Cell Type–specific Roles for Cdc42, Rac, and RhoL in *Drosophila* Oogenesis

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Abstract. The Rho subfamily of GTPases has been shown to regulate cellular morphology. We report the discovery of a new member of the Rho family, named RhoL, which is equally similar to Rac, Rho, and Cdc42. Expression of a dominant-negative RhoL transgene in the *Drosophila* ovary caused nurse cells to collapse and fuse together. Mutant forms of Cdc42 mimicked this effect. Expression of constitutively active RhoL led to nurse cell subcortical actin breakdown and disruption of nurse cell–follicle cell contacts, followed by germ cell apoptosis. In contrast, Rac activity was specifically required for migration of a subset of follicle cells called border cells. All three activities were necessary for normal transfer of nurse cell cytoplasm to the oocyte. These results suggest that Rho protein activities have cell type–specific effects on morphogenesis.

A DYNAMIC actin cytoskeleton is required for the cell shape changes, alterations in cell contacts, and cell migrations that occur during morphogenesis (Letourneau and Shattuck, 1989). Reorganization of the actin cytoskeleton can be catalyzed by the activities of small Ras-like GTPases of the Rho family, which is comprised of Rho, Rac, Cdc42, TC10, and RhoG. These proteins display ~30% sequence identity with Ras and 50–60% identity with each other.

Distinct functions for Rho, Rac, and Cdc42 have been characterized in Swiss 3T3 cells. Actin stress fibers and focal adhesions are rapidly induced after microinjection of constitutively active Rho (Ridley and Hall, 1992), whereas the same cells respond to activated Rac by forming lamellipodia and membrane ruffles (Ridley et al., 1992). On the other hand, constitutively active Cdc42 induces the formation of filopodia (Nobes and Hall, 1995). Furthermore, in 3T3 cells, Cdc42 activation is followed by Rac activation, which in turn stimulates Rho, suggesting a cascade of regulation among these proteins (Nobes and Hall, 1995; Chant and Stowers, 1995).

There are some indications that distinct cell types may display different responses to a given Rho family member. For example, neuronal tissue culture cells require RhoA activity for their response to growth factor stimulation, which includes neurite retraction and cell rounding rather than stress fiber formation (Jalink et al., 1994). In another tissue culture cell type, KB cells, both Rac and Rho induce membrane ruffling (Nishiyama et al., 1994), whereas in neutrophils, Rac mediates the activation response, which includes an increase in pinocytosis and stimulation of NADPH oxidase (Abo et al., 1991; Knaus et al., 1991).

In addition to these responses, Rac and Rho have been implicated in the regulation of cell motility. Both proteins can induce lamellipodial structures at the leading edges of motile cells. Furthermore, two specific inhibitors of Rho activity, C3 ADP-ribosyltransferase and Rho-guanine nucleotide dissociation inhibitor, interfere with Swiss 3T3 cell motility, an inhibition that can be rescued by the addition of constitutively active Rho (Takaishi et al., 1993). Further evidence implicating the Rho family in cell migration comes from studies of tumor metastasis. The *Tiam-1* gene was isolated on the basis of its ability to promote invasiveness in murine T lymphoma cells and was found to be highly homologous to a number of GDP–GTP exchange factors that regulate Rho protein activities (Habets et al., 1994). Constitutively active Rac was subsequently shown to mimic the effect of Tiam-1 in promoting invasiveness (Michiels et al., 1995; Qiu et al., 1995).

The ability of Rho protein activities to alter cell shape and affect cell motility raises the question of what roles Rho family proteins play in morphogenesis. To begin to address this question, several genes coding for Rho family members in *Drosophila* have been identified, and characterization of their roles in development has begun (Luo et al., 1994; Harden et al., 1995; Hariharan et al., 1995). For example, regulated Rac activity is needed during embryogenesis for proper axonal outgrowth in peripheral neurons, for myoblast fusion, and for the epithelial sheet movement known as dorsal closure. Overexpression of *Drosophila* Rho has been examined in the developing eye, where it causes defects in cell shape.

Many differentiated cells contain specialized actin-rich structures, such as microvilli, neurites, or muscle spindles.

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Therefore, additional insight into the requirements for Rho proteins in morphogenesis might come from analyzing additional cell types. In particular, the role of Rho proteins in developmentally regulated cell migrations has not yet been explored. *Drosophila* oogenesis affords a relatively simple model system for such analysis, since egg chambers are comprised of three disparate cell types, each of which has a distinct array of actin-rich structures. Egg chambers contain a cluster of 16 germline cells: 15 polyploid nurse cells and one oocyte (Fig. 1). These sibling cells are derived from a single germline precursor following four rounds of cell division. Cytokinesis is incomplete during these divisions, and the 16 cells remain connected throughout oogenesis by actin-rich cytoplasmic bridges known as ring canals (Warn et al., 1985). Filamentous actin (F-actin) is also concentrated at the cortices of the nurse cells and oocyte (see Fig. 1, *G–I*). The 16-cell germline cluster becomes surrounded by a monolayer epithelium of somatic follicle cells early in oogenesis. In the somatic cells, F-actin is found concentrated at adherens type junctions between follicle cells (Peifer et al., 1993) and at the follicle cell–germ cell interface (see Fig. 1).

During oogenesis, several morphogenetic events occur, in some cases accompanied by dramatic changes in the actin cytoskeleton. For example, the rapid transport of nurse cell cytoplasmic contents into the oocyte, via the ring canals in late oogenesis (stage 11), is preceded by the polymerization of a meshwork of actin filaments that creates a cage around the nurse cell nuclei (Peifer et al., 1993; Cooley et al., 1992). Other morphogenetic events during oogenesis that might involve dynamic changes in actin include the rearrangement and migration of follicle cells. The follicle cells are initially uniformly cuboidal in shape (Fig. 1, *A* and *G*). In mid-oogenesis (stage 9), however, the majority of follicle cells rearrange into a columnar epithelium surrounding the oocyte (Fig. 1, *C*, *F*, and *I*), while the few remaining nurse cell–associated follicle cells become squamous in shape (King, 1970; Fig. 1, *C*, *F*, and *I*). During the same 6-h period, 6–10 follicle cells, known as the border cells, migrate from the anterior tip of the egg chamber (Fig. 1 *H*), in between the nurse cells (Fig. 1 *E*), to the nurse cell/oocyte border (King and Koch, 1963; Cummings and King, 1970) (Fig. 1 *I*).

To further examine the functions of the Rho family in development, we investigated the roles of *Drosophila* Rho proteins in oogenesis. We describe here the cloning of a novel Rho family member, RhoL, and the comparison of its function with Cdc42 and Rac throughout oogenesis. Nurse cell collapse and fusion occurred concomitantly with subcortical actin breakdown when mutant forms of Cdc42 and RhoL were expressed. In addition, constitutively active RhoL affected nurse cell–follicle cell contacts. In the somatic cells, only Rac activity was required for border cell migration. All three protein activities were required for normal transfer of cytoplasm from nurse cells to the oocyte. Thus Rho proteins appear to be required in multiple processes and to play distinct roles in each cell type that was examined.

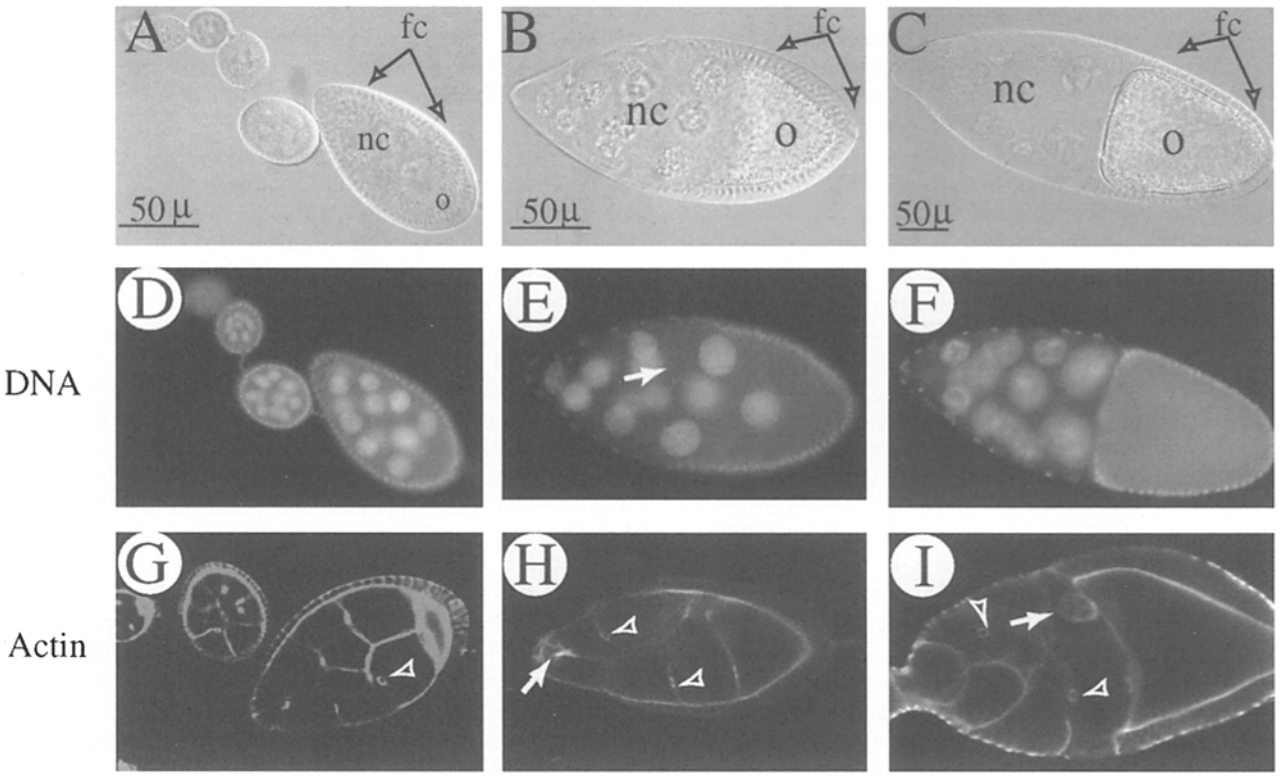

Figure 1. Wild-type oogenesis. (*A–C*) Nomarski optics images of egg chambers. *nc*, nurse cells; *o*, oocyte; *fc*, follicle cells. (*D–F*) Epifluorescence images of nuclear staining with DAPI. Note the regularity of the outer follicle cell nuclei. (*G–I*) Confocal micrographs showing F-actin stained with rhodamine-conjugated phalloidin. (*A*, *D*, and *G*) Early oogenesis through stage 7. (*B*, *E*, and *H*) Stage 9 egg chambers with migrating border cells. (*C*, *F*, and *I*) Stage 10 egg chambers after border cell migration. Where visible, border cells are marked by arrows, and ring canals are marked by arrowheads.

Materials and Methods

Cloning and Sequencing of PCR Product

Degenerate primers were designed to PCR amplify rho-like genes. The 5′ primer 5′GAGGATCCGTGGGCGAYGGCGCCTGYGGHAARAC-NTG3′ corresponded to the highly conserved G1 phosphate-binding region of GTP-binding proteins preceded by BamH1 site for cloning (underlined). The 3′ primer 5′GTAAGCTTRTCRTARTCYTCYTGKC-CGGCSGTRTCCCA corresponded to the slightly less conserved G3 phosphate-binding region followed by a HindIII site for cloning (*underlined*). Both primers were designed with reference to *Drosophila* codon usage, and the 3′ primer was designed to maximize matches with Rho rather than other rho family members or other ras-like genes. Genomic DNA template was prepared by denaturing three fly equivalents of DNA in 8 μl H_2O with 2 μl 2 M NaOH for 5 min at room temperature. Then 7 μl of H_2O and 3 μl of 3 M potassium acetate were added. DNA was EtOH precipitated. PCR conditions were 94°C for 30 s for denaturation, and 42°C for 1 min for annealing and 72°C for 1 min for extension. 100 ng of each primer was used with 0.2 mM dNTPs. 10× PCR buffer used was 200 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 250 mM KCl, 5 μl 2% gelatin, 0.5% Tween 20.

The PCR product was subcloned into pBluescript using the BamH1 and HindIII sites on either end of the PCR product. Double-stranded sequencing was performed according to standard protocol (Sequenase kit; U.S. Biochemical Corp., Cleveland, OH).

Isolation and Sequencing of cDNA and Genomic DNAs

All probes used in these experiments were made using the PrimeIt Kit (Stratagene, La Jolla, CA). The PCR product was used as a probe to isolate a cDNA from an ovary λgt22A library (gift from Peter Tolias, Public Health Research Institute, New York, NY). The cDNA was used as a probe to screen a charon 4 genomic library. Both the cDNA and coding region of the genomic clones were sequenced as above.

Northern Analysis

Standard methods of RNA isolation and blotting were used. The blot was probed with ^{32}P-labeled RhoL cDNA. *Drosophila ras*1 was used as a control for loading.

Site-directed Mutagenesis and DNA Constructs for RhoL and Cdc42

The mutations in $RhoL^{V20}$ and $RhoL^{N25}$ transformant lines were generated by oligonucleotide-directed mutagenesis using the cDNA cloned into Bluescript as the original template. Single-stranded DNA was prepared per the method of Kunkel et al. (1987). Mutagenesis was performed as described (McClary et al., 1989). Intended mutations were identified by sequencing. Mutant RhoLs were digested with Not1 and EcoR1 and cloned into the transformation vectors pUAST(Brand and Perrimon, 1993) and hsCaSpeR. *Saccharomyces cerevisiae* $Cdc42^{V12}$ in pBluescript (Miller and Johnson, 1994) and *Drosophila* $Cdc42^{N17}$ (Luo et al., 1994) were digested with EcoRI and cloned into hsCasper.

Germline Transformation

Standard procedures were used (Rubin and Spradling, 1982).

Phalloidin, DAPI, Antibody and β-Galactosidase Activity Staining

Ovaries were dissected in 1X Ringer's solution. Ovaries for immunohistochemistry were collagenase treated for 1 min in Ringer's solution + 5 mg/ml collagenase + 0.1 mg/ml BSA. Then they were washed once in PBS + 0.1% Tween 20 (PBT)[1]. All ovaries were then fixed for 10 min in devitellinizing buffer (6% formaldehyde, 16.67 mM KH_2PO_4/K_2HPO_4, pH 6.8, 75 mM KCl, 25 mM NaCl, 3.33 mM $MgCl_2$) and then washed twice in PBT.

For antibody staining, egg chambers fixed as described above were incubated in primary antibody overnight in PBT + 0.2% BSA (PBTB). Cdc42 polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used at a dilution of 1:200. 1 h washing was followed by incubation of secondary antibody at a dilution of 1:400 for 2 h at room temperature. Fluorescein-conjugated anti–rabbit IgG secondary (Vector Laboratories, Burlingame, CA) was used. PBTB washes were then done for 1 h, and the egg chambers were mounted in 50% glycerol with 0.01% *p*-phenylenediamine (PPD) (Sigma P-6001; Sigma Immunochemicals, St. Louis, MO) to prevent quenching of fluorescent signal by light.

β-Galactosidase activity staining was carried out in 200 μl of staining solution (10 mM $NaH_2PO_4{\cdot}H_2O/Na_2HPO_4{\cdot}2H_2O$, pH 7.2, 150 mM NaCl, 1.0 mM $MgCl_2$, 3.1 mM $K_4[Fe^{II}(CN)_6]$, 3.1 mM $K_3(Fe^{III}(CN)_6)$, 0.3% Triton X-100μ with 5 μl 8% X-gal in DMF. The reactions were done at 37°C for a period between 1 h and overnight (Bellen et al., 1989).

For actin staining, hand-dissected egg chambers were fixed as described above. Rhodamine-phalloidin (2.5 μl) (R-415; Molecular Probes, Inc., Eugene, OR) was vacuum dried and resuspended in 500 μl PBTB. Egg chambers were incubated in this solution for 20 min at room temperature in the dark and then rinsed in PBTB. Egg chambers were mounted in 50% glycerol/0.01% PPD/50% PBS.

For DAPI staining, fixed egg chambers were incubated for 5 min in 1 μg/ml DAPI in PBTB in the dark. Egg chambers were mounted in 50% glycerol/0.01% PPD/50% PBS.

Terminal Transferase Method for Detection of DNA Fragmentation

Hand-dissected ovaries were fixed in 400 μl heptane, 100 μl 36% formaldehyde, 400 μl Fix solution (2 mM Mg SO_4, 0.1 M Hepes pH 6.9, 1 mM EGTA) for 15 min with rocking/agitation to mix phases. Ovaries were washed three times in PBT.

Terminal Transferase Reaction. 20 μl 5× buffer (0.5 M K-cacodylate, pH 7.2, 10 mM $CoCl_2$, 1 mM DTT), 3 μl biotin–16-dUTP–1 nM/μl stock, 1 μl dNTPs 10 nM stock solution, 74 ml ddH_2O, 1 μl TdT (15 U/ml). Incubated for 3 h at 37°C, rinsed, and washed three times over the course of 1 h in PBT.

Detection. Samples were incubated at room temperature in the dark for 90 min in 2 μl streptavidin-fluorescein (1 mg/ml), 198 μl H_2O, washed four times over the course of 90 min, and equilibrated in 70% glycerol/0.01% PPD/30% PBS before mounting.

Acridine Orange Staining

Ovaries were hand dissected and placed in a mixture of 50% acridine orange/50% heptane for 5 min. The ovaries were then placed on slides covered with halocarbon oil and viewed using the DAPI channel under the microscope.

Results

Cloning of a Novel Member of the Rho Family of Small GTPases

Several *Drosophila* genes encoding Rho family proteins have recently been cloned including two Rac homologues, one Rho homologue and one Cdc42 homologue (Luo et al., 1994; Harden et al., 1995; Hariharan et al., 1995). Before examining the functions of Rho proteins in *Drosophila* oogenesis, we first set out to determine whether additional Rho family members might exist in flies. We performed low stringency reverse transcription PCR using primers from the conserved G1 and G2 phosphate-binding domains of Rho that were designed to minimize Ras homology while maximizing Rho family homology. A product of the expected size was recovered, cloned, and sequenced. The deduced amino acid sequence showed 64% similarity to the Rho family (not shown), and so this 198-bp fragment was used as a probe to screen an ovary cDNA library. The deduced amino acid sequence of one such

1. *Abbreviations used in this paper*: C/EBP, CAAT enhancer binding proteins; DAPI, 1,6-diamidino-2-phenylindole; F-actin, filamentous actin; hsp70, heat shock protein 70; PBT, PBS + 0.1% Tween; PBTB, PBS + 0.2% BSA; PPD, 0.01% *p*-phenylenediamine; UAS, upstream activation sequences.

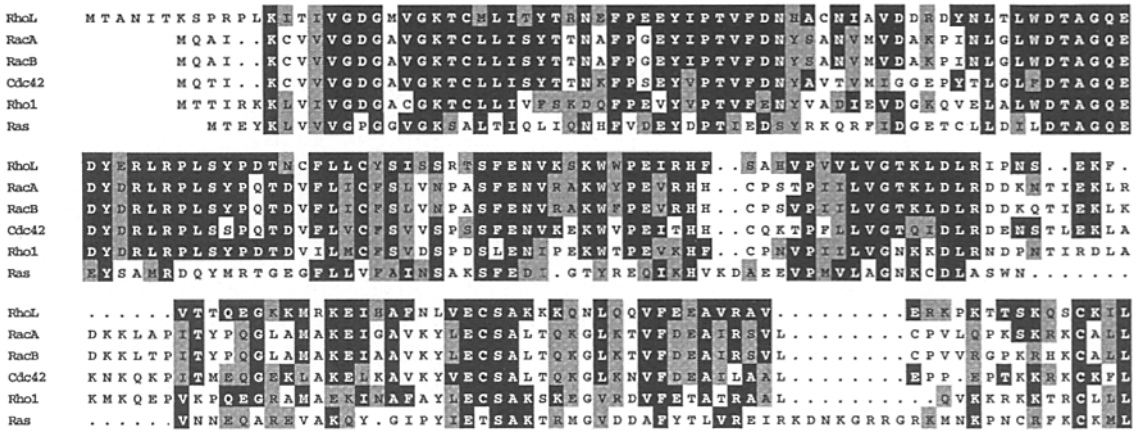

Figure 2. Sequence comparison of *Drosophila* Rho family proteins. Black boxes denote amino acid identity with RhoL, gray boxes denote amino acid similarity with RhoL. The Blosum-62 amino acid similarity matrix was used. GenBank accession No. U50314.

cDNA is shown in Fig. 2. The sequence was ~50% identical to each of the Rho family members and 30% identical to Ras (Fig. 2). Since Rac proteins are 85–90% identical to each other and Cdc42 proteins are likewise highly similar to one another, the new protein appeared to define a novel Rho family member, which we named Rho-like (RhoL). All of the GTP binding domains were highly conserved, and the COOH-terminal CXXX sequence required for lipid modification, which is typical of Ras proteins (Bourne et al., 1991), was also conserved; only the hypervariable carboxy-tail region showed significant divergence from the other Rho proteins. Since the length of the protein was very similar to other family members, it appeared that the entire coding region was represented by this clone. This sequence was confirmed by sequencing the corresponding genomic DNA.

To examine the expression of *RhoL* during development, the cDNA was used to probe a developmental Northern blot. Like other *Drosophila* Rho genes, *RhoL* was expressed at all stages of development; the 1.2-kb transcript displayed no more than a twofold difference in abundance between the stages that were analyzed (Fig. 3).

Generation of Constitutively Active and Dominant-Negative RhoL, Cdc42, and Rac

To compare RhoL functionally with other Rho family members, we generated mutations in RhoL that were predicted to render it either constitutively active or dominant-negative. These mutations caused amino acid substitutions that have been demonstrated in other studies to produce constitutively active or dominant-negative forms of other GTP-binding proteins. Glycine 20 was changed to valine to generate a constitutively active protein ($RhoL^{V20}$). This is analogous to the substitution of valine for glycine 12 found in oncogenic Ras, which interferes with GTPase activity, thus maintaining the protein in its GTP-bound active state. To generate a dominant-negative form of RhoL, threonine 25 was changed to asparagine ($RhoL^{N25}$). The analogous change in Ras causes preferential binding of GDP as compared to GTP (Feig and Cooper, 1988). This is thought to result in the sequestration of GEF, therefore leading to a dominant negative phenotype (Farnsworth and Feig, 1991; Schweighoffer et al., 1993). Both these mutated forms of the RhoL gene were cloned into the pCaSpeR-hs and pUAST vectors, which are described below, and introduced into flies by P element–mediated germline transformation.

In the vector pCaSpeR-hs, genes are placed under the control of the heat shock protein 70 (hsp70) heat-inducible promoter. After heat shock at 37°C, expression from the hsp70 promoter is induced up to 100-fold over background expression. To determine the pattern of expression induced by the hsp70 promoter in the ovary, we stained egg

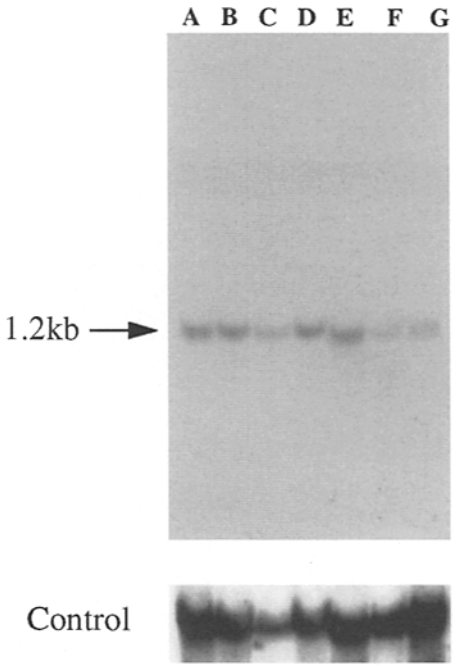

Figure 3. Developmental Northern blot showing the expression of the RhoL transcript. (*A*) Embryos, (*B*) first instar larva, (*C*) second instar larva, (*D*) third instar larva, (*E*) early pupae (days 1–2), (*F*) late pupae (days 3–5), and (*G*) adults. 15 μg of poly A^+ was loaded in each lane. The blot was probed with the *ras1* gene, shown below, as a control for mRNA loading.

chambers dissected from females carrying a heat shock-C/EBP transgene using anti-C/EBP antibodies, since adequate antibodies against Rho proteins were not available. After a 1-h heat shock at 37°C, high levels of C/EBP protein were found in follicle cells through stage 10, with lower levels induced in later stages. Expression was also seen in the germline through stage 9, although at levels lower than that seen in the follicle cells (Fig. 4, *A* and *B*).

The pUAST vector can be used to drive tissue- and stage-specific transgene expression (Brand and Perrimon, 1993). This vector contains Gal4 responsive upstream activation sequences (UAS) and thus restricts expression of the transgene to cells that contain the yeast transcription factor Gal4. A variety of fly lines are available with Gal4 under the control of different tissue- and stage-specific enhancer elements (Brand and Perrimon, 1993; Manseau, L., personal communication). We used lines in which Gal4 was expressed either specifically in the border cells at the time of their migration (Gal4-458), in border cells and posterior follicle cells during the migration (Gal4-306), in all the follicle cells including the border cells (Gal4-198Y), or in outer follicle cells only (Gal4-212). To illustrate these patterns of expression, each Gal4 line was crossed to a line carrying a UAS-lacZ reporter transgene (Brand and Perrimon, 1993). Ovaries were dissected and stained for β-galactosidase activity (Fig. 4, *C–F*). We also used a heat-inducible Gal4 (hs-Gal4). For unknown reasons, the Gal4 protein is not expressed detectably in the germline. Thus, the hs-Gal4 induces the UAS transgene to high levels only in follicle cells.

For comparison with RhoL, we generated transgenic flies bearing pCaSpeR-hs constructs of Cdc42 (hs-Cdc42^{V12} [constitutively active] and hs-Cdc42^{N17} [dominant-negative]). In addition, we took advantage of the constitutively active and dominant-negative forms of both Cdc42 and Rac in the pUAST vector reported by Luo et al. (1994). pCaSpeRhsRac^{N17} (hs-Rac^{N17}) was also available (Harden et al., 1995). However, hs-Rac^{V12} transformants were not obtained, possibly because of the toxicity of even low expression levels of constitutively active Rac (Harden et al., 1995). The transgenes used in this study are summarized in Table I. Most of the Gal4 lines with ovary expression also displayed expression during embryonic and/or larval stages, and many combinations of constructs, especially the activated proteins, were inviable or displayed female-specific lethality. The effects on viability are summarized in Table II.

Analysis of Rho Family Functions in Border Cell Migration

Rhodamine-phalloidin staining of wild-type stage 9–10 egg chambers revealed a dramatic reorganization of actin filaments within the border cells as they initiated migration (Figs. 1 *H* and 5 *C*). One or more of the border cells extended a process between the nurse cells. These processes were full of F-actin filaments and stained brightly with rhodamine-phalloidin. Thin filopodia-like structures were also seen jutting from the main concentration of F-actin (Fig. 5 *C*). After migration, the actin cytoskeleton was completely reorganized. F-actin staining was generally less intense, appearing as diffuse cytoplasmic and cortical staining (Fig. 5 *D*).

To test the roles of the Rho family in border cell migration, we expressed dominant-negative forms of Rac, RhoL, and Cdc42 in the border cells. Migration defects were seen exclusively with Rac^{N17}. Gal4-198Y and Gal4-306, when combined with UAS-Rac^{N17}, resulted in 80% of

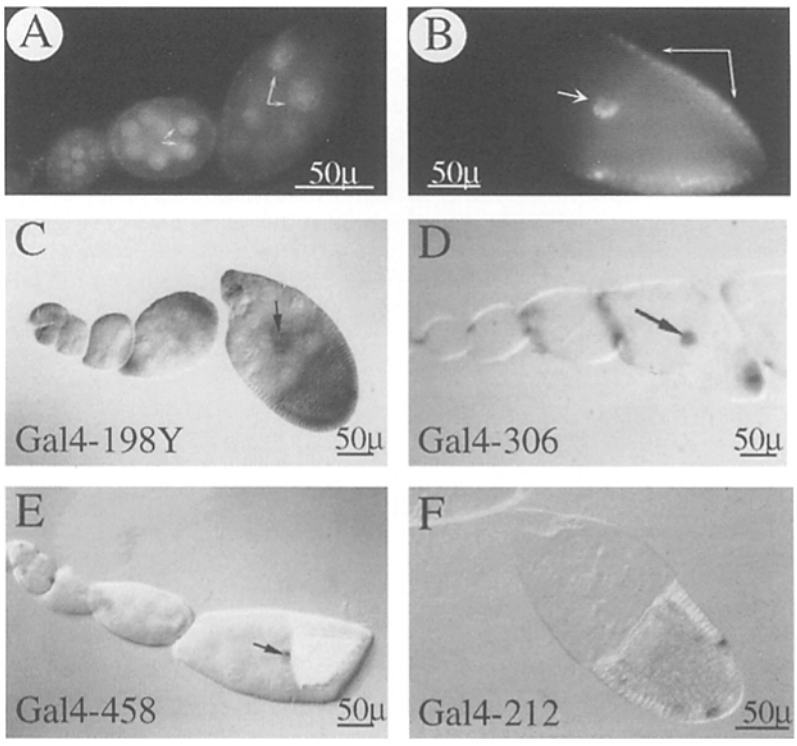

Figure 4. Patterns of expression from Gal4 lines and heat shock vectors used. (*A*) Stages 1–6 hs-C/EBP egg chambers after 1 h heat shock induction and detection of induced protein with anti-C/EBP polyclonal antibody (Montell et al., 1992). Arrows indicate nurse cell nuclei. (*B*) Stage 9 hs-C/EBP as described in *A*. Note at this stage that the nurse cell expression is barely detectable. Single arrow points to border cells. Double arrow shows outer follicle cells. (*C–F*) Gal4 lines crossed to UAS-LacZ, stained for β-galactosidase activity. Arrows point to border cells. (*C*) Gal4-198Y, (*D*) Gal4-306, (*E*) Gal4-458, and (*F*) Gal4-212.

Table I. Summary of Defects Caused by Altered Rho Family Proteins

Transgene	Summary of defects	Reference
Rac		
pUAST-RacN17	Border cell migration	Luo et al., 1994
pUAST-RacV12	None	Luo et al., 1994
pCaSpeR-hs-RacN17	Border cell migration; cytoplasm transfer	Harden et al., 1995
Cdc42		
pUAST-Cdc42N17	None	Luo et al., 1994
pUAST-Cdc42V12	None	Luo et al., 1994
pCaSpeR-hs-Cdc42N17	Nurse cell fusion (mild); cytoplasm transfer	This paper
pCaSpeR-hs-Cdc42V12	Nurse cell fusion (severe)	This paper
RhoL		
pUAST-RhoLN25	None	This paper
pUAST-RhoLV20	Inviable with most GAL4 lines	This paper
pCaSpeR-hs-RhoLN25	Nurse cell–follicle cell contacts; nurse cell fusion (mild); cytoplasm transfer	This paper
pCaSpeR-hs-RhoLV20	Nurse cell–follicle cell contacts; outer follicle cell monolayer; abnormal apoptosis	This paper

stage 10 egg chambers with little or no border cell migration (Fig. 5, *E–H*), and the cells typically did not extend actin-rich processes in between the nurse cells (Fig. 5 *G*). The identity of the cells at the anterior tip of the egg chamber was confirmed by crossing in a border cell enhancer trap marker (*slbo*^{1}) and staining for β-galactosidase activity (Fig. 5 *F*). Gal4-458;UAS Rac^{N17} showed 10% of stage 10 egg chambers with arrested migration (not shown), compared with wild type, where migration is complete in 100% of the egg chambers by this stage (Fig. 5, *A* and *B*). Thus, the degree of border cell migration arrest in the UAS-Rac^{N17}; border cell Gal4 flies correlated with the level of transgene expression induced in each line (see Fig. 4 for relative levels of expression). No border cell migration defects were observed when any Gal4 line was crossed to UAS-RhoL^{N25} (Fig. 5, *I* and *J*) or Cdc42^{N17} (not shown).

The border cells are required to generate the micropyle pore (King, 1970; Montell et al., 1992). This pore is used by sperm for entry into the mature egg. Thus, if the border cells fail to migrate, an unfertilizable egg that does not hatch into a larva is generated (Montell et al., 1992). We therefore measured the hatching frequency of eggs laid by Gal4-198Y;UAS-Rac^{N17} females or Gal4-306;UAS-Rac^{N17} females. 19% (5 out of 26) and 22% (7 out of 32) of the laid eggs hatched, respectively, as compared to the wild-type hatching frequency of 95%. In addition, we observed a micropyle pore defect equivalent to that found in *slbo* mutant egg chambers. Thus, it is likely that the fertility defects were primarily caused by border cell migration defects.

To investigate whether Rac activity was only required to initiate the migration, or whether its activity would also be required during migration, hs-Gal4;UAS-Rac^{N17} flies were analyzed. Females were heat shocked for 1 h at 37°C and then left for 10 h at 25°C or 20 h at 18°C before dissection, fixation, and staining of egg chambers. Egg chambers that were in stage 8 at the time of induction would have developed to late stage 9 or early 10 by the time of dissection (stages 8, 9, and 10A each last 6 h, while stage 10B lasts 4 h; see Lin and Spradling, 1993). Late stage 9 and stage 10A egg chambers showed complete failure of migration, confirming that Rac activity was required for initiation of the migration. In addition, stage 10B egg chambers, which had been in mid–stage 9 during the induction, showed delayed migration (not shown). Thus, removing Rac activity during the migration appeared to halt further progress. Expression of hs-Gal4;UAS-Rac^{N17} presumably inhibited Rac activity in all follicle cells. No discernible effect on the other follicle cells was observed, however, except for an increased frequency of degenerating egg chambers. Thus, the requirement for Rac activity appeared to be specific for border cell migration. We also examined the effects of inducing Rac^{N17} ubiquitously, using hs-Rac^{N17}. A single 1-h heat shock followed by 10 h at 25°C resulted in >98% border cell delay (not shown).

To test whether Rac activity was a limiting factor in regulating border cell migration, UAS-Rac^{V12} was expressed in all follicle cells, including border cells, using the Gal4-198Y line. No sign of either precocious or accelerated migration was observed, though increased levels of F-actin

Table II. Lethality/Viability of Gal4 Constructs with UAS-Rac, Cdc42, or RhoL

	GAL4 line tested				
UAS construct	458	306	198Y	212	hs-GAL4
RhoLV20	Lethal	Lethal	Lethal	Viable	Lethal
RhoLN25	Viable	Viable	Viable	Viable	Viable
RacV12	Female lethal	Female lethal	Viable	Lethal	Lethal
Rac N17	Viable	Viable	Viable	Viable	Viable
Cdc42V12	Viable	Female lethal	Lethal	Lethal	Semi-lethal
Cdc42N17	Viable	Viable	Lethal	Viable	Viable
Embryonic expression	None	Salivary gland	Salivary gland	Not tested	Not tested
Larval expression	Moderate, general	Strong, general	Strong, general	Not tested	Not tested

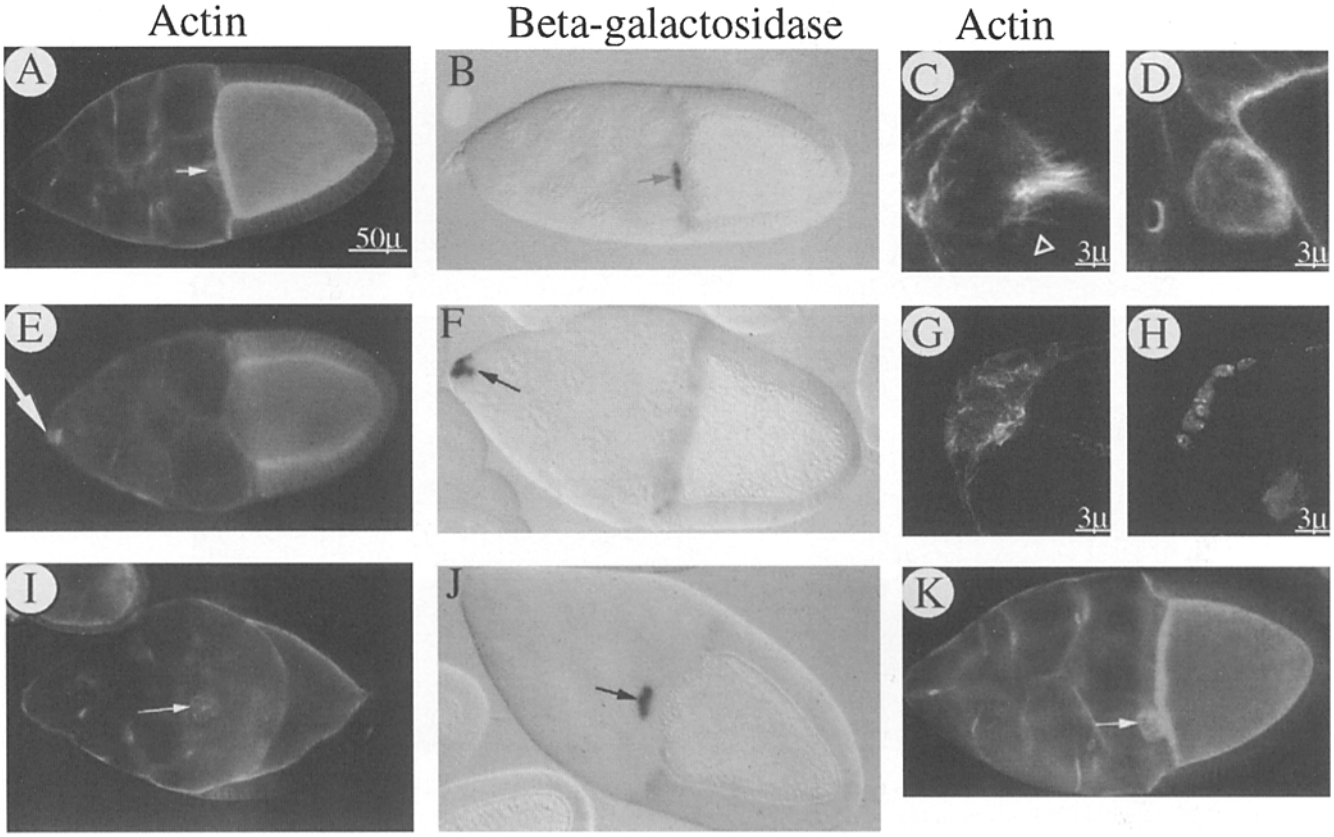

Figure 5. Changes in F-actin distribution during normal border cell migration and effects of altered Rho proteins on border cells. (*A*) Wild-type stage 10 egg chamber stained for F-actin. (*B*) Nomarski optics image of *slbo*^{1}/*CyO* stage 10 egg chamber stained for β-galactosidase activity. (*C*) High power confocal micrographs of wild-type border cells initiating migration in stage 9 (*C*) and after migration in stage 10 (*D*), stained for F-actin. Triangle indicates one filopodium in *C*. *C* and *D* are high power images of the same egg chambers shown in Fig. 1, *H* and *I*. (*E*) F-actin staining of a representative egg chamber expressing Rac^{N17} in border cells and posterior follicle cells (Gal4-306;UAS-Rac^{N17}). (*F*) β-Galactosidase activity staining of a stage 10 egg chamber (Gal4-306;UAS-Rac^{N17};*slbo*^{1}/+) showing that the cells at the anterior tip are border cells. (*G*) High power confocal micrograph of stage 10 border cells from GAL4-306;UAS-Rac^{N17} stained for F-actin. Note the lack of cellular extensions or filopodia. (*H*) Nuclear staining of *G*. (*I*) F-actin staining of a stage 9 egg chamber expressing dominant-negative RhoL. (*J*) β-Galactosidase activity staining of a similar egg chamber. (*K*) F-actin staining of an egg chamber in which Rac^{V12} is expressed in all follicle cells, including border cells. Arrows indicate border cells.

within border cells could be detected (Fig. 5 *K*) compared with wild type (Fig. 5 *A*). No effect on border cell migration was observed in either Cdc42^{V12};Gal4-458 or hs-Gal4; and UAS-Cdc42^{V12} (not shown). UAS-RhoL^{V20} was inviable with all Gal4 lines, but no effect on border cell migration was observed in hs-RhoL^{V20} (not shown).

We tested whether expression of Rac^{V12} was sufficient to rescue the border cell migration mutant, *slow border cells* (*slbo*). The *slbo* locus encodes a *Drosophila* homologue of the transcription factor CAAT enhancer binding protein (C/EBP), which is required for normal border cell migration (Montell et al., 1992). No rescue of border cell migration was observed in flies of the genotype *slbo*^{1}/*slbo*^{1}; UAS-Rac^{V12}/Gal4-198Y (not shown).

Requirement for Regulated Cdc42 Activity in the Germline

Although alterations in Cdc42 activity showed no detectable effect on somatic cells in the ovary, germline cells might have distinct requirements. To determine the effect of Cdc42 on germline cells, a 1-h heat shock was applied to hs-Cdc42^{V12} flies, followed by incubation at 25°C for 5, 6, 7, 8, 9, or 10 h. Ovaries were dissected, fixed, and stained with rhodamine-conjugated phalloidin and DAPI. A complete reorganization of nurse cell cytoarchitecture was observed. In the wild-type, nurse cell nuclei appeared evenly spaced, and subcortical actin outlined the plasma membrane, within which were embedded actin-rich ring canals (Fig. 6, *A* and *B*). In hs-Cdc42^{V12} flies, 5 h after heat shock, defects were apparent in stage 6 and older egg chambers, which became progressively more severe at later time points. F-actin staining in nurse cells appeared irregular and reduced in intensity (Fig. 6 *D*). Discontinuities in subcortical F-actin were apparent, particularly in the middle of the egg chambers, where nurse cell–nurse cell contacts are normally found. Ring canals, often with wisps of F-actin still attached, appeared to be no longer anchored to the plasma membrane. These ring canals remained intact and even formed aggregates and concatamers (Fig. 6, *D* and *F*), though they were somewhat misshapen (Fig. 6 *F*). At later time points, the F-actin was further disrupted and

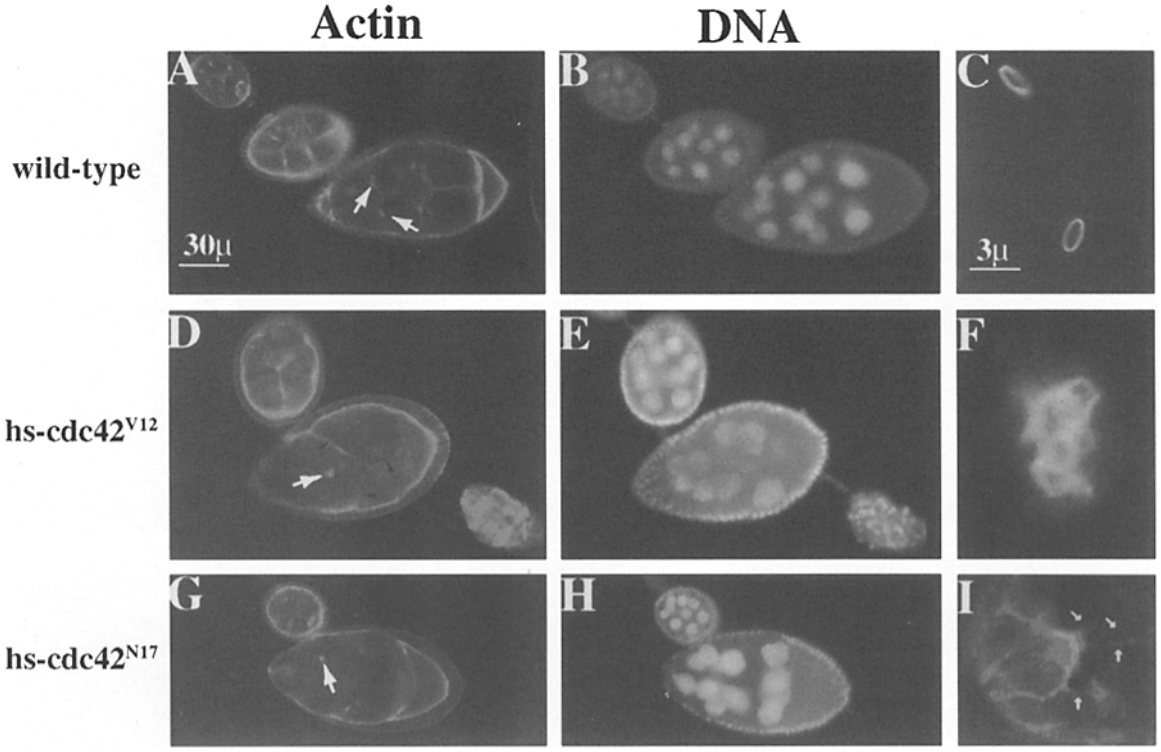

Figure 6. Effects of altered Cdc42 activity on nurse cells. (*A*) Wild-type egg chambers up to stage 8. Note the regular arrangement of subcortical actin around the outside of nurse cells. (*B*) Nuclear staining of *A*. (*C*) Wild-type ring canals stained with anti-hts antibody. (*D*) hs-Cdc42^{V12} after heat shock; arrow points to ring canal aggregates and arrowheads point to the few remaining actin filaments. (*E*) Nuclear staining of *D*. Note that nuclear morphology looks normal despite the absense of most nurse cell membranes and F-actin. (*F*) Anti-hts staining of ring canal aggregates from hs-Cdc^{V12} mutant egg chambers. (*G*) Cdc42^{N17} after multiple heat shocks; arrows point to free ring canals. (*H*) Nuclear staining of *G*. (*I*) Stage 9 hs-Cdc42^{N17} egg chamber in which ring canal aggregates are present (not shown), but filopodial extensions from border cells (*arrows*) are still present.

multinucleation was apparent (Fig. 6, *D* and *E*). Only the membrane and ring canals between the nurse cells and the oocyte were resistant to this breakdown (Fig. 6 *D*). Stage 10 egg chambers were severely affected and showed signs of degeneration (not shown). Later stage egg chambers (stages 11–14), however, were much less abnormal, and the actin filament network that forms around stage 11 nurse cell nuclei still formed (see section on nurse cell cytoplasmic transfer in Fig. 8 *B*). The same heat shock regime, followed by a 24-h incubation before dissection, resulted in the stage 10 and older egg chambers showing signs of degeneration. Thus, the multinucleated egg chambers did not appear to develop past stage 9–10. 2–3 d after induction of hs-Cdc42^{V12}, egg chambers again appeared normal, implying that early stages, including egg chamber formation in the germarium, had not been adversely affected.

To investigate whether the aggregated ring canals retained previously characterized ring canal antigens (Robinson et al., 1994), we performed immunohistochemistry using antibodies to Hts (an adducin-like protein), Kelch, and phosphotyrosine. The aggregated ring canals stained for all of these components (Fig. 7 *F* shows Hts staining). The staining appeared more diffuse than normal, however, suggesting that ring canal structure had been somewhat disorganized.

To examine the normal role for Cdc42 in the germline, multiple heat shocks were applied to flies that had two copies of the pCaSpeR-hs-Cdc42^{N17} transgene to maintain as high a level of product as possible (1 h at 37°C, 2 h at 25°C, 20 min at 37°C, 3 h at 25°C, 1 h at 37°C, and 5 h at 25°C). Egg chambers were then dissected and stained. In most egg chambers, the nurse cell subcortical actin appeared reduced in thickness (Fig. 6 *G*) relative to wild-type (Fig. 6 *A*). In more severe cases, some of the filaments appeared shorter than normal and free ring canals were visible. In 10% of stage 6–8 egg chambers, ring canal aggregates similar to those found with Cdc42^{V12} were seen (Fig. 6 *G*). No defects were seen in the outer follicle cell layer (Fig. 6, *G* and *H*), and we noted that filopodia could be observed emanating from the border cells that were initiating migration (Fig. 6 *I*). Overall, these defects appeared to be a milder version of those seen with Cdc42^{V12}. Thus, increasing or decreasing the normal level of Cdc42 activity appeared to cause similar defects.

Effects of RhoL Activity on Subcortical Actin in Both Germline and Somatic Cells

To gain insight into the normal role for RhoL during oogenesis, the effects of expressing RhoL^{N25}, in the presence of a deficiency for RhoL, were examined. A single 1-h heat shock of hs-RhoL^{N25};Df(3L)997/+ females, followed by a 10-h incubation at 25°C, showed mild defects in the

contacts between the nurse cells and follicle cells (not shown). When repeated 1-h heat shocks were applied every 5 h for 15 h, the outer follicle cells no longer formed a monolayer and signs of nuclear condensation in the nurse cells were apparent in stage 10 egg chambers (Fig. 7, *A* and *B*). In addition, egg chambers of earlier stages contained multinucleate nurse cells with aggregated ring canals similar to those observed with Cdc42^{V12} (Fig. 7, *C* and *D*). Milder defects were observed in the absence of the Df(3L)997, indicating that the phenotype correlated with the amount of RhoL activity and was specific to this protein. A further indication of specificity was that similar treatment of hs-Rac^{N17} did not produce any of these abnormalities.

Constitutively active RhoL (RhoL^{V20}) was examined in both the germline and somatic cells, using the hs-RhoL^{V20} transgenic flies. Egg chambers were examined without heat shock, or 1, 3, or 10 h after heat shock. There are several indications that the hsp70 promoter is leaky and that a low level of expression is present, even at 25°C (for example, see Schweisguth and Posakony, 1994). In the absence of heat shock, irregularities in the contacts between the nurse cells and the follicle cell layer could be seen in hs-RhoL^{V20} ovaries. In ~5% of egg chambers, the nurse cells appeared to have separated from the outer follicle cell layer, leaving a gap between the two cell types (Fig. 7, *E* and *F*). However, the monolayer of outer follicle cells remained intact and nuclear morphology, as visualized with DAPI staining, appeared normal (Fig. 7 *F*).

1 h after heat shock, there appeared to be less discrete F-actin in the nurse cell, and subcortical actin in the outer follicle cells looked irregular (Fig. 7 *G*). The boundary between the nurse cells and follicle cells, which is normally smooth (Fig. 1 *G*), appeared irregular (Fig. 7, *G* and *H*). 3 h after heat shock, nurse cell subcortical actin was no longer detectable, and the nurse cell–follicle cell interface was even more irregular (Fig. 7, *I* and *J*). Signs of nuclear condensation and fragmentation were also apparent (Fig. 7 *J*).

10 h after heat shock, many degenerating egg chambers were found. In the stage 10 egg chambers that remained, the outer follicle cells failed to form the single layer columnar epithelium around the oocyte found in the wild type (Fig. 1, *C*, *F*, and *I*). DAPI staining of wild-type stage 10 egg chambers reveals the normal follicle cell alignment (Fig. 1 *F*). In hs-RhoL^{V20} egg chambers, 10 h after heat shock, follicle cell nuclei were no longer in register, and the epithelium appeared several cells thick at the posterior pole (Fig. 7, *K* and *L*). 20 h after the induction of hs-RhoL^{V20}, even when induction was as short as 10 min, the whole ovary had degenerated, leaving merely a few mature eggs.

The cells within the degenerating egg chambers appeared to be undergoing cell death. DAPI staining showed nuclear condensation and pycnotic DNA suggestive of apoptosis (Fig. 7, *J* and *L*), and nuclear fragmentation could be seen, even at early time points. Staining with acridine orange (Abrams et al., 1993) and the TUNEL technique confirmed that these cells were undergoing apoptosis (data not shown). This was in contrast to the effects of hs-Cdc42^{V12}, where the nurse cell nuclei appeared normal (Fig. 6 *E*), indicating that loss of nurse cell subcortical actin was not sufficient to initiate apoptosis. Somatic expres-

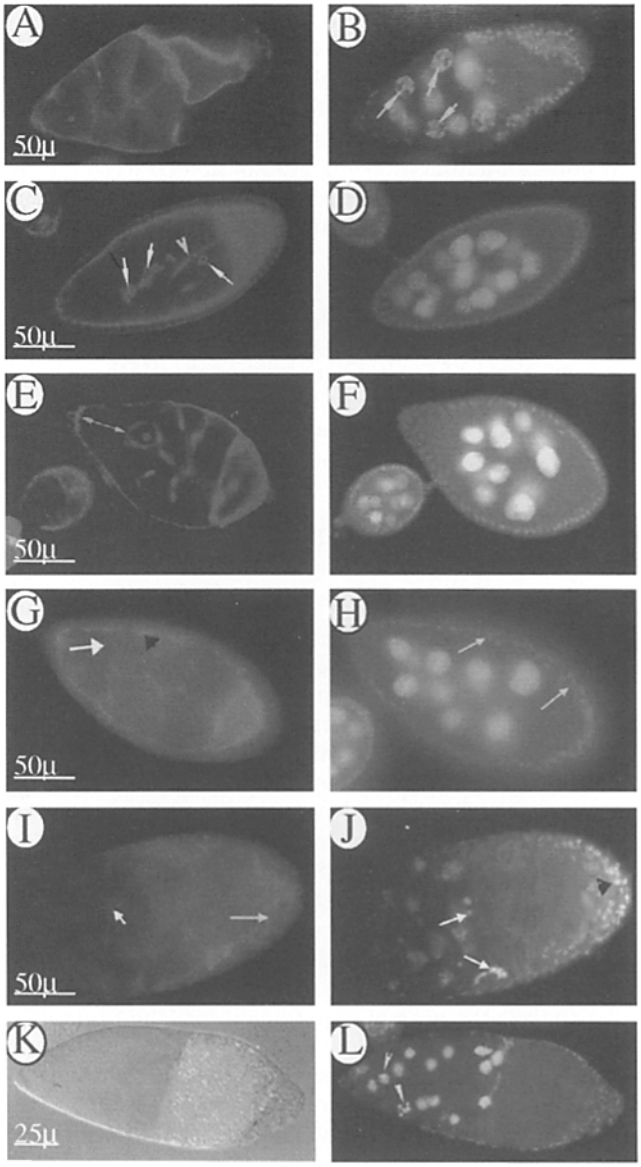

Figure 7. Effects of altered RhoL activity on oogenesis. (*A*) F-actin staining of stage 10 egg chamber of hs-RhoL^{N25} after multiple heat shocks. Note the irregular and unusually thick layer of oocyte-associated follicle cells. (*B*) Nuclear staining of *A*, arrows point to nurse cell nuclei beginning to undergo condensation. (*C*) F-actin staining of hs-RhoL^{N25};Df997 after multiple heat shocks. Arrows show free fing canals, and arrowhead shows remaining F-actin in nurse cells. (*D*) Nuclear staining of *C*. (*E*) F-actin staining of a stage 7 hs-RhoL^{V20} egg chamber without heat shock. The arrow shows the gap between the follicle and nurse cell layers. Also note the uneveness of the F-actin between these two layers. (*F*) Nuclear staining of *E*. (*G*) F-actin staining of a stage 8 hs-RhoL^{V20} egg chamber 90 min after heat shock. The arrow shows thinning nurse cell subcortical actin, and the arrowhead shows punctate F-actin irregular staining between the nurse cells and outer follicle cells whose shape is abnormal. (*H*) Nuclear staining of *G*. (*I*) F-actin staining of a stage 9 hs-RhoL^{V20} egg chamber 3 h after induction. Arrow shows F-actin remnant. (*J*) Nuclear staining of *I*; note nurse that the cell nuclei are condensed and pycnotic. (*K*) Nomarski image of a stage 10 hs-RhoL^{V20} egg chamber 10 h after heat shock. Note that the oocyte-associated follicle cells have moved posteriorward and do not form a monolayer. (*L*) Nuclear staining of *K*; arrows show condensed nurse cell nuclei.

sion of RhoL^{V20} via Gal4-212 did not induce any of these effects. However, only low level and highly mosaic expression of RhoL^{V20} could be analyzed because of the lethality caused by most GAL4;UAS-RhoL^{V20} combinations (Fig. 4 *F*; Table 2). Thus, it was not clear if the follicle cell death was non–cell-autonomous because of defects in contact or signaling with the germline, or whether higher, more uniform levels of RhoL^{V20} protein were required to induce the effect.

Since both the subcortical actin and ring canals seemed to be abnormal in nurse cells, we stained egg chambers at early time points for ring canal– and membrane-associated antigens. None of the antigens examined, which included Armadillo (a *Drosophila* β-catenin homologue), Hts, Kelch, and phosphotyrosine was detectably affected by the increase in RhoL activity (not shown).

Effects of the Rho Family on Nurse Cell Cytoplasm Transport

As previously mentioned, at stage 11 of oogenesis, a network of actin filaments polymerizes to form a cage around the nurse cell nuclei, apparently preventing their movement during the transfer of nurse cell cytoplasm to the oocyte (Fig. 8 *A*). This transfer process, known as "dumping," appears to result from the myosin-based contraction of subcortical actin (Wheatley et al., 1995). Constitutively active forms of Cdc42 and RhoL did not appear to prevent the formation of the actin cage, and though it was not as symmetrical as in the wild type (Fig. 8, *B* and *C*), nurse cell nuclei were held in place properly during the transport process. Hs-Cdc42^{V12} did occasionally disrupt nurse cell subcortical actin and resulted in mild defects in cytoplasm transfer (not shown).

To analyze the normal requirement for Rho protein activities in stage 11, each dominant-negative transgene was crossed to a deficiency for the corresponding wild-type locus. Reducing the amount of wild-type protein by half would be predicted to enhance the dominant-negative effect. Since the hsp70 promoter did not seem to be expressed at a high level in the germline after stage 8 (Fig. 4 *B*), it seemed that such enhancement might be necessary to observe a phenotype in later stages. Flies were heat shocked for 1 h, incubated at 25°C for 5 h, heat shocked again, and incubated at 25°C once more before fixation and staining with DAPI and rhodamine-conjugated phalloidin. The effects on late stage egg chambers were dramatic (Fig. 8, *D–F*). In each case, Rac^{N17} (Fig. 8 *D*), Cdc42^{N17} (Fig. 8 *E*), or RhoL^{N25} (Fig. 8 *F*), the actin cage that normally surrounds stage 11 nurse cell nuclei was absent. This resulted in ring canals becoming obstructed by nurse cell nuclei (Fig. 8, *G* and *H*) and failure of complete cytoplasm transfer, as has been described for the *chickadee*, *singed*, and *quail* mutants (Cooley et al., 1992; Cant et al., 1994; Mahajan-Miklos and Cooley, 1994). In addition, defects in nurse cell subcortical actin may have contributed to cytoplasm transfer defects (Fig. 9, *H* and *I*). In addition to incomplete cytoplasm transport, there may have been defects in the degeneration of nurse cells as the cells persisted at the anterior ends of the egg chambers through the end of oogenesis.

Similar defects were observed with each of the Rho family members; however, the effects appeared to be specific, since only mild defects were observed in the absence of the appropriate deficiency for each locus.

Discussion

RhoL, A New Member of the Rho Subfamily of Small GTPases

The Rho gene, first isolated by spurious cross-hybridization with a neuropeptide cDNA (Madaule and Axel, 1985), was noted for its 30% amino acid sequence identity with Ras. Since that time, the Ras superfamily has grown to include the families Ras, Rho, Ran, Rab, and Arf. Within the Rho family, Rho, Rac, Cdc42, Tc10, and RhoG comprise subfamilies that display 50–60% amino acid sequence identity with each other. We have identified a protein that is equally similar to all of the known Rho subfamilies, and RhoL would therefore appear to define a new subfamily. Families and subfamilies of Ras-related proteins are defined primarily on the basis of amino acid sequence similarity, particularly in the G2 domain (residues 32–40 in p21Ras), which is highly conserved within a family but less well-conserved between different families (Bourne et al., 1991). In addition, functional similarities appear between members of each family. Rho proteins are often associated with changes in cytoarchitecture, whereas Rab proteins mediate vesicle traffic. The functional distinctions are not strict, however: for example, Rho1, Rho3, and Rho4 have visible effects on the cytoskeleton but also seem to affect cell growth (Bender and Pringle, 1989; Matsui and Toh-e, 1992; Yamochi et al., 1994); RhoG and RhoB were both isolated on the basis of their increased expression after growth factor stimulation (Jahner and Hunter, 1991; Vincent et al., 1992); and Ral, which is most similar to Ras in sequence, is found on vesicles (Bielinski et al., 1993; Volknandt et al., 1993) and is expressed at highest levels in secretory tissues (Bhullar, 1992; Wildey et al., 1993). It is possible that these functional overlaps reflect the ability of the Ras superfamily to coordinate various processes, such as morphology and growth. By both sequence identity and functional criteria, RhoL would appear to define a new subfamily within the Rho family.

Rho Proteins in Cell Migration during Development

Previous studies, primarily using cultured cells, have implicated Rac and Rho in the control of cell motility. This involvement varies from cell type to cell type. In Swiss 3T3 cells, Rho but not Rac was found to be involved in cell motility (Takaishi et al., 1993), whereas in the invasion assay, Rac rather than Rho promotes motility (Michiels et al., 1995; Qiu et al., 1995). In our study, Rac activity was required throughout border cell migration. Although migration proceeded to some extent in a fraction of egg chambers where the dominant negative Rac was expressed cell type specifically, it is likely that this was caused by residual Rac activity, because when high levels of Rac^{N17} were expressed using the heat shock promoter, inhibition of migration was complete. The cells prevented from migrating appeared not to extend actin-rich processes into the nurse cell cluster, although they were not always completely

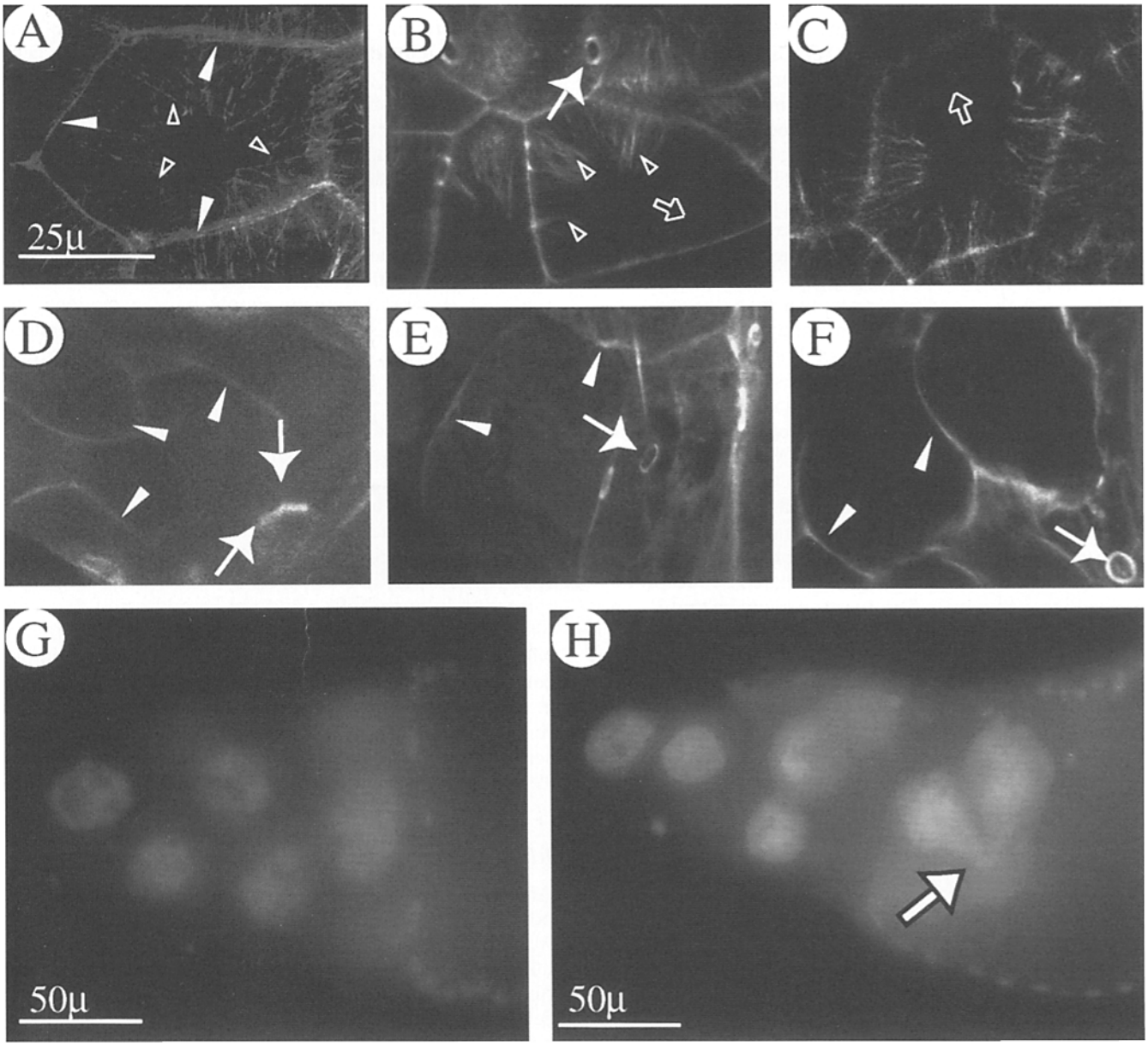

Figure 8. Cytoplasmic transfer defects produced by mutant Rho proteins. (*A*) Confocal micrograph of F-actin staining of a wild-type stage 11 egg chamber, focusing on one nurse cell. (*B*) F-actin staining of hs-Cdc42^{V12} stage 11 egg chamber 2 h after induction. (*C*) F-actin staining of hs-RhoL^{V20} stage 11 egg chamber 2 h after heat shock. (*D*) F-actin staining of hs-Rac^{N17}/Df egg chamber after multiple heat shocks. (*E*) F-actin staining of hs-Cdc42^{N17}/Df stage 11 egg chamber after multiple heat shocks. (*F*) F-actin staining of hs-RhoL^{N25}/Df stage 11 egg chamber after multiple heat shocks. Open arrowheads indicate the actin filament network that surrounds nurse cell nuclei, filled arrowheads indicate subcortical actin, and the open arrows indicate patches lacking in filaments in *B* and *C*. Note the complete absence of filaments in *D–F*. (*G*) Nuclear staining of wild-type stage 11 egg chamber. Note the regular spacing and round shape of the nuclei. (*H*) Nuclear staining of Rac^{N17}/Df showing pear-shaped nuclei (*arrow*) blocking ring canals. This is the same egg chamber as that in *D*.

round in shape. The simplest interpretation of the failure of border cell migration would be that Rac activity is required for the formation of pseudopods and that these processes are essential for migration.

As stated previously, the proposed mechanism of action of the dominant-negative proteins is that they sequester exchange factor. Thus, one caveat of using this approach is that the activities of more than one Rho-like protein may be inhibited after expression of dominant-negative Rac. Since only dominant-negative Rac inhibited border cell migration, however, it does not appear that Rac, Cdc42, and RhoL bound the same exchange factor. There are two *Drosophila* Rac proteins that are 92% identical in amino acid sequence. It is possible that these proteins bind the same exchange factor and, therefore, we cannot distinguish at this point which of the *Drosophila* Rac proteins is required for border cell migration.

We have not yet analyzed Rho activity in this system, and it will be of interest to know whether both Rac and Rho activities are required in these cells, or whether Rac

activity alone is required. What is clear is that neither RhoL^{N25} nor Cdc42^{N17} caused any perturbation of migration. This was a little surprising because Cdc42 promotes filopodia formation in Swiss 3T3 cells (Nobes and Hall, 1995), and we observed filopodia jutting from the border cells as they initiated migration. Reducing Cdc42 activity in egg chambers by expression of Cdc42^{N17} did not cause failure of filopodia formation in border cells, nor did expression of Cdc42^{V12} detectably increase the number of filopodia in border cells as they migrated. Consequently, it would appear that filopodial structures in this cell type form independently of Cdc42 activity.

Rac is not the only protein known to be required for border cell migration. Mutations in the *Drosophila* C/EBP gene result in delay or failure of border cell migration (Montell et al., 1992). C/EBP is a transcription factor that is expressed in terminally differentiating cells in both mammals (Christy et al., 1989; Kaestner et al., 1990) and flies (Montell et al., 1992; Rørth and Montell, 1992). Several putative downstream targets of C/EBP in the border cells have been identified (our unpublished results), including the *breathless* (*btl*) locus, which encodes a *Drosophila* homologue of the FGF receptor (Murphy et al., 1995). Given that Rac activity is stimulated by receptor tyrosine kinase activity in fibroblasts, it could be that Rac activation in border cells is controlled to some extent by FGF receptor activity. It is also possible that Rac expression in the border cells might be affected by C/EBP; however, in general, Ras superfamily proteins seem to be regulated at the level of activity rather than at the level of gene expression.

The lack of any effect of Rac or Cdc42 on the outer follicle cell rearrangement was surprising. This epithelial sheet movement would at first appear somewhat similar to dorsal closure during embryogenesis, a process that is inhibited by hs-Rac^{N17} (Harden et al., 1995). The only effects we observed on outer follicle cells were those caused by both mutant forms of RhoL. Although, as mentioned earlier, it was not possible to distinguish whether these effects resulted from cell-autonomous defects in cytoarchitecture or non–cell-autonomous defects caused by loss of contact with the germline, it is clear that the proper level of RhoL activity is required for the maintenance of a columnar epithelium by the follicle cells.

Rho Proteins and Nurse Cell Fusion

Increases or decreases in Cdc42 activity, or decreasing RhoL activity appeared to cause nurse cells to fuse, leaving unfused membrane connected to ring canals, which aggregated over time. Several examples of cell fusion occur during normal development. For instance, fertilization is a key cell–cell fusion event. Another normal fusion event is the fusion of myoblasts to form myotubes. Previous studies have demonstrated a requirement for Rac activity but not Cdc42 activity in myoblast fusion during *Drosophila* embryogenesis (Luo et al., 1994). The roles of Rho proteins in cell fusion have not been investigated in other organisms; however, mammalian cells have been reported to respond to Cdc42^{V12} by making huge multinucleated masses (Barfod, 1993). Cell fusion is accompanied by changes in cell adhesion and rearrangement of adherens junctions (Podbilewicz and White, 1994), so it is possible that Rho proteins induce changes in cell adhesion that lead to fusion in some cells. It is not clear why some cells respond to Cdc42 activity by fusing while other cells do not. It has been shown that the communication of myoblasts via gap junctions is a prerequisite for fusion (Mege et al., 1994). Perhaps the interconnection of nurse cells via ring canals provides the necessary connection to allow these cells to fuse in response to Cdc42 activity. An alternative hypothesis is that the nurse cells are not actively fusing, but rather are undergoing collapse as a result of the disruption of cortical actin. After cellular collapse, the ruptured membranes might simply have a tendency to fuse. This mechanism has been proposed to explain multinucleation defects caused by protein kinase A or armadillo function. At this point, we cannot distinguish these two mechanisms.

Rho proteins have been implicated in cytokinesis, yet we did not observe defects in cytokinesis or egg chamber formation in response to changes in Rho family activities. One explanation, however, is that at least in the germline, our constructs were not expressed early enough in oogenesis to affect cytokinesis. Thus, the question of what role Rho proteins play in incomplete cytokinesis and ring canal formation in nurse cells could not be addressed. It seems likely that some member or members of the Rho family will play a role in these events, since Rho has been implicated in cytokinesis in *Xenopus* and sanddollar eggs (Kishi et al., 1993; Mabuchi et al., 1993). Follicle cell division continues through stage 6, thus we would have expected to observe defects in follicle cell cytokinesis, yet we did not. Perhaps Rho or as yet undiscovered members of this family participate in cytokinesis in the follicle cells.

Rho Proteins in Cytoplasm Transfer

All three proteins tested perturbed the cytoplasm transport from the nurse cells to the oocyte; however, there were differences in the details of their effects on stage 11 nurse cells. Altered Cdc42, Rac, and RhoL proteins perturbed the formation of the actin cage and disrupted nurse cell subcortical actin. Thus, dumping in those egg chambers might have been prevented, not only because nuclei could be seen obstructing the ring canals, but also because of the structural irregularity of the subcortical actin. Cdc42 seemed to have the most potent effect on late stage egg chambers because defects were observed even in the absence of a deficiency for the locus. In each case, however, the presence of a deficiency for the appropriate locus caused the defects to be more severe, indicating that the level of each of the wild-type proteins was important. Constitutively active Ccd42 and RhoL had less severe effects on actin polymerization. The actin network still formed, but it was less regular than that observed in wild type. Thus, as in other cells and tissues that have been examined, regulation of Rho protein activities may be the key to proper actin organization, and the different Rho subfamilies seem to have related yet distinct functions.

Cellular and Molecular Mechanism of Rho Protein Activities

At the present time, little is known about the mechanisms

by which Rho proteins affect cytoarchitecture. Serine/threonine kinases that bind to activated Rac and Cdc42 have been identified (Manser et al., 1994). In addition, by analogy with Ras, the possibility exists that the GTPase activating proteins, which also bind the GTP-bound form, act as effectors for these proteins. It has been proposed that Rho proteins may influence the actin cytoskeleton by causing changes in the activities of actin-binding proteins. As of yet, however, there is no direct evidence for this, in part because the number of actin-binding proteins is large and it is not clear which ones are the best candidates for regulation by the Rho family. In stage 11 nurse cells, the best actin-binding protein candidates are more obvious. *Drosophila* relatives of the actin-binding proteins villin, profilin, and fascin are known to be required for polymerization of the actin cage (Cooley et al., 1992; Cant et al., 1994; Mahajan-Miklos and Cooley, 1994), a process that we found to also require Rho protein activities. Thus in this system, there are three candidate actin-binding proteins to examine for changes in activity in response to Rho proteins.

It is striking that many of the morphological changes that are observed in cells in response to Rho protein activities are accompanied by alterations in membrane and/or vesicle traffic, as well as actin reorganization. The Cdc42 gene was originally identified in yeast as a protein required for budding, a process that involves the localized insertion of new vesicles (Adams et al., 1990; Johnson and Pringle, 1990; Miller and Johnson, 1994). In mammalian cells, membrane ruffles that form in response to Rac contain distinctive arrays of actin filaments and are active in pinocytosis (Dowrick et al., 1993). Filopodia, which form in response to Cdc42 activity, also form when a synaptotagmin gene is expressed in fibroblasts (Feany and Buckley, 1993). Furthermore, the loss of stress fibers seen when Rho activity is inhibited in fibroblasts can be replicated by the inhibition of coated vesicle formation (Altankov and Grinnell, 1993).

Rho proteins are also often found in close proximity to vesicles. For example, in yeast, Cdc42 is normally localized at the plasma membrane in association with secretory vesicles (Ziman et al., 1993). Likewise, Rho1 has been localized to the cell periphery in regions proposed to contain post-Golgi vesicles (McCaffrey et al., 1991). One possibility is that in addition to regulating the cytoskeleton, Rho proteins may influence various membrane–vesicle fusion and budding activities. Clearly, there is much to be learned concerning the molecular mechanisms underlying the effects of Rho proteins on cells.

Rho proteins appear to play multiple essential functions in morphogenesis, and their effects vary significantly from one cell type to another. Their activities are called upon for dynamic processes, such as cell migration and dumping, but are also required for maintenance functions, for example, in nurse cells. In some cases, loss of activity and overactivity produce similar phenotypic consequences, suggesting that regulation of activity levels is crucial. Although Rho proteins would be unlikely to turn up in a mutant screen for specific developmental defects, because of the variety of processes in which they are required, study of their effects in genetically tractable organisms may allow us to relate their functions to those of the proteins with more restricted functions.

We are grateful to the following colleagues who sent us reagents: Alan Hall, Doug Johnson for *S. cervisiae* $Cdc42^{V12}$ and Cdc42 antibody, Lee-Ann Leshko for a developmental Northern blot and control data for loading, Liqun Luo for UAS-Drac and UAS-Dcdc42 flies, Nick Harden for $hsRac^{N17}$ flies, Lynn Manseau for ovary staining Gal4 lines, Mark Peifer for the armadillo antibody, and Lynn Cooley for hts and kelch antibodies. We heartily thank Sandie Baldauf for her help with the evolutionary tree drawing and Kathleen Margelot for helpful advice about acridine orange staining and TUNEL. Thanks also go to Drs. Alan Hall, Lynn Cooley, Larry Feig, and Craig Montell for helpful discussions and critical reading of the manuscript.

This work was supported by National Institutes of Health grant R296M46425 and a Lucille P. Marley Scholar Award to D.J. Montell.

Received for publication 27 October 1995 and in revised form 7 March 1996.

References

Abo, A., E. Pick, A. Hall, N. Totty, C.G. Teahan, and A.W. Segal. 1991. The small GTP-binding protein, p21Rac1, is involved in the activation of the phagocyte NADPH oxidase. *Nature (Lond.).* 353:668–670.

Abrams, J.M., K. White, L.I. Fessler, and H. Steller. 1993. Programmed cell death during *Drosophila* embryogenesis. *Development (Camb.).* 117:29–43.

Adams, A. E.M., D.I. Johnson, R.M. Longnecker, and B.F. Sloat. 1990. *CDC42* and *CDC43*, two additional genes involved in budding and the establishment of cell polarity in the yeast *Saccharomyces cerevisiae. J. Cell Biol.* 111:131–142.

Altankov, G., and F. Grinnell. 1993. Depletion of intracellular potassium disrupts coated pits and reversibly inhibits cell polarization during fibroblast spreading. *J. Cell Biol.* 120:1449–1459.

Barfod, E.T. 1993. Cloning and expression of a human CDC42 GTPase-activating protein reveals a functional SH3-binding domain. *J. Biol. Chem.* 268: 26059–26062.

Bender, A., and J.R. Pringle. 1989. Multicopy supression of *CDC24* budding defect in yeast by *CDC42* and three newly identified genes including Ras-related *RSR1* gene. *Proc. Natl. Acad. Sci. USA.* 86:9976–9980.

Bhullar, R.P. 1992. Identification of the brain Gn27 as the ral gene product. Comparison between the brain and platelet Gn-proteins. *FEBS Lett.* 298:61–64.

Bielinski, D.F., H.Y. Pyun, K. Linko-Stenz, I.G. Macara, and R.E. Fine. 1993. Ral and Rab3a are major GTP binding proteins of axonal rapid transport and synaptic vescicles and do not redistribute following depolarization stimulated synaptosomal exocytosis. *Biochim. Biophy. Acta.* 1151:246–256.

Bourne, H.R., D.A. Sanders, and F. McCormick. 1991. The GTPase superfamily: conserved structure and molecular mechanism. *Nature (Lond.).* 349:117–127.

Brand, A.H., and N. Perrimon. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development (Camb.).* 118:401–415.

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

Chant, J., and L. Stowers. 1995. GTPase cascades choreographing cellular behavior: movement, morphogenesis, and more. *Cell.* 81:1–4.

Christy, R.J., V.W. Yang, J.M. Ntambi, D.E. Geiman, W.H. Landschultz, A.D. Friedman, Y. Nakabeppu, T.J. Kelly, and M.D. Lane. 1989. Differentiation-induced expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes. *Genes & Dev.* 3:1323–1335.

Cooley, L., E. Verheyen, and K. Ayers. 1992. *chickadee* encodes a profilin required for intercellular cytoplasm transport during *Drosophila* oogenesis. *Cell.* 69:173–184.

Cummings, M.R., and R.C. King. 1970. The cytology of the vitellogenic stages of oogenesis in *Drosophila melanogaster.* I. General staging characteristics. *J. Morphol.* 128:427–442.

Dowrick, P., P. Kenworthy, B. McCann, and R. Warn. 1993. Circular ruffle formation and closure lead to macropinocytosis in hepatocyte growth factor/scatter factor-treated cells. *Eur J. Cell Biol.* 61:44–53.

Farnsworth, C.L. and L.A. Feig. 1991. Dominant inhibitory mutations in the Mg^{2+}-binding site of Ras^{H} prevent its activation by GTP. *Mol. Cell. Biol.* 11: 4822–4829.

Feany, M.B., and K.M. Buckley. 1993. The synaptic vesicle protein synaptotagmin promotes formation of filopodia in fibroblasts. *Nature (Lond.).* 364:537–540.

Feig, L.A., and G.M. Cooper. 1988. Inhibition at NIH313 cell proliferation by a mutant ras protein with preferential affinity for GDP. *Mol. Cell. Biol.* 8: 3235–3243.

Habets, G.G., E.H. Scholtes, D. Zuydgeest, R.A. van der Kammen, J.C. Stam, A. Berns, and J.G. Collard. 1994. Identification of an invasion-inducing gene, Tiam-1, that encodes a protein with homology to GDP-GTP exchang-

ers for Rho-like proteins. *Cell.* 77:537–549.

Harden, N., H.Y. Loh, W. Chia, and L. Lim. 1995. A dominant inhibitory version of the small GTP-binding protein Rac disrupts cytoskeletal structures and inhibits developmental cell shape changes in *Drosophila. Development (Camb.).* 121:903–914.

Hariharan, I.K., K.Q. Hu, and J. Settleman. 1995. Characterization of Rho GTPase family homologues in *Drosophila melanogaster*: overexpressing Rho 1 in retinal cells causes a late developmental defect. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:292–302.

Jahner, D., and T. Hunter. 1991. The ras-related gene *rho*B is an immediate-early gene inducible by v-FPS, epidermal growth factor, and platelet-derived growth factor in rat fibroblasts. *Mol. Cell. Biol.* 11:3682–3690.

Jalink, K., E.J. van Corven, T. Hengeveld, N. Mori, S. Narumiya, and W.H. Moolenaar. 1994. Inhibition of isophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylatin of the small GTP-Binding protein Rho. *J. Cell Biol.* 126:801–810.

Johnson, D.I., and J.R. Pringle. 1990. Molecular characterization of *CDC42*, a *Saccharomyces cerevisiae* gene involved in the development of cell polarity. *J. Cell Biol.* 111:143–152.

Kaestner, K.H., R.J. Christy, and M.D. Lane. 1990. Mouse insulin-responsive glucose transporter gene: characterization of the gene and trans-activation by the CCAAT/enhancer binding protein. *Proc. Natl. Acad. Sci. USA.* 87: 251–255.

King, R.C., and E.A. Koch. 1963. Studies on ovarian follicle cells of *Drosophila. Quart. J. Microscop. Sci.* 104:297–320.

King, R.C. 1970. Ovarian Development in *Drosophila melanogaster.* Academic Press, New York.

Kishi, K., T. Sasaki, S. Kuroda, T. Itoh, and Y. Taki. 1993. Regulation of cytoplasmic division of the *Xenopus* embryo by Rho p21 and its inhibitory GDP/GTP exchange protein (Rho GDI). *J. Cell Biol.* 120:1187–1195.

Knaus, U.G., P.G. Heyworth, T. Evans, J.T. Curnutte, and G.M. Bokoch. 1991. Regulation of phagocyte oxygen radical production by the GTP-binding protein Rac2. *Science (Wash. DC).* 254:1512–1515.

Kunkel, T.A., J.D. Roberts, and R.A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotype selection. *Methods Enzymol.* 154:367–382.

Letourneau, P.C., and T.A. Shattuck. 1989. Distribution and possible interactions of actin-associated proteins and cell adhesion molecules of nerve growth cones. *Development (Camb.).* 105:505–519.

Luo, L., Y.J. Liao, L.Y. Jan, and Y.N. Jan. 1994. Distinct morphogenetic functions of similar small GTPases: *Drosophila* Drac1 is involved in axonal outgrowth and myoblast fusion. *Genes & Dev.* 8:1787–1802.

Mabuchi, I., Y. Hamaguchi, H. Fujimoto, N. Morii, M. Mishima, and S. Naurumiya. 1993. A Rho-like protein is involved in the organization of the contractile ring in dividing sand dollar eggs. *Zygote.* 1:325–331.

Madaule, P., and R. Axel. 1985. A novel ras-related gene family. *Cell.* 42:31–40.

Mahajan-Miklos, S., and L. Cooley. 1994. The villin-like protein encoded by Drosophila *quail* gene is required for actin bundle assembly during oogenesis. *Cell.* 78:291–301.

Manser, E., T. Leung, H. Salihuddin, Z.S. Zhao, and L. Lim. 1994. A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature (Lond.).* 367:40–46.

Matsui, Y. and A. Toh-e. 1992. Yeast *RH03* and *RH04 ras* superfamily genes are necessary for bud growth, and their defect is suppressed by a high dose of bud formation genes CDC42 and BEM1. *Mol. Cell. Biol.* 12:5690–5699.

McCaffrey, M., J.S. Johnson, B. Goud, A.M. Myers, J. Rossier, M.R. Popoff, P. Madaule, and P. Boquet. 1991. The small GTP-binding protein Rholp is localized on the Golgi apparatus and post-Golgi vesicles in *Saccharomyces cerevisiae. J. Cell Biol.* 115:309–319.

Mege, R.M., D. Goudou, C. Giaume, M. Nicolet, and F. Rieger. 1994. Is intercellular communication via gap junctions required for myoblast fusion? *Cell Adhes. Commun.* 2:329–343.

Michiels, F., G.G. Habets, J.C. Stam, R.A. van der Kammen, and J.G. Collard. 1995. A role for Rac in Tiam 1-induced membrane ruffling and invasion. *Nature (Lond.).* 375:338–340.

Miller, P.J., and D.I. Johnson. 1994. Cdc42p GTPase is involved in controlling polarized cell growth in *Schizasaccharomyces pombe. Mol. Cell. Biol.* 14: 1075–1083.

Montell, D.J., P. Rørth, and A.C. Spradling. 1992. *slow border cells*, a locus required for a developmentally regulated cell migration during oogenesis, encodes *Drosophila* C/EBP. *Cell.* 71:51–62.

Murphy, A.M., T. Lee, C.M. Andrews, B.Z. Shilo, and D.J. Montell. 1995. The Breathless FGF receptor homolog, a downstream target of *Drosophila* C/EBP in the developmental control of cell migration. *Development (Camb.).* 121:2255–2263.

Nishiyama, T., T. Sasaki, K. Takaishi, M. Kato, H. Yaku, K. Araki, Y. Matsuura, and Y. Takai. 1994. *rac* p21 is involved in insulin-induced membrane ruffling and Rho p21 is involved in hepatocyte growth factor- and 12-*O*-tetrade-canoylphorbol-13-acetate (TPA)-induced membrane ruffling in KB cells. *Mol. Cell. Bio.* 14:2447–2456.

Nobes, C.D., and A. Hall. 1995. Rho, Rac and Cdc42 GTPases regulate the assembly of multi-molecular focal complexes associated with actin stress fibers, lamellipodia and filopodia. *Cell.* 81:53–62.

Peifer, M., S. Orsulic, D. Sweeton, and E. Wieschaus. 1993. A role for the *Drosophila* segment polarity gene *armadillo* in cell adhesion and cytoskeletal integrity during oogenesis. *Development (Camb.).* 118:1191–1207.

Podbilewicz, B., and J.G. White. 1994. Cell fusions in the developing epithelial of *C. elegans. Dev. Biol.* 161:408–424.

Qiu, R.G., J. Chen, D. Kirn, F. McCormick, and M. Symons. 1995. An essential role for Rac in Ras transformation. *Nature (Lond.).* 374:457–459.

Rørth, P. and D. J. Montell. 1992. *Drosophila* C/EBP: a tissue-specific DNA-binding protein required for embryonic development. *Genes & Dev.* 6:2299–2311.

Ridley, A.J., H.F. Paterson, C.L. Johnston, D. Diekmann, and A. Hall. 1992. The small GTP-binding protein Rac regulates growth factor-induced membrane ruffling. *Cell.* 70:401–410.

Ridley, A.J., and A. Hall. 1992. The Small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell.* 70:389–399.

Robinson, D.N., K. Cant, and L. Cooley. 1994. Morphogenesis of *Drosophila* ovarian ring canals. *Development (Camb.).* 20:2015–2025.

Rubin, G.M., and A.C. Spradling. 1982. Genetic transformation of Drosophila with transposable element vectors. *Science (Wash.DC).* 218:348–353.

Schweighoffer, F., H. Cai, M.C. Chevallier-Multon, I. Fath, G. Cooper, and B. Tocque. 1993. The *Saccharomyces cerevisiae* SDC25 C-domain gene product overcomes the dominant inhibitory activity of Ha-Ras asn-17. *Mol. Cell. Biol.* 13:39–43.

Schweisguth, F., and J.W. Posakony. 1994. Antagonistic activities of suppressor of Hairless and Hairless control alternative cell fates in the *Drosophila* adult epidermis. *Development (Camb.).* 120:1433–1441.

Takaishi, K., A. Kikuchi, S. Kuroda, K. Kotani, T. Sasaki, and Y. Takai. 1993. Involvement of *rho* p21 and its inhibitory GDP/GTP exchange protein (*rho* GDI) in cell motility. *Mol. Cell. Biol.* 13:72–79.

Vincent, S., P. Jeanteur, and P. Fort. 1992. Growth-related expression of *rhoG*, a new member of the *ras* homolog gene family. *Mol. Cell. Biol.* 12:3138–3148.

Volknandt, W., J. Pevsner, L.A. Elferink, and R.H. Scheller. 1993. Association of three small GTP binding proteins with cholinergic synaptic vesicles. *FEBS Lett.* 317:53–56.

Warn, R.M., H.O. Gutzeit, L. Smith, and A. Warn. 1985. F-actin rings are associated with the ring canals of the *Drosophila* egg chamber. *Exp. Cell Res.* 157:355–363.

Wheatley, S., S. Kulkarni, and R. Karess. 1995. *Drosophila* nonmuscle myosin II is required for rapid cytoplasmic transport during oogenesis and for axial nuclear migration in early embryos. *Development (Camb.).* 121:1937–1945.

Wildey, G.M., M. Viggeswarapu, S. Rim, and J.K. Denker. 1993. Isolation of cDNA clones and tissue expression of rat ralA and ralB GTP-binding proteins. *Biochem. Biophys. Res. Commun.* 194:552–559.

Yamochi, W., K. Tanka, H. Nonaka, A. Maeda, and T. Musha. 1994. Growth site localization of Rho1 small GTP-binding Protein and its involvement in bud formation in *Saccharomyces cerevisiae. J. Cell Biol.* 125:1077–1093.

Ziman, M., D. Preuss, J. Mulholland, J.M. O'Brien, D. Botstein, and D.I. Johnson. 1993. Subcellular localization of cdc42p, a Saccharomyces cerevisiae GTP-binding protein involved in the control of cell polarity. *Mol. Biol. Cell.* 4:1307–1316.