *Drok*^{2} (or *Drok*^{1}) mutant egg chambers and results in the generation of either small eggs (50%) (Fig. 5B, Table 1) or normal size eggs (50%) that appear "deflated" and presumably lack much of their normal cytoplasmic content (Fig. 5C).

As has been previously observed in other dumpless mutants, including *chickadee*, *quail* or *singed*, failure of cytoplasmic transport is often due to the obstruction of the ring canals by nurse cell nuclei that fail to be properly tethered by actin filaments (Cant et al., 1994; Mahajan-Miklos and Cooley, 1994; Verheyen and Cooley, 1994). Therefore, we determined whether *Drok*^{2} egg chambers exhibit an abnormal actin cytoskeleton and whether ring canal obstruction potentially accounts for the dumpless-like phenotype. The Anti-Hts-RC (Hu li tai shao-Ring Canals) antibody was used to detect the adducin cytoskeletal protein, a specific actin-binding component of the ring canals (Yue and Spradling, 1992). In contrast to anti-Hts-RC staining in wild-type, *Drok*^{2} GLCs at stage 10b or 11 revealed a somewhat irregular and square shape of the ring canals in most GLCs (93%) with between one and four abnormally shaped canals per egg chamber, but no major difference in the overall adducin distribution and number of ring canals (Figs. 5J, K, Table 1). Phalloidin staining to reveal F-actin in ring canals yielded similar findings (Figs. 5D, E). These findings indicate that DRok is not required for the formation of ring canals but appears to play some role in either establishing or maintaining their normal morphology.

Additional analysis of F-actin organization within the nurse cells revealed that while wild-type egg chambers typically exhibit thick F-actin filaments extending from the plasma membranes towards the nurse cell nuclei in order to anchor them, the F-actin network, although present in some clones, is perturbed in nurse cells within the vast majority of *Drok*^{2} or *Drok*^{1} GLCs (86% and 72%, respectively) (Table 1). Specifically F-actin filaments are generally much thinner and shorter in those rare cells where they do form (Figs. 5D, E) and do not span the nurse cell cytoplasm from the plasma membrane to the nucleus (Figs. 5F, G). This suggests two potential defects. On the one hand, the acto-myosin contractile apparatus may be affected, which would prevent nurse cells from contracting properly, thereby altering cytoplasmic transport. On the other hand, actin-mediated anchoring of the nuclei may be defective, which could obstruct the ring canals, consequently preventing cytoplasmic transport. To address the latter possibility, we used double staining of F-actin and nuclear membranes to reveal the F-actin network which normally tethers nuclei to the center of each nurse cell (Fig. 5H). In *Drok*^{2} GLCs, staining clearly reveals the lack of functional F-actin filaments within nurse cells, as well as the presence of nuclei of increased size (about 30% increased volume compared to wild-type nuclei) that are not properly localized near the center of the nurse cells. Significantly, while these enlarged nuclei are in close proximity to the ring canals, they do not appear to obstruct them (Fig. 5I). DRok-deficient nurse cells (in *Drok*^{2} or *Drok*^{1} GLCs) also exhibit a less structured and "looser" subcortical F-actin organization (Fig. 5I) when compared to wild-type nurse cells (Fig. 5H). Taken together, these results indicate that while DRok is not required for the formation of ring canals, it is essential for cytoplasmic transport. Moreover, lack of cytoplasmic transport in *Drok*^{2} GLCs does not result from obstruction of the ring canals by untethered nurse cell nuclei, which clearly distinguishes *Drok* from the "*chickadee, singed* or *quail*" class of dumpless mutants. In addition, DRok is required for maintaining the plasma membrane integrity of nurse cells.

DMoesin membrane localization is disrupted in DRok-deficient oocytes

In mammalian cells, Rho-kinase has been shown to directly phosphorylate the ERM (Ezrin–Radixin–Moesin) family protein Moesin in vitro (Matsui et al., 1998; Oshiro

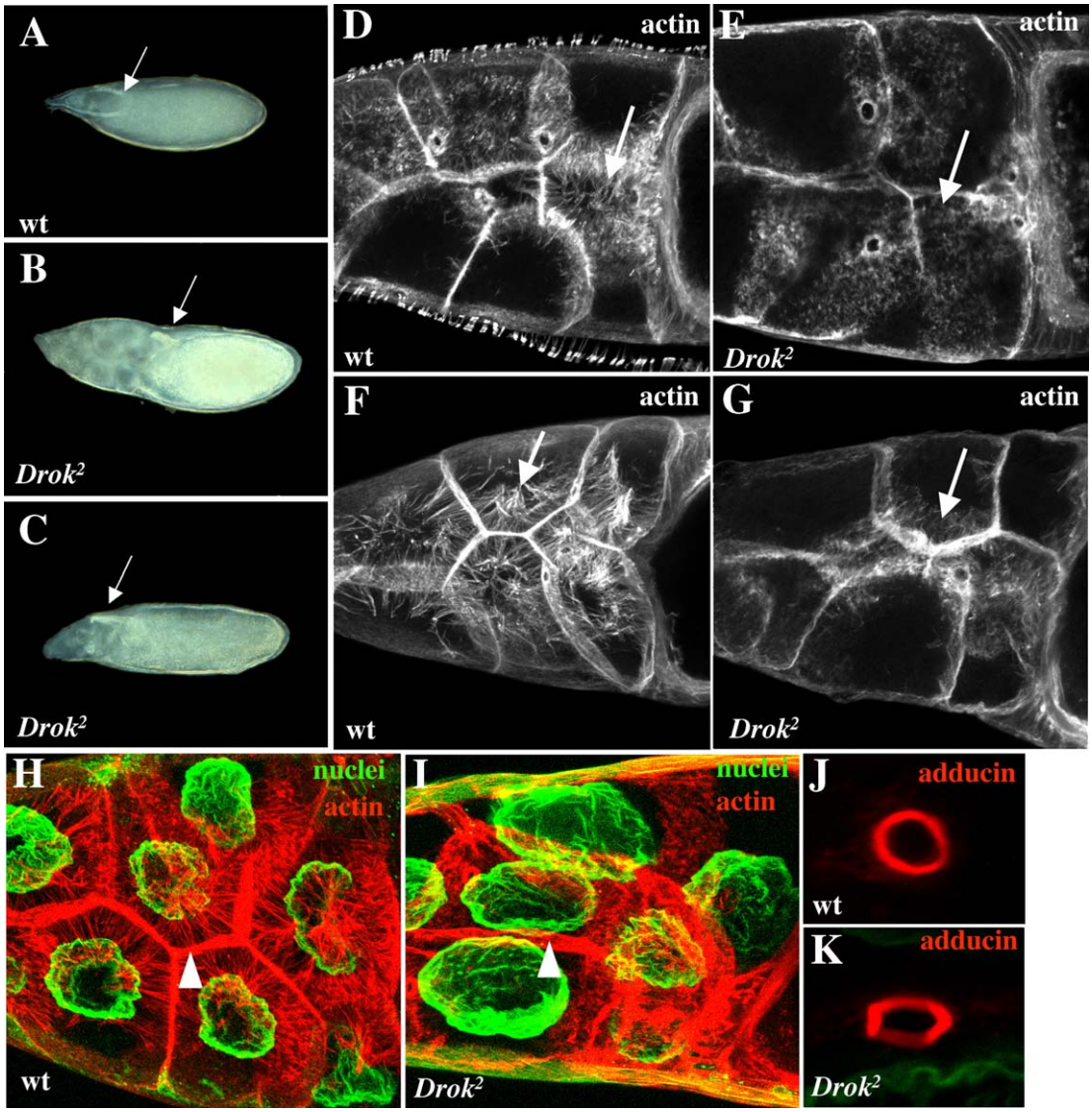


Fig. 5. DRok is required for cytoplasmic transport during oogenesis. (A–C) Phase contrast images of wild-type (A) and *Drok*^{2} GLC (B, C) stage 13 egg chambers. The arrows denote the newly forming dorsal appendages, a marker of stage 13 egg chambers. In panel (A), the nurse cells have dumped their entire cytoplasmic contents into the oocyte and are no longer visible at the anterior region of the egg chamber, whereas DRok-deficient nurse cells retain most of their cytoplasm, resulting in a much smaller egg (B). (C) An example of a less severe dumpless-like phenotype where the mutant oocyte is about the size of a wild-type oocyte but appears somewhat "deflated". (D–G) Phalloidin staining of wild-type stage 10b (D) and stage 11 (F), and *Drok*^{2} mutant stage 10b (E) and stage 11 (G) nurse cells. The rapid phase of cytoplasmic transport takes place from stage 10b until the end of stage 11. In wild-type nurse cells, thick transverse F-actin filaments initially form around the nurse cell cortex at stage 10b (D, arrow) and assemble in bundles all around the nucleus, extending into the nurse cell cytoplasm between the nucleus and the plasma membrane at stage 11 (F, arrow). By contrast, in DRok-deficient nurse cells, F-actin filaments are much thinner and shorter when they do form, and they fail to extend from the plasma membrane to the center of the cells (E, G, arrows). (H, I) Double F-actin and nuclear staining of wild-type and *Drok*^{2} mutant stage11 nurse cells. In wild-type nurse cells, the nuclei remain anchored at the center of each cell by the surrounding F-actin filament bundles. In mutant nurse cells, the nuclei, which appear larger, are clearly displaced from their normal centered localization within the nurse cells (I). The arrowheads point to the subcortical F-actin network which appears more diffuse and disorganized in the mutant nurse cells. (J, K) High magnification images of a ring canal from a wild-type (J) or a *Drok*^{2} mutant (K) nurse cell stained with an anti-adducin antibody (anti-Hts-RC). These doughnut-shaped actin and actin-binding protein-rich structures of wild-type nurse cells are somewhat abnormally shaped and are more "squared" in *Drok*^{2} mutant nurse cells, but their size and number do not vary between the two genotypes. Anterior is to the left and dorsal to the top.

et al., 1998). In vivo, Rho-kinase-dependent phosphorylation of ERM proteins has been reported to vary according to cell type, and it is presently unclear as to whether Rho-kinase acts directly or indirectly to modify ERM phosphorylation (Ivetic and Ridley, 2004; Jeon et al., 2002; Matsui et al., 1999; Oshiro et al., 1998). In *Drosophila*, genetic disruption of the closely related ortholog, *Dmoesin*, results in a phenotype in developing oocytes that resembles the F-actin defects seen in *Drok2* GLCs, including the accumulation of F-actin clumps within the oocyte cytoplasm (Polesello et al., 2002). ERM proteins have been implicated in several biological processes, including cell–cell adhesion, maintenance of cell shape, and cell motility. Upon carboxy-terminal threonine phosphorylation, they are recruited to the plasma membrane, where they function as cross-linkers between the cell membrane and the actin cytoskeleton

(Bretscher, 1999; Mangeat et al., 1999). DMoesin is required for anchoring F-actin filaments to the plasma membrane in the oocyte, and this function requires phosphorylation of T559 in DMoesin, which is analogous to a Thr site in mammalian Moesin (Fig. 6D) that can be directly phosphorylated by Rho-kinases in vitro (Jankovics et al., 2002; Matsui et al., 1998; Oshiro et al., 1998; Polesello et al., 2002). Therefore, we tested the possibility that membrane localization of DMoesin is disrupted in the oocytes of *Drok*^{2} GLCs.

Using an antibody directed against the conserved T558 phospho-peptide site in Moesin, we observe that phospho-DMoesin localizes to the oocyte plasma membrane in a wild-type egg chamber (Fig. 6A). However, in *Drok*^{2} GLCs, there is a significant reduction in phospho-DMoesin at the oocyte cortex (Fig. 6B, Table 1). In most clones, the cortex still retains some phospho-DMoesin staining which appears more diffuse relative to the staining seen in wild-type oocytes (Fig. 6C). In addition, in ~20% of the GLCs, phospho-DMoesin is also found mislocalized in patches within the ooplasm (Fig. 6C). Figures B and C represent relatively strong and weak phenotypes, respectively, with regard to decreased phospho-DMoesin at the oocyte plasma membrane. However, the overall distribution and expression level of total DMoesin is unchanged in *Drok*^{2} mutant oocytes, exhibiting a diffuse staining pattern throughout the ooplasm that is indistinguishable from the staining seen in wild-type oocytes (Figs. 6D, E). Taken together, these observations suggest that proper membrane localization of DMoesin requires DRok activity.

We then determined whether Rho-kinase can directly phosphorylate DMoesin on the T559 residue using an in vitro kinase assay with purified recombinant proteins. In this experiment, we used the commercially available purified kinase domain corresponding to mammalian Rho-kinase (mRok), which is highly conserved with the kinase domain of DRok (Fig. 6E). In the in vitro assay, mRok undergoes autophosphorylation, as expected, and additionally, is able to directly phosphorylate DMoesin (Fig. 6F). The observed phosphorylation of DMoesin is virtually abolished when using a GST-DMoesin-TA mutant in which the putative phosphorylation site was substituted with an alanine residue (Fig. 6F). Taken together, these findings suggest that direct phosphorylation of DMoesin by DRok on a single site facilitates the association of DMoesin with the cortical oocyte membrane during oogenesis to maintain its proper integrity.

Overall, the similarity of antero-posterior polarity, microtubule cytoskeleton integrity and F-actin distribution phenotypes between *Drok*^{2} and *Dmoe* GLCs, along with the cell biology and biochemical phosphorylation data suggests that DRok and its putative effector protein DMoesin interact in the developing oocyte and that DRok mediates some, but not all, of its biological effects through DMoesin. However, *Drok*^{2} and *Dmoe* GLCs are not identical. For example, *Drok*^{2} GLCs exhibit a yolk granule transport phenotype, whereas *Dmoe* GLCs do not. Moreover, the oocyte plasma membrane integrity is more severely affected in *Drok*^{2} GLCs than in *Dmoe* GLCs. These differences imply that DRok must signal through effector proteins other than DMoesin to exert additional distinct effects during oogenesis.

Discussion

In this study, we have determined that the single closely related *Drosophila* ortholog of the mammalian Rho-kinases, DRok, is required for oogenesis and participates in several distinct aspects of this complex developmental processes, including organization of the oocyte cortex, cytoplasmic transport from nurse cells, and germline-soma cross-signaling necessary for establishment of the antero-posterior and dorso-ventral axis of the resulting mature egg. In addition, we found that DRok is required for a developmental process around stages 7–8 of oogenesis, which enables the yolk granules to move freely in the ooplasm after they have been internalized or transferred to the oocyte and before cytoplasmic streaming can take place (stage 10b and higher). These findings, when considered in the context of a variety of other previously reported mutants that exhibit oogenesis phenotypes, suggest that DRok represents a novel class of oogenesis regulators.

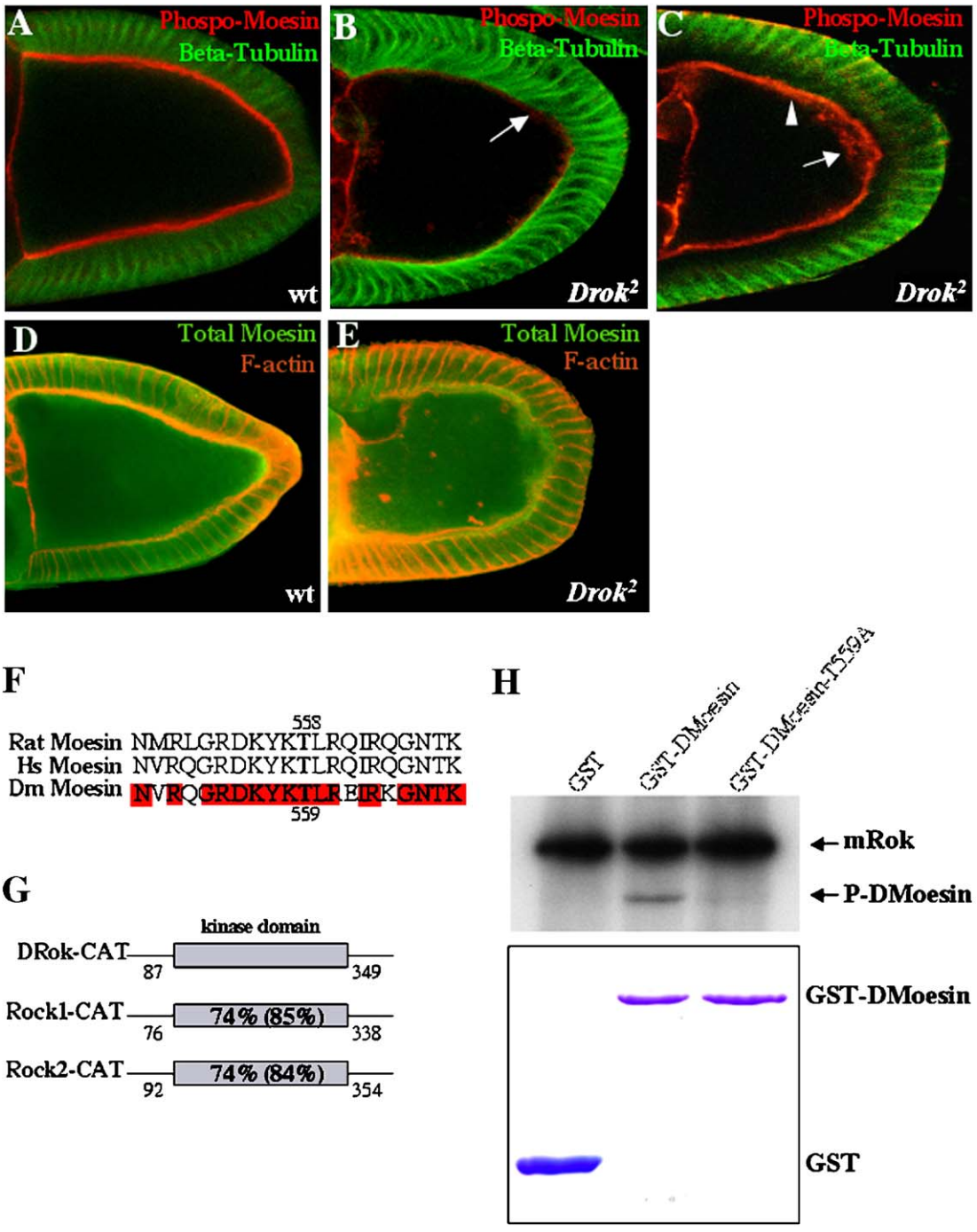
DRok has been previously implicated as an effector of the DRho1 GTPase in the regulation of planar cell polarity in the eye and in the wing, downstream of Frizzled-Dishevelled signals (Winter et al., 2001). In germline cells, DRok and DRho1 mutants exhibit some overlapping actin defects; e.g., the oocyte cortex exhibits a more diffuse F-actin distribution in both *Drok*^{2} GLCs and *Rho1* loss-of-function egg chambers in which Rho1 levels have been reduced in a heterozygous mutant *Rho1* and *wimp* background (*Rho1* GLCs are not viable) (Magie et al., 1999). In addition, wild-type oocytes injected with the Rho-inhibitory C3 toxin exhibit the same ooplasmic streaming defects as *Drok*^{2} mutant oocytes, as discussed below, strongly suggesting that the germline clone phenotypes reflect the disruption of DRho1-DRok signaling in germ cells.

DRok regulates oocyte polarity

Previous analysis of the polarity of *Dmoe* GLC oocytes indicated that DMoesin is specifically required for the localization of posterior determinants such as *oskar* mRNA and Oskar protein but not the formation of the dorso-ventral axis nor the anterior pole. DMoesin appears to function in maintaining posterior polarity by anchoring actin to the membrane cortex which in turn anchors microtubule-delivered *oskar* mRNA and its protein product Oskar to the posterior pole (Polesello et al., 2002). Similarly, in *Drok*^{2} GLCs, the localization of anterior (*bicoid*) or dorso-ventral determinants (*gurken*) is not altered although most *oskar* mRNA is found mislocalized within the ooplasm starting at stage 9. While the establishment of oocyte polarity generally depends upon microtubule cytoskeleton organization (Theurkauf et al., 1992), it has been reported that *Dmoe* null mutations do not disrupt the microtubule cytoskeleton and do not perturb its polarity (Polesello et al., 2002). Similarly, we observed that microtubules in *Drok*^{2} mutant oocytes appear normal. Taken together with the fact

that some *oskar* mRNA remains anchored at the posterior tip of the oocyte plasma membrane in *Drok*^{2} GLCs, as is seen in *Dmoe* GLCs, this indicates that *oskar* mislocalization, and consequently, the alteration of posterior polarity in *Drok*^{2} GLCs is not due to an abnormally organized microtubule cytoskeleton. Moreover, unlike other germline clone mutants with oocyte polarity defects, such as *chic* or *capu*, the *Drok*^{2} and *Dmoe* polarity defects most likely reflect the incapacity of the disorganized subcortical actin cytoskeleton to properly anchor *oskar* at the posterior membrane of the oocyte.

The similarity between the *oskar* polarity phenotype of *Drok*^{2} and *Dmoe* GLCs is also consistent with a likely role for DMoesin as an essential DRok substrate that mediates its effects on the formation of posterior polarity and further supports the functional significance of a signaling pathway from DRok to DMoesin to the actin cytoskeleton in oocyte development. Moreover, the proper localization of Gurken, as defined by the position of the oocyte nucleus, which migrates in a microtubule-dependent manner from the posterior to the anterior and then to the antero-dorsal side of the oocyte starting at stage 8, is consistent with the



presence of a grossly normal appearing microtubule cytoskeleton in DRok-deficient oocytes.

DRok plays a role in germline-soma cross-signaling

A majority of mutations resulting in egg chambers with dorso-ventral axis patterning defects, such as dorsal appendages aberrations, have been associated with genes encoding components of the Gurken-EGFR signaling pathway (Neuman-Silberberg and Schupbach, 1994, 1996). Cross-signaling between the oocyte and the surrounding follicle cells at the antero-dorsal side of the oocyte has been extensively studied and involves the binding of the secreted Gurken ligand to the EGFR present on the apical membrane of follicle cells and subsequent activation of downstream signaling to control the formation of follicle cell-derived dorsal structures (Nilson and Schupbach, 1999). Although Gurken localization is correct in *Drok*^{2} mutant oocytes, loss of Gurken secretion (in 80% of the *Drok*^{2} mutant oocytes) in the intercellular space between the oocyte membrane and the follicle apical membranes indicates the likelihood of altered communication between the oocyte and surrounding follicle cells, possibly resulting in a disruption of the EGFR signaling pathway leading to dorsal appendage defects. The observed requirement for DRok in Gurken secretion may reflect a well established role of Rho signaling in the control of vesicular trafficking and secretion (Symons and Rusk, 2003). However, it remains possible that the apparent absence of Gurken secretion into the intercellular space reflects a consequence of the observed disruption of oocyte plasma membrane integrity.

DRok and the trafficking of yolk granules in the early oocyte

The unexpected observation in our time-lapse confocal microscopy studies that most autofluorescent yolk granules in *Drok*^{2} mutant oocytes or C3-treated wild-type egg chambers accumulate at the oocyte membrane suggests a role for DRho1 and DRok in early vitellogenesis. Vitellogenesis is a process that begins around stage 8 and is defined by the co-secretion of vitelline membrane and yolk material by the surrounding follicle cells leading to the eventual formation of chorionic structures of the egg and normal oocyte growth, respectively. After their secretion, yolk proteins are internalized into the oocyte through endocytosis and are swirled around the ooplasm at later stages, when microtubule-dependent streaming occurs (Spradling, 1993). The high concentration of yolk granules at the oocyte membrane from early vitellogenesis underlies a possible defect in endocytosis of the yolk granules. Together with the fact that C3-treated egg chambers and *Drok*^{2} GLCs exhibit an identical yolk granule phenotype, this suggests that DRok mediates Rho1's role in the trafficking of yolk granules at the oocyte plasma membrane. In addition, nurse cells also normally accumulate yolk material and transfer it to the oocyte. The detection of yolk granules moving to the plasma membrane of *Drok*^{2} mutant oocytes or oocytes in C3-treated egg chambers after they are deposited by the nurse cells is an intriguing phenotype that has not been previously reported and may reflect a trafficking defect in the ooplasm. Further studies to examine molecular components of the endocytic machinery will be required to develop a better understanding of the roles of Rho1 and DRok in yolk granule trafficking within the ooplasm. Notably, it is also conceivable that alteration of oocyte plasma membrane integrity through disruption of actin cytoskeleton organization in most *Drok*^{2} GLCs, as we have observed, could exert a secondary effect on the endocytosis of yolk granules.

Because of the yolk granule phenotype in *Drok*^{2} GLCs in early oogenesis, it is not possible to visualize microtubule cytoskeleton dynamics at later stages in time-lapse confocal microscopy. Thus, it is difficult to determine whether *Drok*^{2} mutant oocytes would undergo normal or premature ooplasmic streaming at stages 10b–11. As a functional relationship between actin and microtubule cytoskeletons has been suggested based on findings with several mutants with oogenesis defects, it is quite conceivable that the abnormalities of the actin cytoskeleton in *Drok*^{2} mutant oocytes could affect microtubule cytoskeleton dynamics. Indeed, it has been demonstrated that some aspect of the actin cytoskeleton normally represses microtubule-based streaming within the oocyte (Manseau et al., 1996). Thus, it is possible that the accumulation of yolk granules near the plasma membrane of *Drok*^{2} mutant oocytes

Fig. 6. DMoesin membrane localization is disrupted in DRok-deficient oocytes. (A–C) Double immunostaining of wild-type (A) and *Drok*^{2} mutant stage 10 oocytes (B, C), using an antibody specific for phospho-T558 mammalian Moesin and an antibody against β-tubulin. Phospho-DMoesin localizes to the oocyte plasma membrane in the wild-type oocyte (A). In *Drok*^{2} GLCs, phospho-DMoesin is significantly decreased at the oocyte cortex (B, arrow). In most clones, the cortex still retains some phospho-DMoesin staining, but it appears more diffuse (C, arrowhead). About 20% of the *Drok*^{2} mutant oocytes also exhibit phospho-DMoesin patches in their ooplasm (C, arrow). (D, E) Double immunostaining of wild-type (D) and *Drok*^{2} mutant stage 10 oocytes (E), using an antibody against total DMoesin and phalloidin. Both wild-type and *Drok*^{2} mutant oocytes exhibit a similar diffuse pattern of total DMoesin throughout the ooplasm with no significant difference in DMoesin expression levels between the two genotypes. Anterior is to the left and dorsal is to the top. (F) Alignment of the amino acid sequences surrounding the specific threonine residue (in bold type) defined at position 558 in human and rat Moesin and position 559 in *Drosophila* Moesin. Note the significant identity between all three sequences (identical residues are shaded in red). (G) Schematic representation of the catalytic domains of DRok (DRok-CAT) and mammalian Rho-kinases, mRok1 and mRok2 (mRok1-CAT, mRok2-CAT) with the numbered start and ending amino acids for each domain. The bold type numbers within the kinase domains correspond to the percentage identity and similarity (in parentheses) between DRok-CAT and either mRok1-CAT or mRok2-CAT. (H) In vitro kinase assay, using the commercially available purified kinase domain of mammalian Rho-kinase (mRok) and GST recombinant proteins. Kinase reactions included ^{32}P-ATP, and the indicated bands correspond to radiolabeled proteins visualized by autoradiography following protein resolution by SDS-PAGE. As expected, autophosphorylation of mRok is detected in all reactions. In the presence of GST-DMoesin, mRok directly phosphorylates DMoesin, and the phosphorylation is abolished when using a GST-DMoesin-T559A mutant (threonine 559 is replaced by an alanine residue) (upper panel). Recombinant proteins were separately assessed after separate SDS-PAGE and Coomassie Blue staining to confirm that equal amounts of protein were used in the assays (lower panel).

reflects a combination of trafficking/endocytosis defects and actin cytoskeleton perturbation-induced alteration of microtubule cytoskeleton dynamics in the ooplasm during early oogenesis.

DRok is required in the nurse cells to ensure their contractility during nurse cell dumping

The oocyte volume in *Drok*^{2} GLCs is frequently smaller than that seen in wild-type oocytes, before the rapid phase of cytoplasmic transport takes place. This suggests a possible defect in the slow phase of cytoplasmic transport. It has been previously reported that transport of some particles towards the oocyte during stages 7–10A depends upon a proper acto-myosin network. In addition, *sqh*^{AX3} GLCs exhibit a similar oocyte size defect. *sqh*^{AX3} is a loss-of-function mutation in the *sqh* locus which codes for the *Drosophila* ortholog of myosin light chain of myosin II (Jordan and Karess, 1997). Taken together with the fact that DRok has been shown to phosphorylate Sqh in vivo (Amano et al., 1996; Winter et al., 2001), these data suggest that DRok mediates, via regulation of Sqh, some aspects of the acto-myosin contractility involved in cytoplasmic transport from early stages of oogenesis.

The observation of dumpless-like oversized nurse cells in most of *Drok*^{2} GLCs also supports a role for DRok in the rapid phase of cytoplasmic transport at stages 10B–11 of oogenesis. Unlike other classes of dumpless mutants including *chickadee*, *singed* or *quail*, failure of rapid cytoplasmic transport from the *Drok*^{2} mutant nurse cells to the oocyte does not result from the obstruction of the ring canals by unanchored nurse cell nuclei, suggesting that *Drok* constitutes a distinct class of dumpless-like mutants. In addition, in *sqh*^{AX3} GLCs, dumpless nurse cells are associated with a lack of acto-myosin contractility by nurse cells, as revealed by mislocalization of myosin II and by absence of the perinuclear organization of actin filaments bundles in the nurse cells. Therefore, *sqh*^{AX3} mutant nurse cells cannot contract properly to expulse their cytoplasm through otherwise weakly damaged ring canals. *Drok*^{2} and *sqh*^{AX3} mutant nurse cells do not share the same actin filament phenotype, as *Drok*^{2} mutant nurse cells exhibit a more dramatic phenotype associated with absence of radial filaments and disorganization of cortical actin. It is, however, likely that DRok and Sqh are part of the same signaling pathway that regulates acto-myosin contractility in nurse cells, as it has already been shown that DRok phosphorylates Sqh in *Drosophila* development. Moreover, the severity of the *Drok*^{2} mutant F-actin phenotypes may reflect DRok's potential to engage multiple distinct downstream substrates, of which Sqh is only one. Significantly, the actin-binding protein, adducin, is also reportedly a direct substrate for mammalian Rho-kinases (Fukata et al., 1999b), and the *Drosophila* Adducin ortholog, Hts, is a major component of ring canals (Yue and Spradling, 1992). Thus, it is possible that the observed defects in ring canal morphology in *Drok*^{2} GLCs involve abnormal regulation of adducin by DRok. However, it is difficult to determine whether this ring canal phenotype contributes to the dumpless-like nurse cell phenotype observed in *Drok*^{2} GLCs.

The observation that nurse cell nuclei are substantially increased in size in *Drok*^{2} GLCs suggests a possible involvement of DRok in increased endoreplication of the nurse cells. The Rho-related Rac and Cdc42 GTPases have previously been associated with endoreplication in porcine aortic endothelial (PAE) cells, although Rho has not been implicated thus far (Muris et al., 2002). Interestingly, this nurse cell nuclei phenotype has not been observed in other previously described GLC mutants of other actin cytoskeleton-regulating signaling components that exhibit oogenesis defects. Thus, *chic* as well as *sqh*^{AX3} GLCs reveal cytokinesis defects associated with the presence of multinucleated nurse cells. In addition, the majority of *sqh*^{AX3} mutant egg chambers harbor less than 15 nurse cells (64% of *sqh*^{AX3} mutant egg chambers have less than 7 nurse cells), a phenotype that is not shared by *Drok*^{2} mutant nurse cells (Jordan and Karess, 1997; Manseau et al., 1996). These findings also suggest that *Drok*^{2} defines a new category of oogenesis mutants that affect the actin cytoskeleton.

DRok is required for oocyte plasma membrane integrity

Both *Dmoe* and *Drok*^{2} GLCs exhibit similar actin defects in the oocyte, associated with a loose uneven cortical actin distribution and the presence of actin clumps in the ooplasm and near the cortex. Moreover, phospho-DMoesin levels are decreased at the cortex or mislocalized within the ooplasm of *Drok*^{2} GLCs and the conserved kinase domain of Rho-kinase phosphorylates DMoesin on threonine 559 in vitro. A potential mechanism for the DRok-DMoesin signal in this setting is that DRok controls actin reorganization through phosphorylation of DMoesin, which has been previously shown to cross-link actin to the plasma membrane when phosphorylated on T559 at the oocyte cortex (Polesello et al., 2002). However, the detection of some phospho-DMoesin in the *Drok*^{2} GLCs indicates that the critical T559 residue can be phosphorylated by other kinases in the oocyte. Indeed, direct phosphorylation of T559 of mammalian Moesin by protein kinase C (PKC)-θ has been shown in vitro (Pietromonaco et al., 1998). In addition, mammalian Rho-kinase and PAK have been reported to both phosphorylate the very conserved T508 residue of LIM-kinase in vitro (Edwards et al., 1999; Ohashi et al., 2000). Therefore, phosphorylation of the conserved T559 residue of Moesin by additional kinases might also occur in *Drosophila*, highlighting the complexity of cross-talk within developmental signaling pathways.

The observation that *Drok*^{2} mutant oocytes are morphologically more affected than *Dmoe* mutant oocytes with regard to the deformed plasma membrane (Fig. 4; 82% of the GLCs) also suggests that to exert its functions at the oocyte cortex, DRok is not only signaling to DMoesin but probably also to additional downstream targets that cooperate with DMoesin in the maintenance of the cortical actin cytoskeleton. The strong phenotype associated with the deformed oocyte plasma membrane, which separates dramatically from the apical plasma membranes of the follicle cell layer in most *Drok*^{2} GLCs (82%, Table 1), raises an intriguing question about DRok's apparent role in an adhesive process. That

specific phenotype has not been previously reported in studies of other oogenesis mutants associated with defective adhesion between the oocyte and the surrounding follicle cells. Previous reports regarding such adhesion largely address cross-signaling between the apical Notch receptor and the germline-derived putative secreted and transmembrane proteins, Brainiac and Egghead, respectively, in which germline loss of either Brainiac or Egghead results in loss of epithelial apico-basal polarity and accumulation of follicular epithelial cells in multiple layers around the oocyte, but does not lead to a physical separation between the oocyte and the follicle cells membranes (Goode et al., 1996). The unique phenotype of *Drok*^{2} GLCs could reflect a role for DRok in mediating a distinct signaling pathway from the oocyte to regulate its shape and its adherence to the surrounding follicle cells. Alternatively, the aberrant morphology of the nurse cells, which appear to "push" against the oocyte without contracting, might produce a mechanical stress on the oocyte itself that prevents it from remaining apposed to the follicle cell layer. Notably, we have also found that the follicle cells themselves also appear to require DRok function for the maintenance of their shape, and it is possible that their ability to signal to the oocyte is also affected by DRok deficiency.

In summary, we have determined that the single closely related *Drosophila* Rho-kinase ortholog, DRok, is required for several aspects of oogenesis, including maintaining the integrity of the oocyte cortex, actin-dependent tethering of nurse cell nuclei, "dumping" of nurse cell contents into the oocyte, establishment of oocyte polarity, and the trafficking of oocyte yolk granules. It is likely that several previously identified direct phosphorylation targets of DRok, including DMoesin, Sqh (myosin light chain), and Hts (adducin), which have each been implicated in various aspects of oogenesis, mediate at least some of the functions of DRok in developing egg chambers. These findings indicate an essential role for Rho-DRok signaling via multiple DRok effectors in several distinct aspects of oogenesis.

Acknowledgments

We are grateful to Laurel Raftery, Andi McClatchey, Lynn Cooley, and members of the Cooley and Settleman laboratories for their helpful discussions. We thank Alexey Veraksa for the assistance with confocal microscopy and Martha Betson for the critical comments on the manuscript. Reagents were generously provided by Liqun Luo, Daniel Kiehart and Lynn Cooley. Some *Drosophila* stocks and antibodies were obtained from the Bloomington Stock Center and Developmental Studies Hybridoma Bank, respectively. This work was supported by NIH grants RO1 GM60466 to J.S. and RO1 GM066847 to S.M.P.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.05.016.

References

Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y., Kaibuchi, K., 1996. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J. Biol. Chem. 271, 20246–20249.

Amano, M., Chihara, K., Nakamura, N., Fukata, Y., Yano, T., Shibata, M., Ikebe, M., Kaibuchi, K., 1998. Myosin II activation promotes neurite retraction during the action of Rho and Rho-kinase. Genes Cells 3, 177–188.

Bretscher, A., 1999. Regulation of cortical structure by the Ezrin–Radixin–Moesin protein family. Curr. Opin. Cell Biol. 11, 109–116.

Burridge, K., Wennerberg, K., 2004. Rho and Rac take center stage. Cell 116, 167–179.

Cant, K., Knowles, B.A., Mooseker, M.S., Cooley, L., 1994. Drosophila singed, a fascin homolog, is required for actin bundle formation during oogenesis and bristle extension. J. Cell Biol. 125, 369–380.

Cha, B.J., Koppetsch, B.S., Theurkauf, W.E., 2001. In vivo analysis of Drosophila bicoid mRNA localization reveals a novel microtubule-dependent axis specification pathway. Cell 106, 35–46.

Chou, T.B., Perrimon, N., 1996. The autosomal FLP-DFS technique for generating germline mosaics in *Drosophila melanogaster*. Genetics 144, 1673–1679.

Edwards, D.C., Sanders, L.C., Bokoch, G.M., Gill, G.N., 1999. Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. Nat. Cell Biol. 1, 253–259.

Fukata, Y., Oshiro, N., Kaibuchi, K., 1999a. Activation of moesin and adducin by Rho-kinase downstream of Rho. Biophys. Chem. 82, 139–147.

Fukata, Y., Oshiro, N., Kinoshita, N., Kawano, Y., Matsuoka, Y., Bennett, V., Matsuura, Y., Kaibuchi, K., 1999b. Phosphorylation of adducin by Rho-kinase plays a crucial role in cell motility. J. Cell Biol. 145, 347–361.

Ghiglione, C., Bach, E.A., Paraiso, Y., Carraway III, K.L., Noselli, S., Perrimon, N., 2002. Mechanism of activation of the *Drosophila* EGF Receptor by the TGFalpha ligand Gurken during oogenesis. Development 129, 175–186.

Goode, S., Melnick, M., Chou, T.B., Perrimon, N., 1996. The neurogenic genes egghead and brainiac define a novel signaling pathway essential for epithelial morphogenesis during *Drosophila* oogenesis. Development 122, 3863–3879.

Gutzeit, H.O., 1986. The role of microfilaments in cytoplasmic streaming in *Drosophila* follicles. J. Cell Sci. 80, 159–169.

Hall, A., 1998. Rho GTPases and the actin cytoskeleton. Science 279, 509–514.

Hill, C.S., Wynne, J., Treisman, R., 1995. The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. Cell 81, 1159–1170.

Hirose, M., Ishizaki, T., Watanabe, N., Uehata, M., Kranenburg, O., Moolenaar, W.H., Matsumura, F., Maekawa, M., Bito, H., Narumiya, S., 1998. Molecular dissection of the Rho-associated protein kinase (p160ROCK)-regulated neurite remodeling in neuroblastoma N1E-115 cells. J. Cell Biol. 141, 1625–1636.

Ivetic, A., Ridley, A.J., 2004. Ezrin/radixin/moesin proteins and Rho GTPase signalling in leucocytes. Immunology 112, 165–176.

Jankovics, F., Sinka, R., Lukacsovich, T., Erdelyi, M., 2002. MOESIN crosslinks actin and cell membrane in *Drosophila* oocytes and is required for OSKAR anchoring. Curr. Biol. 12, 2060–2065.

Jeon, S., Kim, S., Park, J.B., Suh, P.G., Kim, Y.S., Bae, C.D., Park, J., 2002. RhoA and Rho kinase-dependent phosphorylation of moesin at Thr-558 in hippocampal neuronal cells by glutamate. J. Biol. Chem. 277, 16576–16584.

Jiang, W., Sordella, R., Chen, G.C., Hakre, S., Roy, A.L., Settleman, J., 2005. An FF domain-dependent protein interaction mediates a signaling pathway for growth factor-induced gene expression. Mol. Cell 17, 23–35.

Jordan, P., Karess, R., 1997. Myosin light chain-activating phosphorylation sites are required for oogenesis in *Drosophila*. J. Cell Biol. 139, 1805–1819.

Kawano, Y., Fukata, Y., Oshiro, N., Amano, M., Nakamura, T., Ito, M., Matsumura, F., Inagaki, M., Kaibuchi, K., 1999. Phosphorylation of myosin-binding subunit (MBS) of myosin phosphatase by Rho-kinase in vivo. J. Cell Biol. 147, 1023–1038.

Kim, G.H., Han, J.K., 2005. JNK and ROKalpha function in the noncanonical

Wnt/RhoA signaling pathway to regulate Xenopus convergent extension movements. Dev. Dyn. 232, 958–968.
Kim-Ha, J., Smith, J.L., Macdonald, P.M., 1991. oskar mRNA is localized to the posterior pole of the *Drosophila* oocyte. Cell 66, 23–35.
Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A., Kaibuchi, K., 1996. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science 273, 245–248.
Lai, S.L., Chang, C.N., Wang, P.J., Lee, S.J., 2005. Rho mediates cytokinesis and epiboly via ROCK in zebrafish. Mol. Reprod. Dev. 71, 186–196.
Maekawa, M., Ishizaki, T., Boku, S., Watanabe, N., Fujita, A., Iwamatsu, A., Obinata, T., Ohashi, K., Mizuno, K., Narumiya, S., 1999. Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. Science 285, 895–898.
Magie, C.R., Meyer, M.R., Gorsuch, M.S., Parkhurst, S.M., 1999. Mutations in the Rho1 small GTPase disrupt morphogenesis and segmentation during early *Drosophila* development. Development 126, 5353–5364.
Mahajan-Miklos, S., Cooley, L., 1994. The villin-like protein encoded by the *Drosophila* quail gene is required for actin bundle assembly during oogenesis. Cell 78, 291–301.
Mangeat, P., Roy, C., Martin, M., 1999. ERM proteins in cell adhesion and membrane dynamics. Trends Cell Biol. 9, 187–192.
Manseau, L., Calley, J., Phan, H., 1996. Profilin is required for posterior patterning of the *Drosophila* oocyte. Development 122, 2109–2116.
Matsui, T., Maeda, M., Doi, Y., Yonemura, S., Amano, M., Kaibuchi, K., Tsukita, S., 1998. Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association. J. Cell Biol. 140, 647–657.
Matsui, T., Yonemura, S., Tsukita, S., 1999. Activation of ERM proteins in vivo by Rho involves phosphatidyl-inositol 4-phosphate 5-kinase and not ROCK kinases. Curr. Biol. 9, 1259–1262.
Mizuno, T., Amano, M., Kaibuchi, K., Nishida, Y., 1999. Identification and characterization of *Drosophila* homolog of Rho-kinase. Gene 238, 437–444.
Muris, D.F., Verschoor, T., Divecha, N., Michalides, R.J., 2002. Constitutive active GTPases Rac and Cdc42 are associated with endoreplication in PAE cells. Eur. J. Cancer 38, 1775–1782.
Neuman-Silberberg, F.S., Schupbach, T., 1993. The *Drosophila* dorsoventral patterning gene gurken produces a dorsally localized RNA and encodes a TGF alpha-like protein. Cell 75, 165–174.
Neuman-Silberberg, F.S., Schupbach, T., 1994. Dorsoventral axis formation in *Drosophila* depends on the correct dosage of the gene gurken. Development 120, 2457–2463.
Neuman-Silberberg, F.S., Schupbach, T., 1996. The *Drosophila* TGF-alpha-like protein Gurken: expression and cellular localization during *Drosophila* oogenesis. Mech. Dev. 59, 105–113.
Ng, J., Luo, L., 2004. Rho GTPases regulate axon growth through convergent and divergent signaling pathways. Neuron 44, 779–793.
Nilson, L.A., Schupbach, T., 1999. EGF receptor signaling in *Drosophila* oogenesis. Curr. Top. Dev. Biol. 44, 203–243.
Nobes, C.D., Hall, A., 1995a. Rho, rac and cdc42 GTPases: regulators of actin structures, cell adhesion and motility. Biochem. Soc. Trans. 23, 456–459.
Nobes, C.D., Hall, A., 1995b. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 81, 53–62.
Nobes, C.D., Hall, A., 1999. Rho GTPases control polarity, protrusion, and adhesion during cell movement. J. Cell Biol. 144, 1235–1244.
Ohashi, K., Nagata, K., Maekawa, M., Ishizaki, T., Narumiya, S., Mizuno, K., 2000. Rho-associated kinase ROCK activates LIM-kinase 1 by phosphorylation at threonine 508 within the activation loop. J. Biol. Chem. 275, 3577–3582.
O'Neill, J.W., Bier, E., 1994. Double-label in situ hybridization using biotin and digoxigenin-tagged RNA probes. BioTechniques 17 (870), 874–875.
Oshiro, N., Fukata, Y., Kaibuchi, K., 1998. Phosphorylation of moesin by rho-associated kinase (Rho-kinase) plays a crucial role in the formation of microvilli-like structures. J. Biol. Chem. 273, 34663–34666.
Piekny, A.J., Mains, P.E., 2002. Rho-binding kinase (LET-502) and myosin phosphatase (MEL-11) regulate cytokinesis in the early *Caenorhabditis elegans* embryo. J. Cell Sci. 115, 2271–2282.
Piekny, A.J., Wissmann, A., Mains, P.E., 2000. Embryonic morphogenesis in *Caenorhabditis elegans* integrates the activity of LET-502 Rho-binding kinase, MEL-11 myosin phosphatase, DAF-2 insulin receptor and FEM-2 PP2c phosphatase. Genetics 156, 1671–1689.
Pietromonaco, S.F., Simons, P.C., Altman, A., Elias, L., 1998. Protein kinase C-theta phosphorylation of moesin in the actin-binding sequence. J. Biol. Chem. 273, 7594–7603.
Polesello, C., Delon, I., Valenti, P., Ferrer, P., Payre, F., 2002. Dmoesin controls actin-based cell shape and polarity during *Drosophila melanogaster* oogenesis. Nat. Cell Biol. 4, 782–789.
Qualmann, B., Mellor, H., 2003. Regulation of endocytic traffic by Rho GTPases. Biochem. J. 371, 233–241.
Raftopoulou, M., Hall, A., 2004. Cell migration: Rho GTPases lead the way. Dev. Biol. 265, 23–32.
Ridley, A.J., 1996. Rho: theme and variations. Curr. Biol. 6, 1256–1264.
Riento, K., Ridley, A.J., 2003. Rocks: multifunctional kinases in cell behaviour. Nat. Rev., Mol. Cell Biol. 4, 446–456.
Sahai, E., Alberts, A.S., Treisman, R., 1998. RhoA effector mutants reveal distinct effector pathways for cytoskeletal reorganization, SRF activation and transformation. EMBO J. 17, 1350–1361.
Spradling, A.C., 1993. Germline cysts: communes that work. Cell 72, 649–651.
Symons, M., Rusk, N., 2003. Control of vesicular trafficking by Rho GTPases. Curr. Biol. 13, R409–R418.
Symons, M., Settleman, J., 2000. Rho family GTPases: more than simple switches. Trends Cell Biol. 10, 415–419.
Theurkauf, W.E., Hazelrigg, T.I., 1998. In vivo analyses of cytoplasmic transport and cytoskeletal organization during *Drosophila* oogenesis: characterization of a multi-step anterior localization pathway. Development 125, 3655–3666.
Theurkauf, W.E., Smiley, S., Wong, M.L., Alberts, B.M., 1992. Reorganization of the cytoskeleton during *Drosophila* oogenesis: implications for axis specification and intercellular transport. Development 115, 923–936.
Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M., Narumiya, S., 1997. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. Nature 389, 990–994.
Van Aelst, L., D'Souza-Schorey, C., 1997. Rho GTPases and signaling networks. Genes Dev. 11, 2295–2322.
van Eeden, F., St Johnston, D., 1999. The polarisation of the anterior-posterior and dorsal-ventral axes during *Drosophila* oogenesis. Curr. Opin. Genet. Dev. 9, 396–404.
Verheyen, E.M., Cooley, L., 1994. Profilin mutations disrupt multiple actin-dependent processes during *Drosophila* development. Development 120, 717–728.
Wei, L., Roberts, W., Wang, L., Yamada, M., Zhang, S., Zhao, Z., Rivkees, S.A., Schwartz, R.J., Imanaka-Yoshida, K., 2001. Rho kinases play an obligatory role in vertebrate embryonic organogenesis. Development 128, 2953–2962.
Winter, C.G., Wang, B., Ballew, A., Royou, A., Karess, R., Axelrod, J.D., Luo, L., 2001. *Drosophila* Rho-associated kinase (Drok) links Frizzled-mediated planar cell polarity signaling to the actin cytoskeleton. Cell 105, 81–91.
Wissmann, A., Ingles, J., McGhee, J.D., Mains, P.E., 1997. *Caenorhabditis elegans* LET-502 is related to Rho-binding kinases and human myotonic dystrophy kinase and interacts genetically with a homolog of the regulatory subunit of smooth muscle myosin phosphatase to affect cell shape. Genes Dev. 11, 409–422.
Xue, F., Cooley, L., 1993. kelch encodes a component of intercellular bridges in *Drosophila* egg chambers. Cell 72, 681–693.
Yue, L., Spradling, A.C., 1992. hu-li tai shao, a gene required for ring canal formation during *Drosophila* oogenesis, encodes a homolog of adducin. Genes Dev. 6, 2443–2454.