

Rho1 interacts with p120^{ctn} and α -catenin, and regulates cadherin-based adherens junction components in *Drosophila*

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SUMMARY

Rho GTPases are important regulators of cellular behavior through their effects on processes such as cytoskeletal organization. Here we show interactions between *Drosophila* Rho1 and the adherens junction components α -catenin and p120^{ctn}. We find that while Rho1 protein is present throughout the cell, it accumulates apically, particularly at sites of cadherin-based adherens junctions. Cadherin and catenin localization is disrupted in *Rho1* mutants, implicating Rho1 in their regulation. p120^{ctn} has recently been suggested to inhibit Rho activity through an unknown mechanism. We find that Rho1 accumulates in response to lowered p120^{ctn} activity.

Significantly, we find that Rho1 binds directly to α -catenin and p120^{ctn} in vitro, and these interactions map to distinct surface-exposed regions of the protein not previously assigned functions. In addition, we find that both α -catenin and p120^{ctn} co-immunoprecipitate with Rho1-containing complexes from embryo lysates. Our observations suggest that α -catenin and p120^{ctn} are key players in a mechanism of recruiting Rho1 to its sites of action.

Key words: Rho GTPase, *Drosophila melanogaster*, Adherens junction, p120^{ctn}, α -catenin

INTRODUCTION

Members of the Rho family of small GTPases have been shown to be important regulators of cellular behavior, especially actin cytoskeletal organization and acto-myosin based contractility (Hall, 1998). Rho proteins function as molecular switches, cycling between an active GTP-bound state and an inactive GDP-bound state. Their activation state is controlled by regulatory proteins such as guanine dissociation inhibitors (GDIs), which inhibit the release of GDP and keep Rho inactive, guanine exchange factors (GEFs), which catalyze the exchange of GDP for GTP and activate Rho, and GTPase activating proteins (GAPs), which increase the rate at which Rho hydrolyzes GTP and hence becomes inactivated (Maruta, 1998; Symons and Settleman, 2000). Rho GTPases have been implicated in many seemingly disparate cellular processes such as cell cycle progression, MAP kinase signaling, and cell-substrate and cell-cell adhesion (Braga, 1999; Mackay and Hall, 1998; Narumiya, 1996; Van Aelst and D'Souza-Schorey, 1997). Understanding the biochemical mechanisms through which Rho is activated and carries out its various functions has been the focus of much recent work, and a number of downstream targets have been identified from studies in cell culture suggesting that Rho acts through multiple pathways (Bishop and Hall, 2000; Narumiya et al., 1998).

The subcellular localization of Rho proteins has been shown to play an important role in their function (Symons and

Settleman, 2000). Rho is predominantly cytosolic but translocates to the membrane upon ligand stimulation, with different ligands leading to differential localization (Fleming et al., 1996). Membrane localization is dependent on a carboxy-terminal CAAX motif which allows the protein to be isoprenylated (Fleming et al., 1996; Kranenburg et al., 1997). Interfering with this translocation event affects the ability of Rho to carry out a subset of its functions. Prenylation of mammalian RhoB is required for it to transform NIH3T3 cells (Lebowitz et al., 1997), while the RhoA CAAX domain is needed for cytoskeletal contraction in response to ligand stimulation in N1E-115 neuroblastoma cells (Kranenburg et al., 1997). However, RhoB's ability to activate transcription from the *c-fos* serum response element and RhoA's stress fiber induction is not compromised in these systems, indicating that membrane localization is not necessary for all Rho functions.

In epithelial and endothelial cells, Rho is enriched at sites of actin accumulation and membrane ruffling, where it colocalizes with the ezrin/radixin/moesin (ERM) family of proteins (Takaishi et al., 1995). ERM family proteins are involved in mediating association of the plasma membrane and the actin cytoskeleton. The colocalization of Rho with ERM proteins suggests a role for both in the regulation of adhesive complexes. Consistent with this, the formation of cadherin-based adherens junctions (AJs) has been shown to be regulated by Rho and Rac in mammalian cell culture (Braga et al., 1997).

In addition to the cadherins themselves, AJs contain a

number of associated proteins including members of the catenin family: β -catenin (Armadillo), α -catenin, and p120 catenin (p120^{ctn}) (Hatzfeld, 1999; Steinberg and McNutt, 1999). While β -catenin and α -catenin are involved in linking cadherins to the actin cytoskeleton, p120^{ctn} appears to play more of a regulatory role and has been proposed to both positively and negatively regulate adhesion, although the precise mechanisms involved are not yet known (Anastasiadis and Reynolds, 2000; Noren et al., 2000). In addition to their roles at adherens junctions, β -catenin and p120^{ctn} function in the nucleus in conjunction with transcription factors to regulate gene expression (Behrens et al., 1996; Daniel and Reynolds, 1999). Recently p120^{ctn} has been suggested to negatively regulate the activity of Rho when present in the cytoplasm by acting as a GDI and preventing the exchange of GDP for GTP, although p120^{ctn} has no sequence homology to other GDIs (Anastasiadis et al., 2000). p120^{ctn} could also be involved in activating Rho by recruiting it to sites of cadherin localization, where it can become accessible to GEFs and its downstream effectors.

Cadherin-based AJs are responsible for many of the cell-cell contacts found in the *Drosophila* embryo (Tepass et al., 1996; Uemura et al., 1996). We have previously characterized a mutation in the *Drosophila* RhoA homolog, *Rho1* (Magie et al., 1999). This mutation results in a number of maternal and zygotic defects in morphogenetic processes consistent with a role for Rho1 in regulating cytoskeletal dynamics and cell shape changes, as well as in patterning events involving transcriptional activation. Here we show that Rho1 protein accumulates at AJs in the *Drosophila* embryo and ovary and that cadherin and catenin localization is aberrant in *Rho1* mutants. We find that Rho1 interacts physically with p120^{ctn} and α -catenin, components of adherens junction complexes. These data suggest a role for the catenins in recruiting Rho1 to the plasma membrane, and a subsequent role for Rho1 in the regulation of proper AJ formation.

MATERIALS AND METHODS

Fly stocks

Flies were cultured and crossed on yeast-cornmeal-molasses-malt extract medium at 25°C. The Rho1 alleles used in this study were: *Rho1^{rev220}/CyO*, an imprecise P-element excision line (Magie et al., 1999) and *Rho1^{E3.10}/CyO*, a point mutation within the C-terminal CAAX domain of Rho1 (Halsell et al., 2000). Other alleles used: *UAS-Rho1* (Harden et al., 1999); *UAS-dRac^{N17}*, *UAS-Rac^{L89}*, *UAS-Cdc42^{N17}*, and *UAS-Cdc42^{V12}* (Murphy and Montell, 1996); *UAS-actin-GFP* (H. Oda); *puc^{E69}* (A. Martinez-Arias); *hep^{r75}* (S. Noselli); *Actin3-Gal4/TM6B*, *en-Gal4*, *UAS-GFP/CyO*, *In(2R)bw^{Vde2L-CyR}/In(2LR)Gla*, *wg^{Gla-1}* (p120^{ctn} deficiency), *bsk¹* and *w^a Nf^{a-g}*; *Df(2R) Jp8, w⁺/CyO* (*Rho1* deficiency) (Bloomington Stock Center).

Antibody production and characterization

BALB/c BYJ Rb(8.12) 5BNR/J mice (Jackson Labs) were immunized with GST-Rho1. The P1D9 monoclonal line was generated in the FHCRC Hybridoma Production Facility as described (Hoffstrom and Wayner, 1995; Wayner et al., 1989), and has been sent to the Developmental Studies Hybridoma Bank (University of Iowa). Western blotting was used to test antibody specificity using bacterially expressed GST-dRho1, GST-RhoL (obtained by PCR and subcloned into pGEX-3X as a 5'*Bam*HI-3'*Eco*RI fragment), GST-Rac1 and GST-Cdc42. Wild-type *Drosophila* whole cell (from 0- to 2-hour embryos)

and nuclear (from 0- to 12-hour embryos) extracts were a gift from T. Tsukiyama. Polyclonal antiserum against *Drosophila* p120^{ctn} (p120ctn) was generated by immunizing BALB/c BYJ Rb(8.12) 5BNR/J mice with a protein composed of GST fused to the first 222 aa (amino acids) of *Drosophila* p120^{ctn} (CG17484).

Immunofluorescence

Embryos were prepared and detection of proteins was performed as described previously (Parkhurst et al., 1990). Ovaries were fixed for 10 minutes in 6% formaldehyde, 16.7 mM KPO₄ (pH 6.8), 75 mM KCl, 25 mM NaCl and 3.3 mM MgCl₂, then washed 3× in PBS + 0.1% Triton X-100. Ovarioles were dissected by hand, then blocked in 2% goat normal serum for 2 hours before incubation with primary antibody. Antisera used were as follows: anti-DE-cadherin from H. Oda (1:100), anti-neurexinIV from H. Bellen (1:15000), anti- α -spectrin from D. Branton (1:500), anti- β -galactosidase from J. Scully (1:1000), anti-Lava lamp from J. Sisson (1:5000), and anti-Armadillo from M. Peifer and the Developmental Studies Hybridoma Bank (1:100). Anti-Rho1 P1D9 monoclonal antiserum was used at 1:50. Propidium iodide staining was done by incubating embryos in 1 μ g/ml propidium iodide and 50 μ g/ml RNase for 1 hour following antibody staining, then washing as described previously (Parkhurst et al., 1990).

In vitro interactions

cDNAs corresponding to *DE-cadherin* (CG3722) (also known as *shotgun*; *sh*) *α -catenin* (CG17947), *armadillo* (CG11579), *p120^{ctn}* (CG17484), *rok* (CG9774) and *RhoGDI* (CG7823) were obtained from the Berkeley *Drosophila* Genome Project (BDGP). The DE-cadherin cytoplasmic domain was amplified by PCR, then subcloned as a 5'*Bam*HI-3'*Eco*RI fragment into pGEX-3X. A full-length p120^{ctn} clone was generated by overlapping PCR with a truncated cDNA (lacking exon 4) and exon 4 obtained by PCR from genomic DNA. The *α -catenin*, *armadillo*, and *p120^{ctn}* ORFs were subcloned into pCite 4c⁺ (Novagen) as 5'*Sal*I-3'*Not*I, 5'*Bam*HI-3'*Xho*I, and 5'*Sal*I-3'*Not*I fragments, respectively. *rok* and *RhoGDI* ORFs were subcloned into pCite 4a⁺ (Novagen) as 5'*Bam*HI-3'*Not*I, and 5'*Bam*HI-3'*Eco*RI fragments, respectively. They were then expressed from the T7 promoter using the Promega TnT in vitro expression kit. Full-length GST-Rho1 was described previously (Magie et al., 1999). Pieces of Rho1 were amplified by PCR from the Rho1 cDNA (N-term=aa 1-75; C-term=aa 76-192; piece A=aa 1-27; piece C=aa 50-75). Substitutions were made within the full-length Rho1 cDNA using primers that change the codons corresponding to the appropriate amino acids (V14A; N17A; F39A; E40N; KDQ/A, substituting alanines at aa 27-29; KQVE/A, substituting alanines at aa 51-54; RLRP/A, substituting alanines at aa 68-71). The Rho1 pieces and substitutions were cloned into pGEX-3X as 5'*Bam*HI-3'*Eco*RI fragments. His-p120^{ctn} was generated by subcloning the p120^{ctn} ORF into pRSetA (Invitrogen) as a 5'*Xho*I-3'*Kpn*I fragment. GST pulldown assays were performed as described (Lu and Settleman, 1999; Magie et al., 1999).

Amino acid residues corresponding to the α -catenin and p120^{ctn} binding domains were modeled on the GTP- and GDP-bound RhoA crystal structure using the Quanta protein modeling program.

Immunoprecipitations

Embryo lysate was prepared by homogenizing an overnight collection of embryos in 0.5 ml L-buffer (PBS + 0.1% NP-40 + protease inhibitors), followed by sonication and centrifugation to pellet debris. Lysate was incubated with primary antibody in 0.5 ml L-buffer for 1 hour at 4°C. Protein G sepharose was then added and the reaction allowed to proceed overnight. Analysis was conducted using SDS-PAGE followed by western blots.

RNA interference (RNAi)

p120^{ctn}, α -catenin and fushi tarazu (*ftz*) single-stranded RNAs (ssRNA) were transcribed from the T7 and SP6 promoters present on

the pOT2A plasmids (BDGP) using the RiboMAX RNA production system (Promega). RNA preparation and injection was conducted as described previously (Kennerdell and Carthew, 1998). Embryos aged 15-45 minutes were injected at roughly 50% egg length with double-stranded RNA (dsRNA; 5 μ M). The embryos were aged to the appropriate stage, then fixed and stained as described previously (Magie et al., 1999).

Germline transformation

The UAS-p120^{ctn} and UAS- α -catenin expression constructs were made by first amplifying the ORFs by PCR from the appropriate catenin cDNA, then cloning them into the pUASp vector (Rorth, 1998) as 5'Kpn-3'Xba fragments. The UAS-p120^{ctn} and UAS- α -catenin vectors (500 μ g/ml) were injected along with the pTURBO helper plasmid (100 μ g/ml) (Mullins et al., 1989) into isogenic *w*¹¹¹⁸ flies as described (Spradling, 1986). Transgenics were scored by eye color and the insertions were mapped and balanced using standard genetic methods.

RESULTS

Characterization of monoclonal antibodies to *Drosophila* Rho1

A hybridoma fusion was conducted to recover monoclonal antibodies from mice immunized with a full length *Drosophila* Rho1-glutathione-S transferase (GST) fusion protein. Western analysis of GST fusion proteins of the *Drosophila* Rho family members Rho1, RhoL, Rac1, and Cdc42 identified one line that recognized Rho1 with high specificity (P1D9; Fig. 1A). This line, P1D9, recognizes an epitope within the C-terminal 55 amino acids of Rho1, which is the region most dissimilar to other Rho family members (data not shown). Western blots prepared with whole cell and nuclear embryo extracts indicate that P1D9 recognizes one major band of the predicted size, 21 kDa, in whole cell, but not nuclear, extracts (Fig. 1B). We intermittently observe a 130 kDa band in the nuclear extract, the identity of which is not known.

To examine the specificity of P1D9 for Rho1 in vivo we examined P1D9 staining in embryos lacking dRho1. Embryos homozygous for a deficiency that includes the *Rho1* locus exhibit no staining above background, compared with sibling controls (Fig. 1C,D). We also overexpressed Rho1, Rac1, and Cdc42 in embryos using the conditional Gal4-UAS system. An Engrailed-Gal4 driver was used to express the Rho proteins in stripes, along with green fluorescent protein to visualize the overexpression domains. Stripes of ectopic protein accumulation could be seen above the uniform endogenous Rho1 levels in embryos expressing ectopic Rho1 (Fig. 1E), but not in embryos expressing ectopic Rac1 (Fig. 1G) or Cdc42 (Fig. 1I), confirming the in vivo specificity of P1D9.

Rho1 protein is expressed ubiquitously during development

Previous studies in cell culture have suggested that the subcellular localization of Rho can have important effects on some of its functions, particularly those involving cell-cell adhesion. We used the P1D9 monoclonal antibody to examine the localization of Rho1 during *Drosophila* embryogenesis. During the syncytial blastoderm stages, Rho1 subcellular localization changes in a cell-cycle dependent manner. During interphase, Rho1 surrounds nuclei asymmetrically (Fig. 2A),

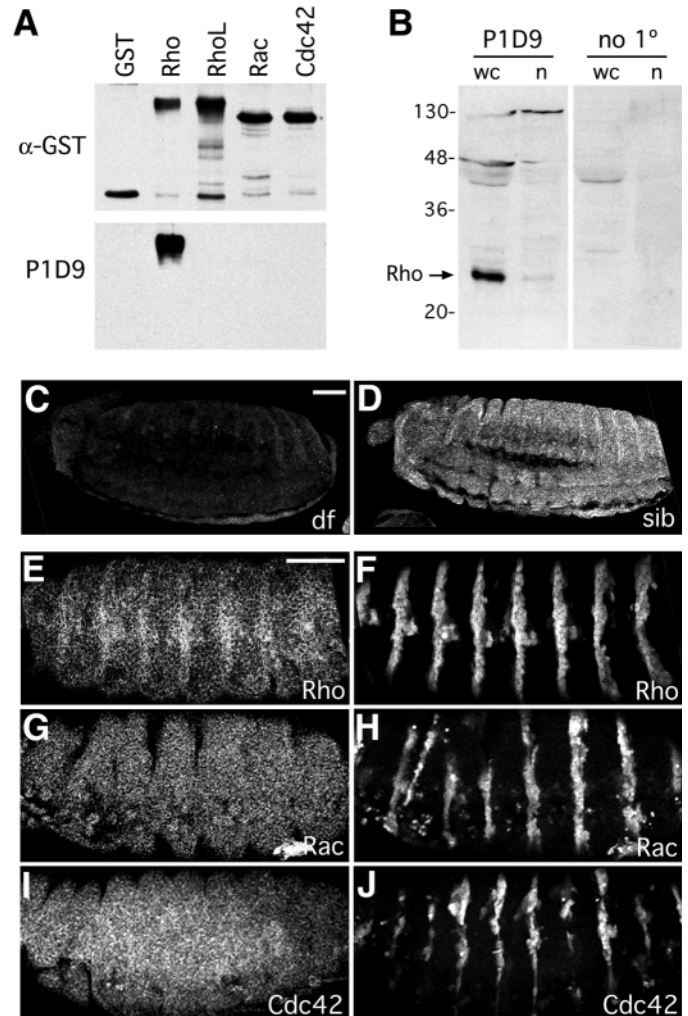


Fig. 1. P1D9 monoclonal antibody is specific for Rho1 protein in vitro and in vivo. (A) Western analysis of *Drosophila* Rho family GST fusion proteins hybridized with α -GST (top) and P1D9 monoclonal (bottom) antiserum. (B) Western analysis of whole cell (wc) and nuclear (n) lysates prepared from 0- to 2-hour and 0- to 12-hour embryos, respectively. P1D9 recognizes a single major band in whole cell but not nuclear lysates. This band is not detected when primary antibody is omitted. (C,D) P1D9 staining of a stage 14 embryo homozygous for a deficiency that includes the *Rho1* locus (C) relative to a sibling control (D). The relative intensity of the staining in these embryos can be directly compared, as both embryos were photographed in the same visual field. (E-J) P1D9 recognizes ectopically expressed Rho1 (E), but not Rac1 (G) or Cdc42 (I). Each protein was overexpressed in the Engrailed domain, as highlighted by green fluorescent protein expression (F,H,J), and embryos were examined at stage 14. For all embryos, anterior is left and dorsal is up. Scale bars: 50 μ m.

while in metaphase it accumulates at the transient furrows that form around each nucleus (Fig. 2B). At the cellular blastoderm stage Rho1 protein is present throughout the cytoplasm of all cells, but concentrated apically (Fig. 2C, arrow in Fig. 2C'). This pattern of apical localization is similar to that of the cell-cell adhesion molecule DE-cadherin, which is concentrated at AJs (Fig. 2D,D') (Oda et al., 1994). Rho1 is excluded from nuclei (Fig. 2E) and localizes to the cytoplasm, as indicated by

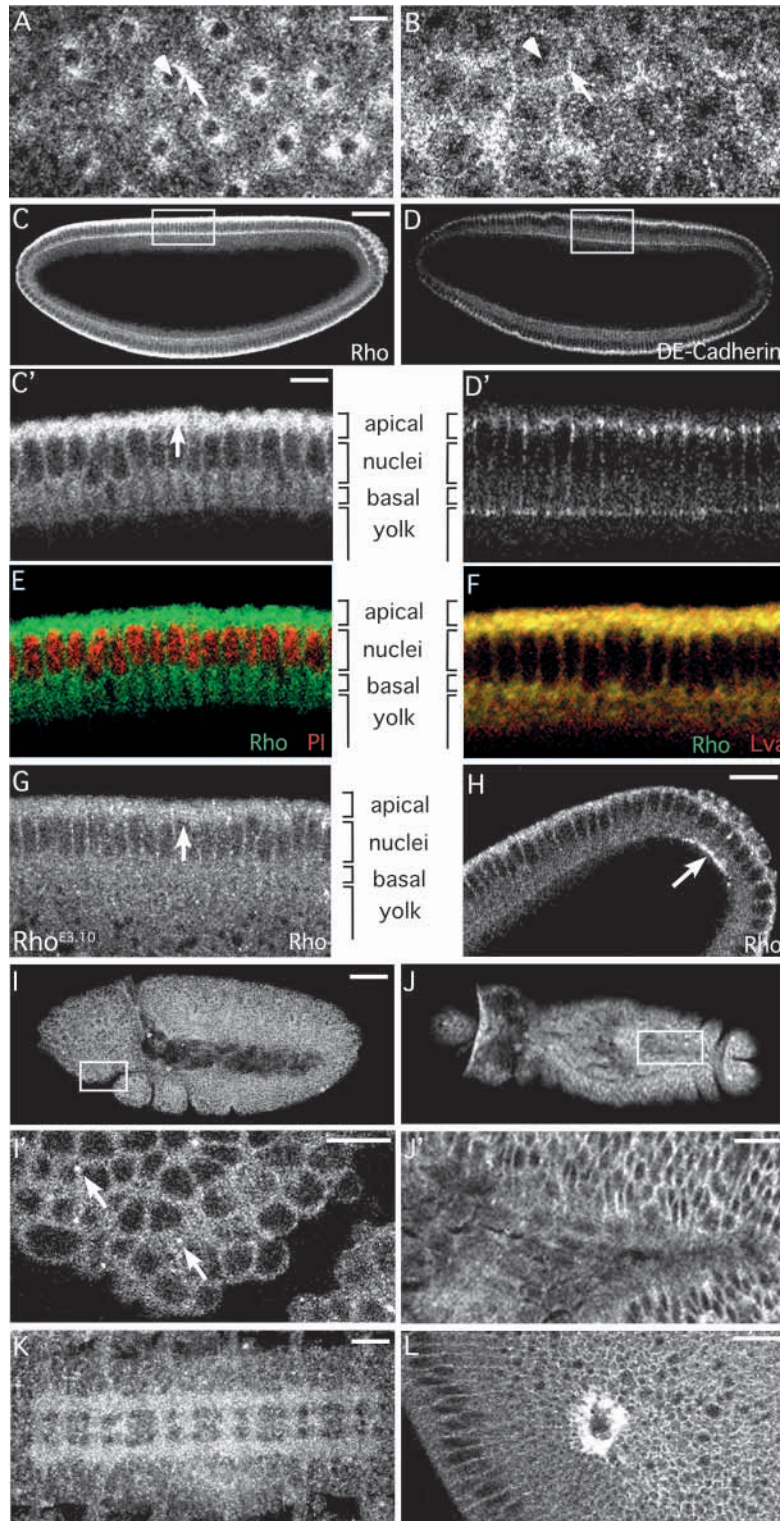


Fig. 2. Rho1 protein is ubiquitously expressed, but concentrated apically, in blastoderm embryos. (A,B) Confocal micrograph showing Rho1 localization at interphase (A) and metaphase (B) in an early syncytial blastoderm stage. Note that the accumulation of Rho protein (arrows) relative to nuclei (arrowheads) changes with cell cycle phase. (C-D') Confocal micrograph showing PID9 (C,C') and DE-cadherin (D,D') staining of embryos at the cellular blastoderm stage. Note apical accumulation of Rho1 protein at this stage (arrow in C'). (E-F) Rho protein is localized cytoplasmically. (E) Double staining with PID9 (green) and propidium iodide (red) to label nuclei. (F) Double staining with PID9 (green) and an antibody against Lava lamp (red), a protein present in the cytoplasm. (G) PID9 staining of *Rho1^{E3.10}* homozygous embryos at the cellular blastoderm stage. Note the lack of apical Rho1 accumulation (arrow, compare with C'). (H) Close-up of an embryo at the cellular blastoderm stage showing basal accumulation of Rho1 protein in cells underlying the pole cells (arrow). (I-J') PID9 staining in stage 11 (I,I') and stage 14 (J,J') embryos. (I',J') Higher magnification views of the portion of the embryos boxed in I and J, respectively. Note subcellular punctate spots of Rho1 accumulation (arrows in I'), and that Rho1 protein does not accumulate in cells at the leading edge during dorsal closure (J'). (K) Neural commissures of a stage 14 embryo stained with PID9. (L) Grazing section of a cellular blastoderm stage embryo showing accumulation of Rho1 protein at a puncture wound site. In all images except (L), anterior is left and dorsal is up. Scale bars: A,C',I': 10 μ m; C, I: 50 μ m; H,J',K,L: 25 μ m.

protein is enriched in the neural commissures of the central nervous system (Fig. 2K), and also accumulates at wound sites, such as those resulting from the microinjection of early embryos (Fig. 2L), implicating Rho1 in the wound healing response.

We observe aberrant Rho1 localization in *Rho1* mutants, particularly in embryos homozygous for *Rho1^{E3.10}*, a point mutation that disrupts the C-terminal isoprenylation site involved in tethering Rho to the plasma membrane (Fig. 2G). In these mutant embryos cytoplasmic staining is present, however apical accumulation of Rho protein is no longer detectable (compare Fig. 2G with 2C').

Rho1 protein accumulates at sites of adherens junction formation in the embryo

Rho has been implicated in the regulation of cell adhesion through its effects on a number of different types of cellular junctions, including integrin-based focal adhesions (Schwartz and Shattil, 2000) and cadherin-based AJs (Kaibuchi et al., 1999). We examined Rho1 localization relative to DE-cadherin, a component of AJs located around the apical margin of cells and Neurexin, a component of septate junctions (SJs), which are thought to be analogous to tight junctions in mammalian cells (Baumgartner et al., 1996). Since AJs and other cell-cell contacts are not yet fully formed at the cellular blastoderm stage, we examined cells of the gut epithelium, which show a clear apical-basal polarity with AJs located at the apical end of the cell and SJs more basal (Fig. 3J) (Tepass and Hartenstein, 1993). As in blastoderm embryos (Fig. 2,

the overlap in staining with the cytoplasmic protein Lava Lamp (Fig. 2F) (Sisson et al., 2000). High levels of protein are also seen at the basal surface of cells underlying the pole cells in the posterior of the embryo (Fig. 2H). At later stages Rho1 protein is also located throughout the cytoplasm of cells, but in addition to showing a diffuse, uniform pattern, it is concentrated in occasional punctate spots (Fig. 2I-J'). Rho1

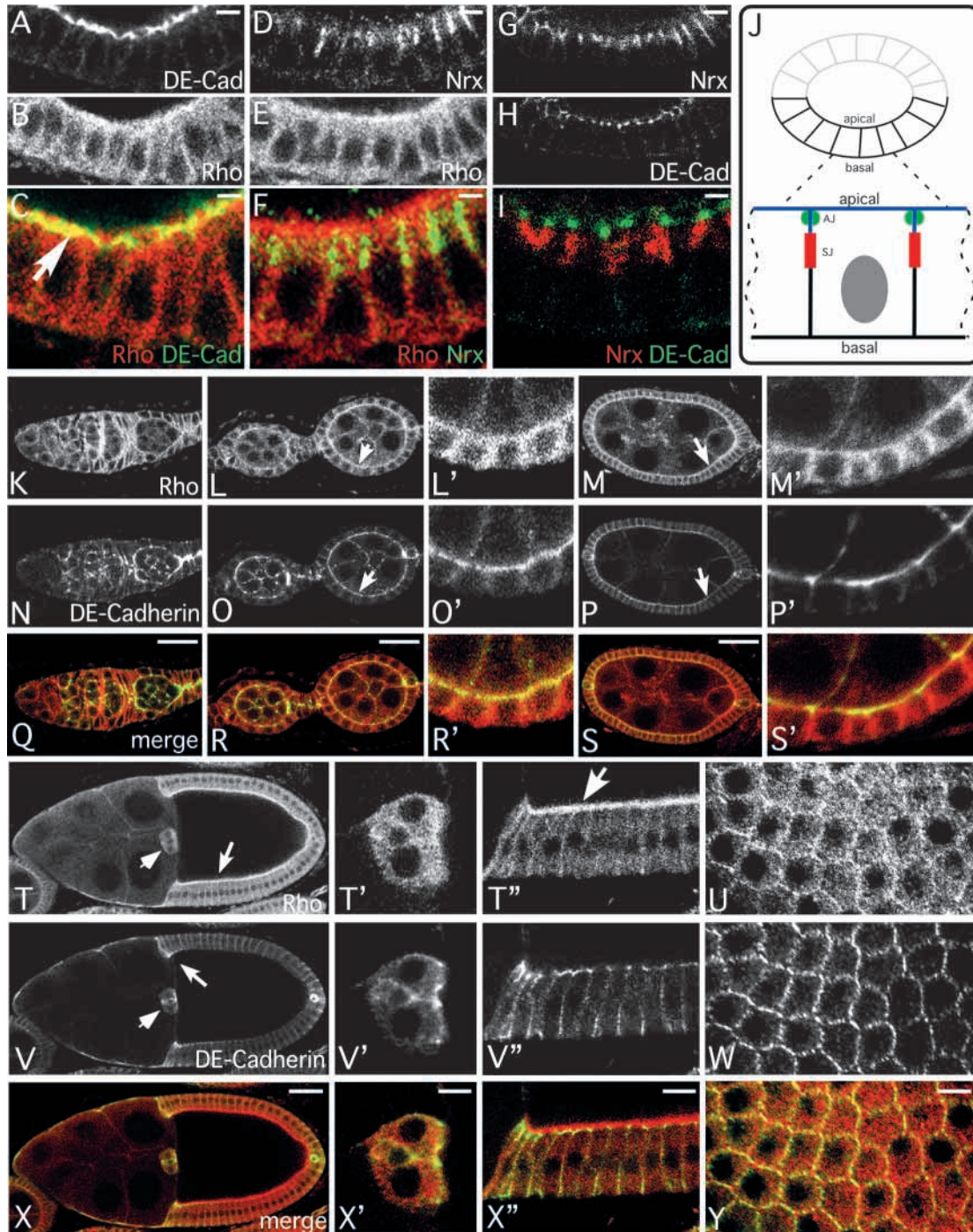


Fig. 3. Rho1 accumulates at sites of DE-cadherin localization in the embryo and ovary. (A-C) Confocal image of a hindgut tube from a stage 14 embryo showing double labeling for both DE-cadherin (A) and Rho1 (B). A higher magnification view of the merged image is shown in C. In all merged images, Rho1 staining is red and the staining for other molecules is green. Apical is up in all images. Note the apical accumulation of Rho1 protein that coincides with DE-cadherin expression (arrow in C). (D-F) A stage 14 hindgut tube double labeled for the septate junction protein neurexin (D), Rho1 (E), and the merged image (F). Note that the neurexin staining (green) is largely exclusive of Rho1 apical accumulation (red). (G-I) Control staining documenting DE-cadherin (green) localization relative to neurexin (red). (J) A schematic diagram of the gut showing the relative locations of the adherens junctions (AJ) and septate junctions (SJ). (K-M', T-U) Expression of Rho1 protein during oogenesis. Rho1 protein is expressed in all stages, including the germarium (K). Note Rho1 accumulation in the apical regions of follicle cells (L', M'; arrows in L, M). Rho1 expression is upregulated in border cells (T') and accumulates at lateral follicle cell contacts (cross section, T''; grazing section, U) and in the cortex of the oocyte (arrows in T, T''). (N-P', V-W) Localization of DE-cadherin during oogenesis. Cadherin is upregulated in the border cells (P', arrowhead in V; V') and also localized to lateral follicle cell contacts (arrow in V; V'', W). (Q-S', X-Y) Merged images of Rho1 and DE-cadherin staining (red: Rho1, green: DE-cadherin). Stages of oogenesis shown are the germarium (K, N, Q), stage 3 and 5 (L, O, R), stage 8 (M, P, S) and stage 10a (T, V, X). Scale bars: A, D, G: 0.6 μ m; C, F, I: 0.2 μ m; Q, R, S, X: 30 μ m; X', X'', Y: 10 μ m.

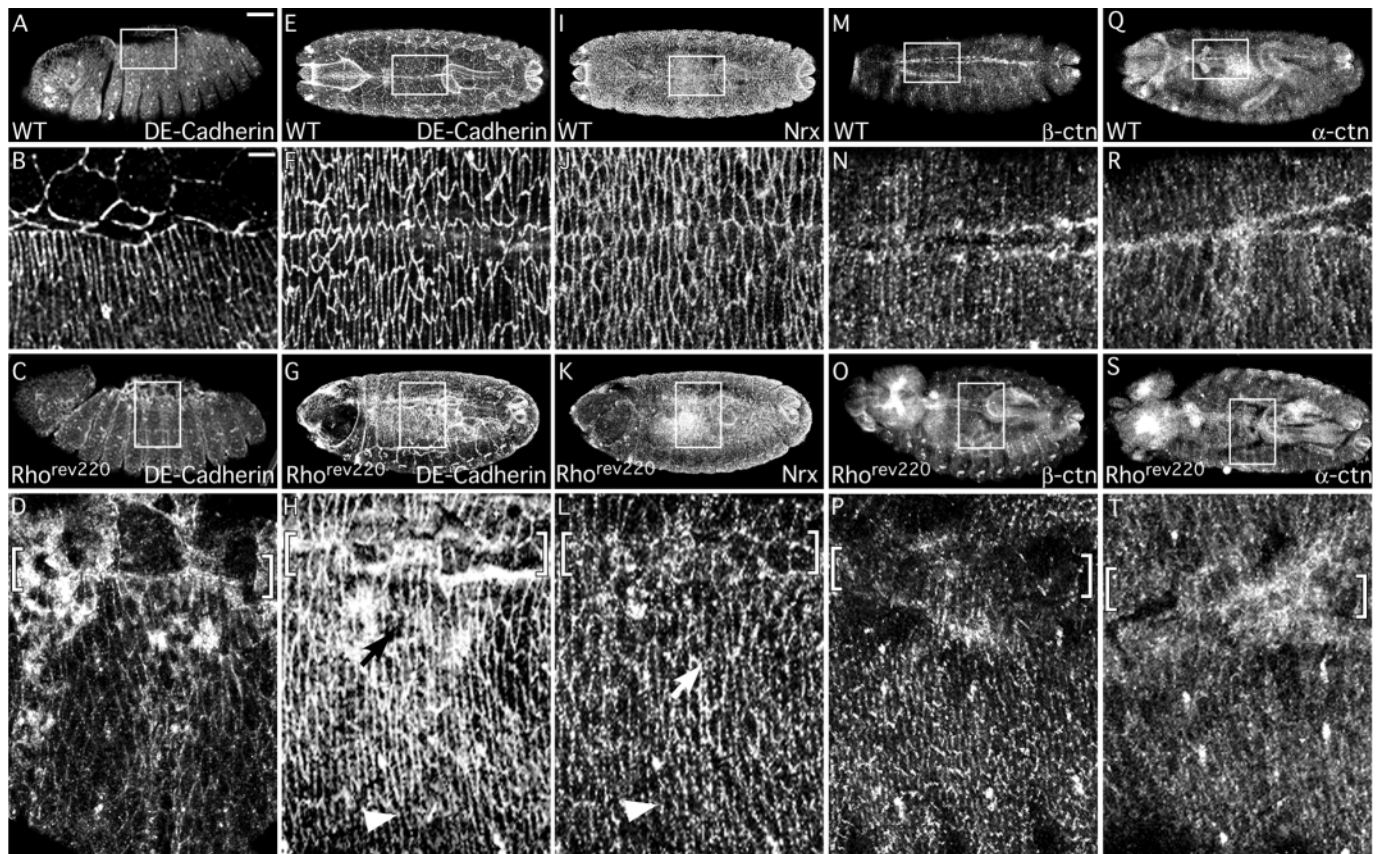


Fig. 4. Embryos mutant for *Rho1* show aberrant DE-cadherin and catenin localization. Confocal micrographs showing junction protein expression in wild-type and *Rho1* mutant embryos. (A-B,E-F) DE-cadherin expression in stage 14 (A,B) and stage 15 (E,F) wild-type embryos. (C-D,G-H) DE-cadherin expression in stage 14 (C,D) and stage 15 (G,H) *Rho1* mutant embryos. Note the disruption of DE-cadherin localization near the leading edge (arrow in H), but not in more lateral regions (arrowhead in H). (I-L) No difference in neurexin expression (septate junctions) is observed in the stage 15 *Rho1* mutant embryo (K,L) compared to wild type (I,J). DE-cadherin and neurexin were simultaneously imaged in the same wild-type and *Rho1* mutant embryos. Brackets in D indicate the leading edge, and in H,L,P,T, the dorsal midline. (M-P) β -catenin expression is disrupted in stage 15 *Rho1* mutants (O,P) compared to wild-type embryos (M,N). (Q-T) α -catenin expression is disrupted in stage 15 *Rho1* mutants (S,T) compared to wild-type embryos (Q,R). In all images, anterior is left. Dorsal is up in A-D. E-T are dorsal views. Boxes in A,C,E,G,I,K,M,O,Q,S indicate the region of the embryo shown in B,D,F,H,J,L,N,P,R,T, respectively. Scale bars: A: 50 μ m, B: 10 μ m.

Rho1 protein is ubiquitously cytoplasmic but concentrated apically. Significantly, the apical cytoplasmic accumulation of *Rho1* protein overlaps with the sites of cadherin localization (Fig. 3C). Neurexin is localized basal to the apical accumulations of *Rho1* and does not show substantial overlap (Fig. 3F).

***Rho1* accumulates at sites of cadherin localization in ovaries**

Previous studies have indicated a role for *Rho1* in the regulation of the actin cytoskeleton during oogenesis (Magie et al., 1999). As in the embryo, *Rho1* is present throughout the cytoplasm of all cells in the ovary, but accumulates in the apical regions of follicle cells (Fig. 3L-M'). *Rho1* also accumulates in specialized subsets of follicle cells such as the border cells (Fig. 3T,T'), in addition to regions of contact between follicle cells (Fig. 3T'',U) and the oocyte cortex (Fig. 3T,T''). As is the case in the embryo, *Rho1* apical accumulation overlaps DE-cadherin localization (Fig. 3N-P',V-W). Cadherin protein is localized to AJs at the apical end of follicle cells (Fig. 3O-P'),

and at lower levels to lateral follicle cell contacts and nurse cell-nurse cell contacts (Fig. 3V'',W). Cadherin is also up-regulated in those same populations of follicle cells that show accumulation of *Rho1* protein, including the border cells (Fig. 3V') (Niewiadomska et al., 1999).

Cadherin and catenin localization is disrupted in *Rho1* mutants

To determine whether the accumulation of *Rho1* protein at sites of DE-cadherin localization has a functional role, we examined DE-cadherin expression in *Rho1* mutants. In wild-type embryos, DE-cadherin protein is localized to AJs in the apical cortex of epithelial cells, resulting in an antibody staining pattern that outlines cells distinctly (Fig. 4A-B,E-F). In zygotic *Rho1* mutant embryos, however, this pattern of cadherin localization is disrupted (Fig. 4C-D,G-H). Rather than being restricted to cell-cell contacts, cadherin protein is diffusely distributed across the cell. This is especially evident in the cells near the leading edge of the epithelia undergoing dorsal closure (arrow in Fig. 4H), the process whereby the lateral epidermal

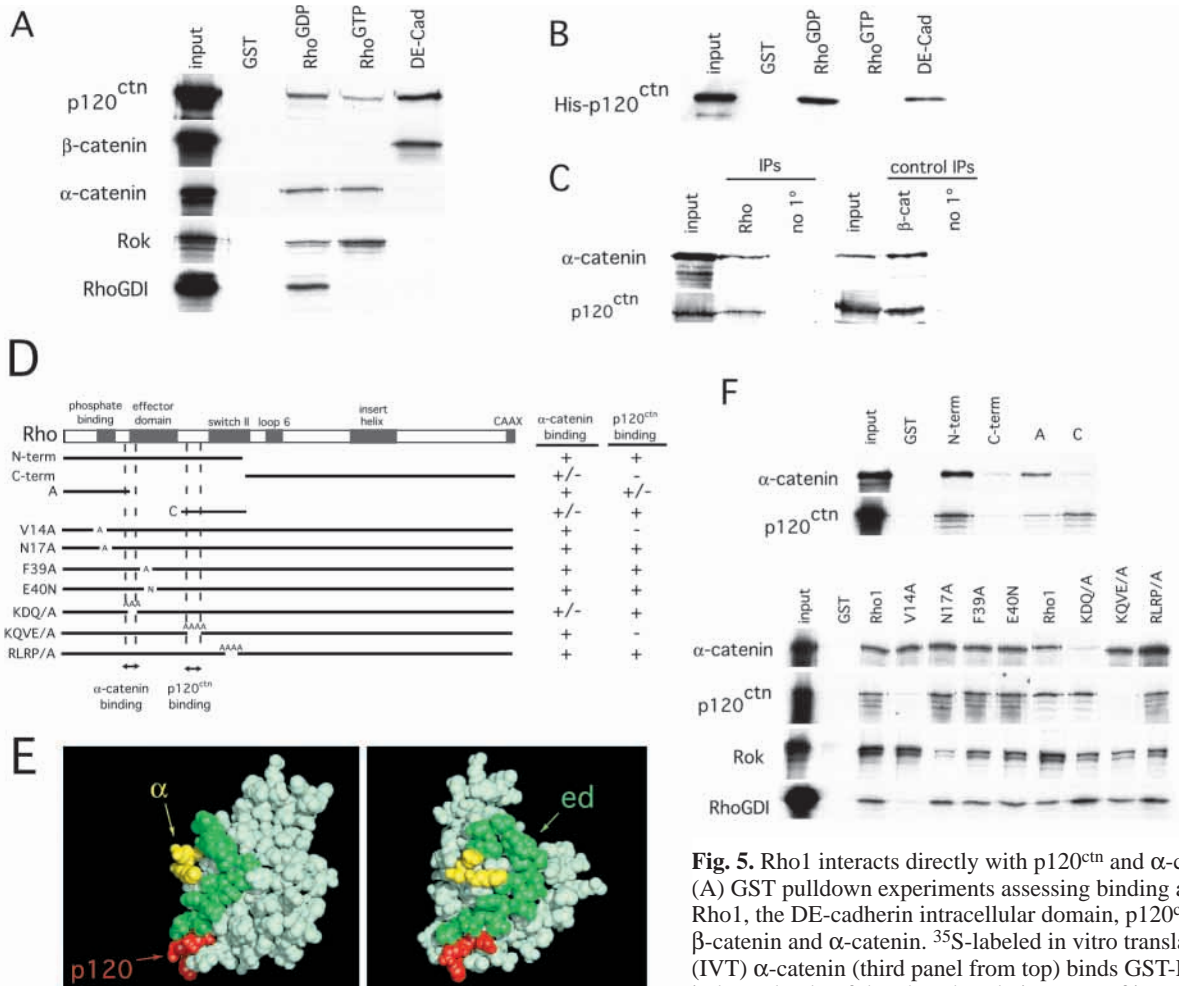


Fig. 5. Rho1 interacts directly with p120^{ctn} and α -catenin. (A) GST pull-down experiments assessing binding among Rho1, the DE-cadherin intracellular domain, p120^{ctn}, β -catenin and α -catenin. ³⁵S-labeled in vitro translated (IVT) α -catenin (third panel from top) binds GST-Rho1 independently of the phosphorylation state of its associated

nucleotide, IVT-p120^{ctn} binds preferentially to GST-Rho1^{GDP} (top panel) and IVT- β -catenin does not interact with either form of GST-Rho1 (second panel from top). Rok and a putative RhoGDI are included as binding controls. 5% input is shown. (B) GST pull-down experiment utilizing purified bacterially-expressed His-p120^{ctn}. His-p120^{ctn} also binds preferentially to GST-Rho1^{GDP}. (C) Immunoprecipitations showing in vivo interaction between Rho1 and α -catenin, and Rho1 and p120^{ctn}. β -catenin immunoprecipitations were performed as a positive control. 5% input is shown. (D) Diagram of the protein fragments, substitutions and point mutants used to map interaction domains on Rho1. (E) Computer model of GDP-bound RhoA crystal structure. Residues required for α -catenin binding are shown in yellow and those required for p120^{ctn} binding are shown in red. For reference the effector domain is highlighted in green. (F) GST pull-down experiments demonstrating the regions of Rho1 required for binding of α -catenin (top panel) and p120^{ctn} (second panel from top). α -catenin binds to region A and its binding is disrupted by replacing aa 27-29 (KDQ) with alanines, whereas p120^{ctn} binds to region C and its binding is disrupted by replacing aa 51-54 (KQVE) with alanines. Rok and RhoGDI bound preferentially to constitutively active (V14A) and dominant negative (N17A) forms of Rho1, respectively, but equally well to all other forms of Rho1 tested. V14A protein was exchanged with GTP and N17A with GDP in all experiments. All other forms of Rho1 in the α -catenin, p120^{ctn} and RhoGDI binding experiments were exchanged with GDP, while the Rho1 proteins used to test Rok binding were exchanged with GTP.

cells zip together at the dorsal midline. The mislocalization of DE-cadherin in *Rho1* mutants does not appear to be simply the result of cellular disorganization, as localization of the septate junction protein Neurexin is not disrupted (compare Fig. 4J with 4L). In addition, expression of other proteins such as β -tubulin and those detected by α -phosphotyrosine antibodies are not disrupted in *Rho1* mutants (Magie et al., 1999). We do not observe a similar disruption of DE-cadherin staining in *Rho1* mutant ovaries, probably because we can only reduce, but not eliminate, *Rho1* activity during oogenesis.

We also examined the localization of the AJ proteins α - and β -catenin in *Rho1* mutants. β -catenin binds to the cytoplasmic

domain of DE-cadherin, and α -catenin binds to β -catenin. Both proteins show expression patterns similar to DE-cadherin in wild-type embryos, although not as tightly localized to the plasma membrane (compare Fig. 4M-N and Q-R with 4E-F). Both catenins are less precisely organized in *Rho1* mutants (Fig. 4O-P,S-T), especially in cells on the dorsal surface near the leading edge. As expected given the mislocalization of DE-cadherin, loss of Rho1 also affects the localization of α - and β -catenin.

Rho1 interacts directly with p120^{ctn} and α -catenin

To determine if the localization of Rho1 at AJs is due to direct

interactions between Rho1 and components of the junctional complex, we examined the ability of Rho1 to bind the catenins using an in vitro GST-pulldown assay. As reported previously, we find that in vitro translated (IVT-) p120^{ctn} and β -catenin, but not α -catenin, bind to the intracellular domain of DE-cadherin (Fig. 5A) (Aberle et al., 1994; Daniel and Reynolds, 1995; Herrenknecht et al., 1991; Hulsken et al., 1994). While Rho1 does not bind β -catenin, we were surprised to find that it binds directly to both p120^{ctn} and α -catenin. As would be expected for a protein reported to keep Rho in a GDP-bound state, p120^{ctn} binds preferentially to GST-Rho1^{GDP}. Unexpectedly, Rho1 also binds directly to α -catenin independently of the phosphorylation state of the nucleotide Rho1 is associated with, suggesting a role for α -catenin in either recruiting Rho1 to AJs, or tethering it there once recruited by p120^{ctn}. We also examined the binding of Rho-kinase (Rok), a known Rho effector, and a putative RhoGDI identified by the Berkeley Drosophila Genome Project (BDGP) based on sequence homology, as controls. As expected, Rok bound preferentially to GST-Rho1^{GTP}, while RhoGDI bound exclusively to GST-Rho1^{GDP} (Fig. 5A).

To test whether the interactions we observe between Rho1 and the catenins are direct or mediated by bridging proteins within the IVT lysate, we purified bacterially expressed His-tagged p120^{ctn}. Consistent with the results using IVT-p120^{ctn}, His-p120^{ctn} binds directly to GST-Rho1 with preference for GST-Rho1^{GDP} (Fig. 5B).

To verify that the in vitro interactions we observed occur in vivo, we co-immunoprecipitated Rho-containing complexes from *Drosophila* embryo lysates. The P1D9 monoclonal antibody immunoprecipitates endogenous Rho1 (data not shown). Western blot analysis of these Rho1-immunoprecipitated complexes reveals the presence of α -catenin and p120^{ctn} (Fig. 5C).

We mapped the domains of Rho1 required for interaction with the two catenins by using a series of Rho1 protein fragments followed by targeted amino acid substitution mutations in the context of full-length Rho1 (Fig. 5D). The Rho1 binding domains for the two catenins are distinct. α -catenin maps to a surface-exposed region between the phosphate-binding loop and the effector domain that has not so far been assigned a function (Fig. 5E). α -catenin binding can be greatly reduced by substituting alanines for 3 amino acids (aa 27-29) within this domain (KDQ/A; Fig. 5D,F), but is not affected by other small substitutions (Fig. 5D,F). We also tested point mutations within the effector loop previously shown to affect binding of RhoA to particular effectors in mammalian cell culture (Sahai et al., 1998), but none of these affected α -catenin binding.

p120^{ctn} binds preferentially to a region between the effector domain and the switch II domain (Fig. 5D-F). This binding can be disrupted by the KQVE/A substitution (Fig. 5F). These residues are part of a surface-exposed loop distinct from that to which α -catenin binds (Fig. 5E). This region changes conformation depending which nucleotide is bound, consistent with the preference of p120^{ctn} for GDP-bound Rho1. Also consistent with this preference, p120^{ctn} binds to dominant negative Rho1 (N17A) but not to constitutively active Rho1 (V14A) (Fig. 5F). As controls, we examined the binding of Rok and RhoGDI to these *Rho* mutants. Rok binding is disrupted by dominant negative Rho1, and RhoGDI by

constitutively active Rho1, as expected given their nucleotide preference. Both bind as well to all other mutants tested as they do to wild-type Rho1 (Fig. 5F).

Rho1 localization is disrupted in catenin mutants

p120^{ctn} has recently been proposed to negatively regulate Rho in the cytoplasm (Anastasiadis et al., 2000; Noren et al., 2000). We find that embryos homozygous for a deficiency that uncovers p120^{ctn} show a severe dorsal open phenotype and, unlike wild type (Fig. 2J'), an accumulation of Rho1 protein in the leading edge cells (Fig. 6A-B'; arrows in A',B'). Since this deficiency removes several genes, and specific p120^{ctn} mutations have not yet been reported, we used dsRNA interference (RNAi) to specifically disrupt p120^{ctn} function (Kennerdell and Carthew, 1998). RNAi-generated p120^{ctn} mutant embryos exhibit severe morphogenetic defects, particularly in head involution. This phenotype is more severe than homozygous deficiency embryos, probably due to removal of maternal as well as zygotic p120^{ctn} by the RNAi method. In cases where the leading edge cells were detectable, an accumulation of Rho1 protein was observed in and around those cells (data not shown). To confirm this result, we injected p120^{ctn} dsRNA into embryos expressing β -galactosidase under the control of the *puckered* promoter, a gene expressed in the leading edge cells. Rho1 protein accumulates in and around the cells expressing β -gal (Fig. 6C-F'). This accumulation is not seen in uninjected embryos (data not shown) or in embryos injected with a control dsRNA (*ftz*, Fig. 6G-H'). Accumulation of Rho1 protein at the leading edge is not a general property of dorsal closure mutants, as Rho1 accumulation is not observed in the dorsal closure mutants *basket* (Fig. 6I,K) and *hemipterous* (Fig. 6J,L).

Since neither deficiencies covering the locus nor specific α -catenin mutations have been reported, we also used RNAi to remove α -catenin activity. RNAi-generated α -catenin mutant embryos cannot carry out the cell movements that accompany the early stages of gastrulation, and fail to form recognizable structures (Fig. 6M-O'). Antibodies recognizing α -spectrin, a component of the membrane cytoskeleton, provide a means of visualizing cell architecture, and α -catenin has been shown to interact with the spectrin cytoskeleton (Pradhan et al., 2001). We therefore examined Rho1 and α -spectrin localization in embryos injected with α -catenin dsRNA. In early stages, cells in the posterior of the α -catenin mutant embryo that would normally be involved in extending over the dorsal surface show a breakdown of the spectrin cytoskeleton, although cells in the anterior remain relatively normal (Fig. 6N',O'). The Rho1 staining pattern mirrors that of α -spectrin, showing disrupted localization in the posterior regions of the embryo (Fig. 6N,O). At later stages, all regions of the embryo show severely reduced DE-cadherin expression, although occasional attempts at forming a polarized epithelium can be seen (Fig. 6Q).

Overexpression of p120^{ctn} or α -catenin enhances the Rho1 phenotype

The observation that localization of the catenins is disrupted in embryos lacking Rho1 and Rho1 localization is aberrant in embryos with reduced p120^{ctn} or α -catenin suggests that the balance of Rho1 to the catenins is important for their proper function. If this is the case, then perturbing this balance by overexpressing the catenins should show effects in a *Rho1*

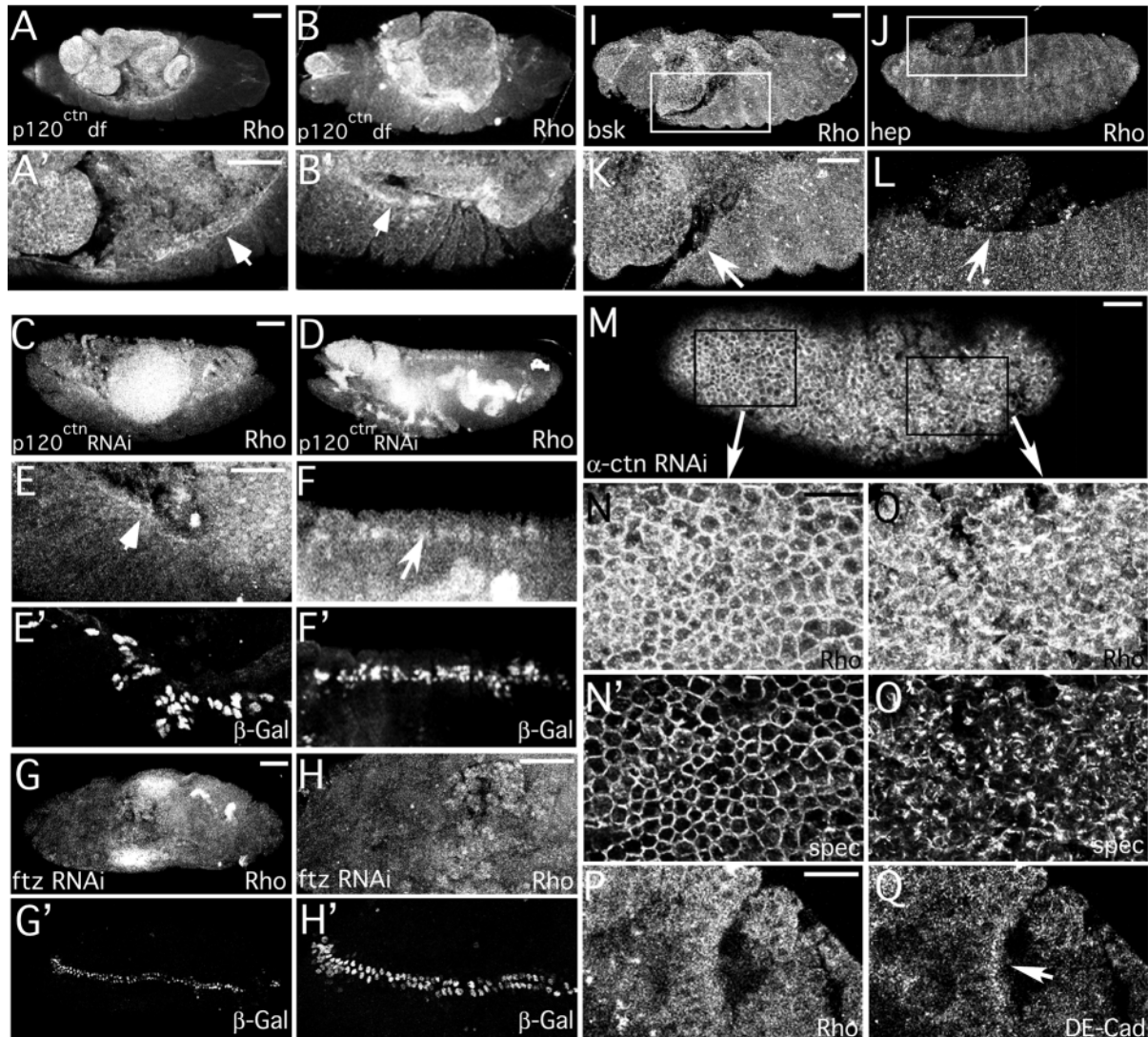


Fig. 6. Rho1 localization is aberrant in *catenin* mutants. (A-B') Rho1 expression in stage 15 embryos homozygous for a deficiency that removes the p120^{ctn} locus. (C-F') Rho1 (C-F) and β -galactosidase (E',F') expression in stage 15 *puc-lacZ/TM3* embryos injected with p120^{ctn} dsRNA. Note the accumulation of Rho1 protein at the leading edge in both deficiency and RNAi embryos (arrows in A',B',E,F), not observed in either *ftz* RNAi embryos (G-H') or other dorsal closure mutants *basket* (I,K) and *hemipterous* (J,L) (arrows in K, L; compare with A',B'). (M-O') Rho1 (M,N,O) and α -spectrin (N',O') expression in stage 6 embryos injected with α -catenin dsRNA. (P,Q) Rho1 (P) and DE-cadherin (Q) expression in embryos injected with α -catenin dsRNA. Note apical localization of DE-cadherin protein in some cells (arrow in Q). In all images, anterior is left. Scale bars: A,C,G,I,M: 50 μ m; A',E,H,K,N,P: 25 μ m.

mutant background. We therefore generated transgenic flies containing p120^{ctn} or α -catenin under the control of the Gal4-inducible UAS promoter that would allow us to ectopically express the catenins. One copy of UAS-p120^{ctn} or UAS- α -catenin overexpressed in a wild-type background using an actin-Gal4 driver results in approximately 15% lethality, with a third of these embryos exhibiting very mild segmentation defects (data not shown). 25% of *Rho1* homozygous mutant embryos show segmentation defects (20% mild, 5% severe; Fig. 7A). p120^{ctn} and α -catenin enhance both the incidence and severity of the segmentation defects observed when overexpressed in a *Rho1* mutant background, with α -catenin exerting the stronger effect (Fig. 7A). The enhancement effects of p120^{ctn} and α -catenin are specific, as overexpression of

GFP-tagged actin using this method had no effect on the severity of the *Rho1* phenotype (Fig. 7A). 42% of *Rho1* mutants overexpressing p120^{ctn} (26% mild, 16% severe) and 51% of *Rho1* mutants overexpressing α -catenin (17% mild, 34% severe) show segmentation defects. Significantly, 22% of embryos overexpressing α -catenin in a *Rho1* mutant background show cuticular holes, whereas only 2% of *Rho1* mutants and 3% of those overexpressing α -catenin in a wild-type background display this phenotype.

DISCUSSION

Subcellular localization of Rho protein has been shown to be

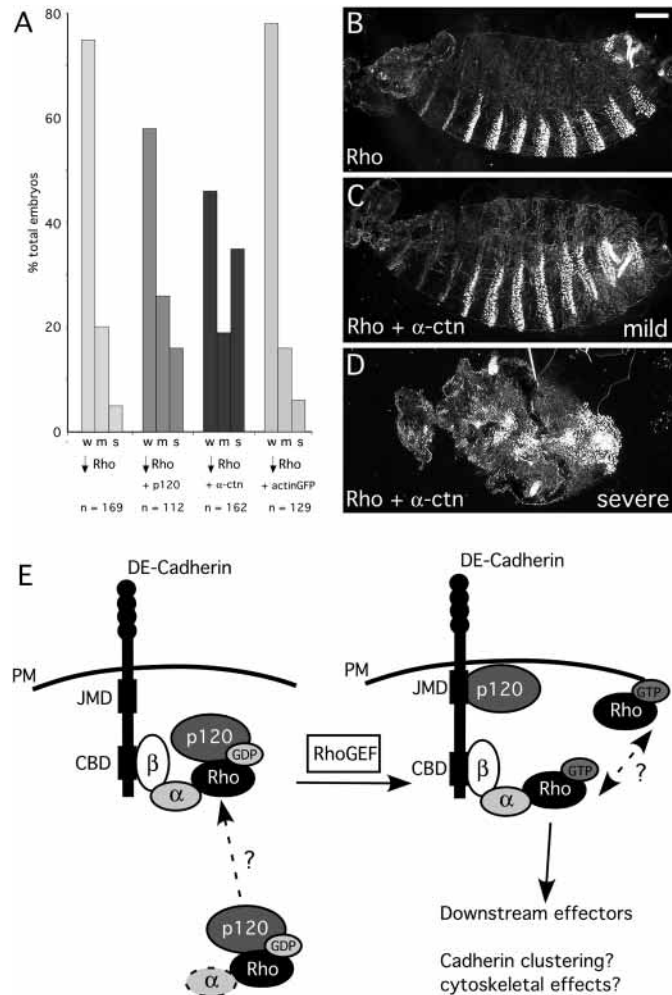


Fig. 7. Overexpression of p120^{ctn} or α-catenin enhances the Rho1 phenotype. The graph shows percentage total embryos (y-axis) with segmental patterning in each phenotypic class (w, wild type; m, mild; s, severe) for the following genotypes: *Rho1* homozygous mutants (↓Rho), *Rho1* mutants with one copy of UAS-p120^{ctn} overexpressed with the actin-Gal4 driver (↓Rho + p120^{ctn}), *Rho1* mutants with one copy of UAS-α-catenin overexpressed with the actin Gal4 driver (↓Rho + α-ctn), and *Rho1* mutants with one copy of UAS-actin-GFP overexpressed with the actin Gal4 driver (↓Rho + actinGFP). The number of embryos scored is indicated beneath each genotype. (B-D) Cuticles depicting representative examples of each phenotypic class: (B) homozygous *Rho1* mutant phenotype exhibiting an anterior dorsal hole but relatively normal anterior-posterior (AP) segmentation, (C) mild disruption of AP segmentation resulting from overexpressed α-catenin in the *Rho1* mutant background, (D) severe disruption of AP segmentation resulting from overexpressed α-catenin in the *Rho1* mutant background. (E) Model depicting the relationship of Rho1 to components of adherens junctions. p120^{ctn} can cycle between the cytoplasm and AJs. In the cytoplasm p120^{ctn} inhibits Rho by preventing the exchange of GDP for GTP. At AJs it binds to the JMD of cadherin and can no longer inhibit Rho. p120^{ctn} and/or α-catenin may be involved in recruiting Rho1 to AJs, allowing it to be activated by GEFs and carry out its downstream functions. Rho1 could be anchored at the AJ through its interaction with α-catenin or by insertion into the PM mediated by its isoprenylation modification. (PM: plasma membrane, CBD: catenin binding domain, JMD: juxtamembrane domain). For cuticles, anterior is left. Scale bar: 50 μm.

important for its proper function. Such localization could bring Rho into close juxtaposition with either activating molecules such as GEFs or downstream effectors that can then carry out local functions. We have used a monoclonal antibody that specifically recognizes *Drosophila* Rho1 to examine its localization in an organismal and developmental context. Our results indicate that in addition to Rho1's ubiquitous cytoplasmic expression, it accumulates at adherens junctions and is involved in regulating the proper localization of AJ components. Further, we have identified direct physical interactions between Rho1 and the catenins, p120^{ctn} and α-catenin. Isoprenylation at the C-terminal CAAX motif is involved in regulating the subcellular localization of Rho (Fleming et al., 1996; Kranenburg et al., 1997), however, binding to the catenins may represent another mechanism of recruiting Rho1 to its sites of action.

We find that Rho1 activity is required to properly localize DE-cadherin during development, consistent with data from mammalian cell culture experiments implicating Rho and Rac in cadherin assembly and maintenance (Braga et al., 1999; Braga et al., 1997). The defects we observe in cadherin localization are most prevalent in and around the leading edge (LE) cells undergoing dorsal closure. Previously Rho1 had been implicated in dorsal closure via its regulation of the LE actin cytoskeleton in cells flanking the segment borders (Harden et al., 1999). However, the disruption we observe in cadherin distribution suggests that regulation of cell-cell adhesion may play a role in the dorsal closure phenotype observed in these embryos. Thus Rho1's effects on cadherin localization could be the result of a direct role in DE-cadherin clustering, or an indirect effect on the cortical actin cytoskeleton. The process of AJ formation in keratinocytes has been shown to require actin polymerization and the interdigitation of filopodia from neighboring cells (Vasioukhin et al., 2000). A similar interdigitation of filopodia is seen during dorsal closure in *Drosophila* and is likely involved in forming adhesive contacts between the two epithelial fronts (Jacinto et al., 2000). Since Rho and Cdc42 have been shown to act antagonistically in the formation of cellular processes in neurons (Kozma et al., 1997), it is possible that disrupting the balance of Rho1 and Cdc42 function in LE cells results in inappropriate regulation of filopodial extensions. This could partially explain the disruption of DE-cadherin localization we observe in *Rho1* mutants. Alternatively, Rho1's primary role could be in directly regulating the adhesion of cells near the LE, with Rac and Cdc42 acting as the major organizers of the acto-myosin network.

In addition to the accumulation of Rho1 protein at sites of cadherin localization, we observe a direct physical interaction between Rho1 and both p120^{ctn} and α-catenin. The catenin family of proteins is important in regulating cadherin-based adhesion and linking cadherins to the actin cytoskeleton (Kemler, 1993). β-catenin binds to the catenin-binding domain of the cadherin molecule as well as to α-catenin. α-catenin, in turn, acts as a link to the actin cytoskeleton, either by directly binding actin filaments or through association with other actin-binding proteins. α-catenin also has been shown to bind spectrin, a major component of the membrane skeleton underlying the plasma membrane involved in stabilizing it and determining cell shape. Human colon carcinoma Clone A cells that contain mutant α-catenin have defects in spectrin assembly

(Pradhan et al., 2001). Consistent with this, we observe a breakdown of the α -spectrin cytoskeleton in embryos injected with α -catenin dsRNA, especially in morphogenetically active cells early in gastrulation. α -catenin protein is enriched at adherens junctions, but is not as strictly localized to them as is DE-cadherin. Binding of α -catenin to Rho1 may be a general mechanism through which Rho1 is recruited to the plasma membrane.

p120^{ctn} regulates the adhesive properties of cadherin complexes through its binding to the juxtamembrane domain of the cadherin molecule, although the precise mechanisms underlying this function are not known. p120^{ctn} also acts in the cytoplasm where it has been proposed to negatively regulate Rho activation in a manner similar to the GDI proteins, which prevent Rho from exchanging GDP for GTP, although it shares no sequence homology with them (Anastasiadis et al., 2000). The binding of p120^{ctn} to cadherins and its effects on Rho function have been shown to be mutually exclusive, such that once p120^{ctn} binds a cadherin molecule, it is no longer capable of inhibiting Rho activity or function (Anastasiadis et al., 2000). Rho would then be accessible to activating regulatory proteins such as GEFs, and could carry out its downstream functions. The physical interaction we observe between Rho1 and p120^{ctn} suggests that this negative regulation of Rho1 is due to direct binding of p120^{ctn} to GDP-Rho1. Interestingly, this is the same face of the Rho protein that has been shown to bind to classical GDIs (Hoffman et al., 2000), consistent with the idea that despite the lack of sequence homology, p120^{ctn} may be acting in a similar way. Overexpression of p120^{ctn} in mammalian cells leads to an inhibition of Rho activity (Anastasiadis et al., 2000; Noren et al., 2000). Overexpression of p120^{ctn} in our system enhances the *Rho1* mutant phenotype, as would be expected for a negative regulator. We find that embryos homozygous for a deficiency uncovering the p120^{ctn} locus show an accumulation of Rho1 protein at the leading edge and exhibit a severe dorsal open phenotype. A similar accumulation of Rho1 protein is observed in embryos injected with p120^{ctn} dsRNA. A positive feedback mechanism may be functioning whereby the relief of p120^{ctn}-mediated regulation in those cells results in the upregulation of Rho1 protein or an increase in Rho1 stability. It has recently been shown that overexpression of a RhoGDI in the hearts of mouse embryos results in the upregulation of RhoA expression, suggesting the existence of a negative feedback mechanism in the regulation of RhoA levels (Wei et al., 2002), although we are not aware of any other instances in which a positive feedback mechanism has been linked to Rho expression. Excess Rho activity disrupts cellular migration (Nobes and Hall, 1999); cells at the leading edge in embryos that lack p120^{ctn} function remain cuboidal, rather than elongating as they would during normal dorsal closure, suggesting that Rho1 may be involved in regulating these cell shape changes. Alternatively, p120^{ctn} has been suggested to activate Rac and Cdc42 in the cytoplasm through an interaction with the GEF Vav2, and this could account for some of its effects on cell morphology (Noren et al., 2000). The observation that Rho1 can bind both p120^{ctn} and α -catenin and that their binding sites are not overlapping suggests that either could be involved in recruiting Rho1 to AJs or the plasma membrane in general. Our data indicating that overexpression of α -catenin enhances the *Rho1* mutant phenotype to a greater degree than p120^{ctn} suggests an

important role for α -catenin in Rho1 function, perhaps as a factor generally involved in localizing Rho1 to its sites of action, while p120^{ctn} plays a more specific role at AJs.

Our data suggest a model (Fig. 7E) in which p120^{ctn} or α -catenin or both are involved in recruiting Rho1 to sites of cadherin localization, where it can then be activated and carry out its functions, including proper AJ formation. If Rho1 is not recruited properly, as in the case of a *Rho1* mutant, this results in mislocalization of AJ components. The binding of p120^{ctn} to Rho1, either in the cytoplasm or while Rho1 is tethered at AJs through its interaction with α -catenin, inhibits the exchange of GDP for GTP and keeps Rho1 in an inactive state. The binding of p120^{ctn} to the juxtamembrane domain may release Rho1, allowing it to be activated by GEFs. GTP-Rho1 could then bind its downstream effectors and either directly regulate DE-cadherin assembly or maintenance, or indirectly affect AJ formation through its effects on the actin cytoskeleton. Rho1 localization at AJs could then be mediated either through continued association with α -catenin or through isoprenylation and insertion into the plasma membrane. Mutational analysis aimed at distinguishing between these models will provide further insight into this important feature of Rho1 function during morphogenesis.

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