Cdc42-GTP is required for apical domain formation in epithelial cells, where it recruits and activates the Par-6–aPKC polarity complex, but how the activity of Cdc42 itself is restricted apically is unclear. We used sequence analysis and 3D structural modeling to determine which Drosophila GTPase-activating proteins (GAPs) are likely to interact with Cdc42 and identified RhoGAP19D as the only high-probability Cdc42GAP required for polarity in the follicular epithelium. RhoGAP19D is recruited by α-catenin to lateral E-cadherin adhesion complexes, resulting in exclusion of active Cdc42 from the lateral domain. rhogap19d mutants therefore lead to lateral Cdc42 activity, which expands the apical domain through increased Par-6–aPKC activity and stimulates lateral contractility through the myosin light chain kinase, Genghis khan (MRCK). This causes buckling of the epithelium and invasion into the adjacent tissue, a phenotype resembling that of precancerous breast lesions. Thus, RhoGAP19D couples lateral cadherin adhesion to the apical localization of active Cdc42, thereby suppressing epithelial invasion.

Introduction

The form and function of epithelial cells depends on their polarization into distinct apical, lateral, and basal domains by conserved polarity factors (Rodriguez-Boulan and Macara, 2014; St Johnston and Ahringer, 2010). This polarity is then maintained by mutual antagonism between apical polarity factors such as atypical PKC (aPKC) and lateral factors such as Lethal (2) giant larvae (Lgl) and Par-1. While many aspects of the polarity machinery are now well understood, it is still unclear how the apical domain is initiated and what role cell division control protein 42 (Cdc42) plays in this process.

Cdc42 was identified for its role in establishing polarity in budding yeast, where it targets cell growth to the bud tip by polarizing the actin cytoskeleton and exocytosis toward a single site (Chiu et al., 2017). It has subsequently been found to function in the establishment of cell polarity in multiple contexts. For example, Cdc42 recruits and activates the anterior PAR complex to polarize the anterior–posterior axis in the Caenorhabditis elegans zygote and the apical–basal axis during the asymmetric divisions of Drosophila neural stem cells (Gotta et al., 2001; Kay and Hunter, 2001; Atwood et al., 2007; Rodriguez et al., 2017).

Cdc42 also plays an essential role in the apical–basal polarization of epithelial cells, where it is required for apical domain formation (Genova et al., 2000; Hutterer et al., 2004; Jaffe et al., 2008; Bray et al., 2011; Fletcher et al., 2012). Cdc42 is active when bound to GTP, which changes its conformation to allow it to bind downstream effector proteins that control the cytoskeleton and membrane trafficking. An important Cdc42 effector in epithelial cells is the Par-6–aPKC complex. Par-6 binds directly to the switch 1 region of Cdc42 GTP through its semi-CRIB domain (Cdc42 and Rac interactive binding; Lin et al., 2000; Joberty et al., 2000; Qiu et al., 2000; Yamanaka et al., 2001). This induces a change in the conformation of Par-6 that allows it to bind to the C-terminus of another key apical polarity factor, the transmembrane protein Crumbs, which triggers the activation of aPKC's kinase activity (Peterson et al., 2004; Whitney et al., 2016; Dong et al., 2020). As a result, active aPKC is anchored to the apical membrane, where it phosphorylates and excludes lateral factors, such as Lgl, Par-1, and Bazooka (Baz; Betschinger et al., 2003; Hurov et al., 2004; Suzuki et al., 2004; Nagai-Tamai et al., 2002; Morais-de-Sá et al., 2010). In addition to this direct role in apical–basal polarity, Cdc42 also regulates the organization and activity of the apical cytoskeleton through effectors such as neuronal Wiskott-Aldrich syndrome protein (N-WASP), which promotes actin polymerization, and myotonic dystrophy kinase-related Wiskott-Aldrich syndrome protein (MRCK; Genghis khan [Gek] in Drosophila), which phosphorylates the myosin regulatory light chain to activate contractility (Padrick and Rosen, 2010; Zihni et al., 2017).
This crucial role of active Cdc42 in specifying the apical domain raises the question of how Cdc42-GTP itself is localized apically. In principle, this could involve activation by Cdc42 guanine nucleotide exchange factors (Cdc42GEFs) that are themselves apical or lateral inactivation by Cdc42GAPs. The Cdc42GEFs Tubα, intersectin 2, and DbIII have been implicated in activating Cdc42 in mammalian epithelia (Otani et al., 2006; Qin et al., 2010; Rodriguez-Fraticelli et al., 2010; Oda et al., 2014; Zihni et al., 2014). Only DbIII localizes apical to tight junctions, however, as Tubα is cytoplasmic and enriched at tricellular junctions and intersectin 2 localizes to centrosomes. Thus, GEF activity may not be exclusively apical, suggesting that it is more important to inhibit Cdc42 laterally. Although nothing is known about the role of GAPs in restricting Cdc42 activity to the apical domain of epithelial cells, this mechanism plays an instructive role in establishing radial polarity in the blastomeres of the early C. elegans embryo. In this system, the Cdc42GAP PAC-1 is recruited by the cadherin adhesion complex to sites of cell-cell contact, thereby restricting active Cdc42 and its effector the Par-6-aPKC complex to the contact-free surface (Anderson et al., 2008; Klompstra et al., 2015).

Here we analyzed the roles of Cdc42GAPs in epithelial polarity using the follicle cells that surround developing Drosophila egg chambers as a model system (Bastock and St Johnston, 2008). By generating mutants in a number of candidate Cdc42GAPs, we identified the Par-1 orthologue, RhoGAP19D, as the GAP that restricts active Cdc42 to the apical domain. In the absence of RhoGAP19D, lateral Cdc42 activity leads to an expansion of the apical domain and a high frequency of epithelial invasion into the germline tissue, a phenotype that mimics the early steps of carcinoma formation.

**Results**

To confirm that Cdc42 regulates apical domain formation in Drosophila epithelia, we generated homozygous mutant clones of cdc42	extsuperscript{2o f1 7}, a null allele, in the follicular epithelium that surrounds developing egg chambers (Fig. 1 A). Mutant cells lose their cuboidal shape, leading to gaps and multilayering in the epithelium, and fail to localize GFP-aPKC apically, indicating that Cdc42 is required for polarity in the follicle cell layer.

There are 22 Rho-GTPase-activating proteins in the Drosophila genome (Table 1), but in most cases, it is unclear whether they regulate Rho, Rac, or Cdc42. We therefore predicted the tendency for each of the Drosophila GAPs to interact with Cdc42 using InterPreTS (Aloy and Russell, 2002). This uses a known structure of a protein complex (in this case, the structure of the human Cdc42–Rho-GTPase–activating protein 1 [ARHGAPI] complex; Nassar et al., 1998) as a template to predict whether homologous proteins (in this case, other Drosophila GAPs and Cdc42) would be able to interact in the same way. The fit of each sequence pair on the structure is assessed via statistical potentials that score the compatibility of each amino acid pair at the (e.g., GAP–Cdc42) interface. The rank for each Drosophila GAP according to its likelihood of interacting with Drosophila Cdc42 is shown in Table 2.

Interestingly, the Drosophila ARHGAPI orthologue, RhoGAP68F, was only second in the ranking behind another known Cdc42GAP, the PAC-1 orthologue, RhoGAP19D. Inspection of the InterPreTS results in detail shows that several key conserved positions mediating interactions in the structure of the human Cdc42–ARHGAPI complex are conserved in RhoGAP19D (and many other) Drosophila GAPs, in addition to three positions that appear to make the interaction stronger (Fig. 1, B and C). Specifically, an Arg in ARHGAPI is replaced by an Ile (1237 in RhoGAP19D), making for a better interaction with Ala13 in Cdc42; a Val is replaced by a Ser (1275), making a more favorable interaction with Glu62; and a Thr is replaced by a Lys (1241), possibly making an additional salt bridge with Glu95 and additional interactions with Asn92.

To test whether any of these putative Cdc42 GAPs play a role in epithelial polarity, we generated null mutants in the seven highest-ranked GAPs using CRISPR-mediated mutagenesis (Table S1) and examined their phenotypes in the follicular epithelium that surrounds developing egg chambers. Mutants in RhoGAP92B are lethal and, therefore we used the flippase (Flp)/flippase recognition target (FRT) system to generate homozygous mutant follicle cell clones, whereas mutants in the other GAPs are homozygous viable or semiviable, allowing us to analyze the follicle cell phenotype in homozygous mutant females. Null mutants in RhoGAP68F, CdGAPr, RhoGAP92B, RhoGAP82C, conundrum (Conu; Neisch et al., 2013), and RhoGAP93B cause no discernible changes in follicle cell shape or polarity, as shown by the localization of aPKC apically and Lgl laterally (Fig. S1). By contrast, 40% of homozygous mutant rhogap19d egg chambers show invasions of regions of the follicular epithelium into the overlying germline cyst (Fig. 2, A–C). This phenotype is not a consequence of overproliferation of the mutant follicle cells, because mutant egg chambers contain the same number of follicle cells as wild-type egg chambers, and the same proportion of homozygous mutant cells and wild-type cells are in mitosis at stages 4 and 5 (Fig. 2, D–F). Introducing endogenously tagged E-cadherin into the rhogap19d mutant background reveals that the invading follicle cells maintain their apical adherens junctions, indicating that they have not undergone an epithelial-to-mesenchymal transition and are still epithelial in nature (Fig. 2 E).

We examined the localization of RhoGAP19D protein by using CRISPR-mediated homologous recombination to insert the mNeonGreen fluorescent tag at the N-terminus of the endogenous RhoGAP19D coding region. Neon::RhoGAP19D localizes laterally in the follicle cells, covering the full length of the domain, including the apical adherens junctions, where it sometimes appears to be slightly enriched (Fig. 2 H). A similar lateral localization was observed in all other epithelia we examined, such as the larval salivary gland, the adult testis accessory gland, the larval midgut, and the cellular blastoderm embryo (Fig. S2, A–F). Thus, RhoGAP19D seems to be a lateral factor in multiple epithelia. This is consistent with the observation that rhogap19d mutants die at several stages. Zygotic rhogap19d mutants are semilethal, with about two-thirds of homozygotes dying before adulthood. Furthermore, all embryos from homozygous mutant mothers either fail to hatch or die as first-instar larvae, indicating that it is a fully penetrant maternal effect lethal.

The C. elegans orthologue of RhoGAP19D, PAC-1, is recruited to cell contacts by the E-cadherin complex through redundant
Figure 1. Cdc42 is essential for the establishment of cell polarity in Drosophila follicular cells. (A) A stage 7 egg chamber containing multiple cdc42 mutant follicular cell clones (marked by the loss of RFP; magenta) stained for Armadillo (Arm; white) and DAPI (blue) expressing endogenously tagged GFP-aPKC (green). The mutant cells are marked by arrows. GFP-aPKC is lost from the apical side of cdc42 mutant follicular cells. In some cells, GFP-aPKC colocalizes with Armadillo in puncta. Cells lacking Cdc42 become round and often lose contact with neighboring cells, resulting in breaks in the epithelial layer. In other cases, the cdc42 mutant cells lie basally to wild-type cells. Scale bars, 10 µm. (B) A diagram showing the interface between Cdc42 and bound ARHGAP1/RhoGAP19D. Key amino acids that mediate the interaction are shown in purple for Cdc42 and in green for ARHGAP1. Amino acid changes that are predicted to strengthen the interaction between Cdc42 and RhoGAP19D are shown in parentheses. (C) Alignment of Drosophila GAPs and human ARHGAP1. Conserved amino acids involved in the interaction with Cdc42 are indicated by black arrows. The green arrows mark the variable amino acids that are predicted to strengthen the interaction between Cdc42 and RhoGAP19D.
interactions with α-catenin and p120-catenin (Klompstra et al., 2015). We observed no change in the lateral recruitment of RhoGAP19D in p120 catenin–null mutants, but the junctional signal was almost completely lost when α-catenin was depleted by RNAi (Fig. 2, I–L). Thus, RhoGAP19D is localized to the lateral membrane by a nonredundant interaction with α-catenin, which links it to cadherin adhesion complexes. The lateral localization of RhoGAP19D was strongly reduced in clones homozygous for shotgun, an Antennapedia shotgun (E-cadherin), whereas clones homozygous for a deletion of N-cadherin 1 and N-cadherin 2 had no effect (Figs. 2 M and S2 K; Tepass et al., 1996; Prakash et al., 2005). The weaker phenotype of shotgun clones compared with α-catenin knockdown is presumably because N-cadherin is upregulated in E-cadherin mutants, and either E- or N-cadherin can recruit α-catenin and RhoGAP19D (Grammont, 2007).

α-Catenin and E-cadherin are concentrated in the apical adherens junctions, whereas RhoGAP19D shows only a slight apical enrichment and is much more uniformly distributed along the lateral membrane. Because this suggests that other factors may modulate the recruitment of RhoGAP19, we examined whether any lateral polarity factors affect its distribution, but we observed no change when the lateral adhesion proteins, FasII, FasIII, or Neuroglian, were knocked down by RNAi or in null mutant clones for the lateral polarity factors Lgl, Scribble (Scrib), and Coracle (Fig. 2 N; and Fig. S2, H–N).

The localization of RhoGAP19D suggests that it may function to inhibit Cdc42 laterally. We therefore examined where Cdc42 is active by following the localization of an endogenously tagged version of the Cdc42 effector, N-WASP (Kim et al., 2000). N-Wasp-Neon is expressed at very low levels in the follicle cells, with slightly higher expression in the posterior cells. In wild-type cells, N-WASP-Neon localizes exclusively to the apical domain, consistent with the apical localization of active Cdc42. By contrast, N-Wasp-Neon also localizes along the lateral membrane in rhogap19d mutant clones at the posterior (Fig. 3 A). Although N-Wasp-Neon is harder to detect in lateral follicle cells, horizontal sections through regions containing clones also reveal lateral localization in the mutant cells, but not in wild-type cells (Fig. 3 B). Thus, RhoGAP19D is required to exclude active Cdc42 from the lateral domain.

To confirm that RhoGAP19D represses Cdc42 activity, we used upstream activating sequence (UAS)-GrabFP-Aint to mislocalize the protein to the apical domain (Harmansa et al., 2017). The GrabFP-Aint construct consists of an N-terminal Cherry, a transmembrane domain, and an anti-GFP nanobody fused to the localization signal of Baz (Par-3; Fig. 3 C). When this construct is expressed in the follicle cells under the control of Tj-Gal4, the fusion protein localizes to the apical membrane and apical junctions without any apparent effect on the appearance of the cells (Fig. 3 D). Similarly, overexpression of UAS-GFP-RhoGAP19D alone results in higher levels of RhoGAP19D along the

Table 1. Drosophila GTPase-activating proteins

| Gene symbol | Gene name | Other names | UniProt accession no. |
|-------------|-----------|-------------|-----------------------|
| CdgAPr      | CdGAPr    | Cd GTPase activating protein--related | Q9VIS1 |
| cont        | Conundrum |                         | Q8T0G4 |
| cv-c        | Crossveinless c | RhoGAP8BC | A8JR05 |
| Graf        | GRAF orthologue (Homo sapiens) |                         | X2J0Y8 |
| Ocrl        | Oculocerebrorenal syndrome of Lowe | EG.86E4.5 | Q95R90 |
| RacGAP84C   | Protein at 84C | RhoGAP | P40809 |
| RhoGAP1A    | Protein at 1A | EG.23E12.2 | Q6W436 |
| RhoGAP5A    | RhoGTPase activating protein at 5A |                         | Q9W4A9 |
| RhoGAP5B    | RhoGTPase activating protein at 5B |                         | Q6W2H5 |
| RhoGAP16F   | RhoGTPase activating protein at 16F |                         | Q9VWY8 |
| RhoGAP18B   | RhoGTPase activating protein at 18B | whir | Q9VWL7 |
| RhoGAP19D   | RhoGTPase activating protein at 19D |                         | Q9VRA6 |
| RhoGAP54D   | RhoGTPase activating protein at 54D |                         | A1ZAW3 |
| RhoGAP58F   | RhoGTPase activating protein at 58F | CG 6811 | M9PC96 |
| RhoGAP71E   | RhoGTPase activating protein at 71E | l(3)6B9 | B72058 |
| RhoGAP92B   | RhoGTPase activating protein at 92B |                         | A0A0B4LHC1 |
| RhoGAP93B   | RhoGTPase activating protein at 93B | CrGAP | Q9VDE9 |
| RhoGAP100F  | RhoGTPase activating protein at 100F | Syd-1 | Q9VR97 |
| RhoGAP102A  | RhoGTPase activating protein at 102A | Dm.4.1183 | H9XV11 |
| RhoGAP190   | RhoGTPase activating protein at 190 | p190RhoGAP, p190 RhoGAP | Q9VX32 |
| Rhlip       | Ral interacting protein | dRalBP, D-RLIP | Q9VZC3 |
| tum         | Tumbleweed | racGAP50C, acGAP, RacGAP, DRacGAp | Q9N9Z9 |
lateral membrane but has no effect on cell polarity or morphology during stages 1–8 of oogenesis. When GFP-RhoGAP19D and GrabFP-Aint are coexpressed, however, the apical recruitment of GFP-RhoGAP19D by the anti-GFP nanobody disrupts polarity and epithelial organization, as shown by the failure to concentrate aPKC apically and the irregular cell shapes (Fig. 3 E).

To investigate the cellular basis for the invasive behavior of rhoap19d mutant follicular cells, we compared the phenotypes of mutant and wild-type cells in the same epithelium by generating homozygous mutant clones. Live imaging revealed that of mutant cells in invasions show that the mutant cells expand and contract along the lateral domain to generate taller cells that protrude into the germline. A similar apical expansion is also observed in rhoap19d mutant testis accessory glands and in the primary epithelium of cellular blastoderm embryos derived from rhoap19d mutant mothers, suggesting that this is a general phenotype of loss of RhoGAP19D in Drosophila epithelia (Fig. S3).

To gain insight into how rhoap19d mutant follicle cells invade the germline, we imaged living egg chambers at stages 5–7, the stages when invasions are most likely to occur (Video 1; Fig. 5 A). The mutant cells are not only taller than wild-type cells with domed apical surfaces but are also more motile. Temporal projections show that the mutant cells expand and contract along their apical–basal axes, whereas wild-type cells are static (Fig. 5 B). The apical expansion of the mutant cells and the up and down movements are likely to increase strain in the epithelium and raise the probability of regions of the follicle cell layer invading the germline (Fig. 5 A). More rarely, we observed clusters of cells that had detached from the basement membrane and were beginning to invade (Fig. 5 C).

The higher motility suggests that myosin activity is increased in mutant cells, and we therefore examined the distribution of nonmuscle myosin II (NMYII) using a protein trap insertion in the heavy chain (Zipper). This revealed that the mutant cells have more numerous and larger NMYII foci along their lateral membranes and reduced levels of apical NMYII (Fig. 5 D). This increase in lateral NMYII is likely to account for the apical–basal contractions in mutant cells. In MDCK cells, Cdc42 recruits and activates NMYII apically through its effector, MRCK, which phosphorylates the myosin regulatory light chain to stimulate contractility (Zihni et al., 2017; Zhao and Manser, 2015). This suggests that the Drosophila orthologue of MRCK, Gek, might play a similar role in coupling Cdc42 to the activation of NMYII in the follicular epithelium. Antibody staining revealed that Gek

Table 2. Drosophila GTPase-activating proteins ranked by the predicted strength of their interactions with Cdc42

| Protein 1 | Gene 1 | % Id 1 | Protein 2 | Gene 2 | % Id 2 | PDB accession no. | Z-score |
|-----------|--------|--------|-----------|--------|--------|-----------------|---------|
| Q9VRA6-RhoGAP | RhoGAP19D | 24 | P40793-RAS | Cdc42 | 94 | IAM4 | 3.329 |
| M9PC96-RhoGAP | RhoGAP68F | 40 | P40793-RAS | Cdc42 | 92 | 1GRN | 3.122 |
| Q9VIS1-RhoGAP | CdGPr | 26 | P40793-RAS | Cdc42 | 92 | 1GRN | 2.999 |
| A0A0B4LHC1-RhoGAP | RhoGAP92B | 36 | P40793-RAS | Cdc42 | 92 | 2NGR | 2.9 |
| P40809-RhoGAP | RacGAP84C | 26 | P40793-RAS | Cdc42 | 92 | 1GRN | 2.88 |
| Q8T0G4-RhoGAP | conu | 26 | P40793-RAS | Cdc42 | 92 | 1GRN | 2.835 |
| Q9VDE9-RhoGAP | RhoGAP93B | 30 | P40793-RAS | Cdc42 | 92 | 1GRN | 2.823 |
| X2JQY8-RhoGAP | Graf | 29 | P40793-RAS | Cdc42 | 94 | IAM4 | 2.748 |
| Q9KH05-RhoGAP | RhoGAP15B | 28 | P40793-RAS | Cdc42 | 94 | IAM4 | 2.723 |
| A8JR05-RhoGAP | cv-c | 29 | P40793-RAS | Cdc42 | 94 | IAM4 | 2.642 |
| Q9VWY7-RhoGAP | RhoGAP18B | 28 | P40793-RAS | Cdc42 | 94 | IAM4 | 2.488 |
| Q9VWY8-RhoGAP | RhoGAP16F | 22 | P40793-RAS | Cdc42 | 94 | IAM4 | 2.142 |
| Q9NH29-RhoGAP | tum | 22 | P40793-RAS | Cdc42 | 94 | IAM4 | 1.951 |
| Q9VX32-RhoGAP | RhoGAPp190 | 28 | P40793-RAS | Cdc42 | 92 | 1GRN | 1.93 |
| Q9VDG2-RhoGAP | Rip | 32 | P40793-RAS | Cdc42 | 92 | 2NGR | 1.683 |
Figure 2. RhoGAP19D is required for the integrity of the epithelial layer. (A) Comparison of the domain structure of Drosophila RhoGAP19D with its orthologues, human ARHGAP23/21 and C. elegans PAC-1. RhoGAP19D contains PDZ and GAP domains but lacks a PH domain. (B) Diagram showing the CRISPR-induced mutations in RhoGAP19D. The mutations generate proteins that lack the GAP domain but still contain the PDZ domain. (C) A stage 7 rhogp19d mutant...
is predominantly localized to the apical surface of the follicle cells, consistent with its role in MDCK cells (Fig. 5 E). Gek extending along the lateral membrane, however, in all rhogap19d mutant cells (Fig. 5 F). Thus, the ectopic Cdc42 activity in rhogap19d mutants recruits Gek to the lateral cortex, where it can localize and activate NMYII. Our results suggest that the invasive phenotype of rhogap19d mutants depends on a partial disruption of polarity, in which the apical domain expands at the expense of the lateral domain. Because the relative sizes of the apical and lateral domains are determined by mutual antagonism between apical and lateral polarity factors, reducing the dosage of lateral factors should enhance this phenotype, whereas reducing apical factors should suppress it. We therefore tested whether polarity mutants act as dominant modifiers of the rhogap19d phenotype (Fig. 6). Removing one copy of the lateral polarity proteins, lgl and scrib, doubles the frequency of germline invasions, as does removing both copies of fasciclin II (fasc-II) or RNAi-knockdown of neuroglian, both of which encode lateral adhesion factors (Bilder and Perrimon, 2000; Wei et al., 2004; Szafrański and Goode, 2007). By contrast, loss of one copy of apkc or crβ strongly suppresses invasions. Reducing the dosage of geke also decreases the frequency of invasion, consistent with its role in activating NMYII laterally to stimulate the movement of the follicular cells into the germline. Thus, these genetic interactions support the view that the invasive behavior of rhogap19d mutant cells is driven by the expansion of the apical domain and Gek-dependent lateral contractility, both of which will increase the stress on the epithelium without completely disrupting polarity.

Two of the mutants showed unexpected genetic interactions with rhogap19d. First, reducing the dosage of the lateral polarity factor, Par-1, suppressed the invasive phenotype of the rhogap19d mutant, whereas the other lateral factors strongly enhance it. By localizing to the lateral membrane and functions to limit the basal extent of the adherens junctions by phosphorylating and antagonizing Baz (Par-3; Benton and St Johnston, 2003; Wang et al., 2012). The ability of the par-1 mutant to suppress rhogap19d indicates that Par-1 does not function in the same pathway as Scrib, Lgl, FasII, and Nrg and suggests instead that it either negatively regulates these lateral factors or positively regulates apical ones. It is also possible that Par-1 acts through the actin cytoskeleton and is required for the lateral contractility induced by ectopic Gek activity. Second, p21-activated kinase 1 (Pak1) has been reported to function redundantly with apkc to specify the apical domain downstream of active Cdc42 (Aguilar-Aragon et al., 2018). Although one would therefore expect the pak1 mutant to suppress the invasive phenotype like mutants in the other apical factors, it acts as a strong enhancer of invasion. This is consistent with the role of Pak1 as a component of the lateral Scribble complex and argues against the proposal that it functions as an apical Cdc42 effector kinase (Bahri et al., 2010).

Discussion

Here we report that RhoGAP19D restricts Cdc42 activity to the apical side of the follicle cells and probably many other Drosophila epithelial tissues. In the absence of RhoGAP19D, both N-WASP and Gek are recruited to the lateral membrane, indicating that Cdc42 is ectopically activated there. This implies that RhoGAP19D is the major Cdc42GAP that represses Cdc42 laterally, because no other GAPs can compensate for its loss. This also suggests that the GEFs that activate Cdc42 are not restricted to the apical domain and can turn it on laterally once this repression is removed. This is consistent with the identification of multiple vertebrate GEFs with different localizations that contribute to apical Cdc42 activation (Otani et al., 2006; Qin et al., 2010; Rodríguez-Fraticelli et al., 2010; Oda et al., 2014; Zihni et al., 2014). Our results therefore identify RhoGAP19D as a new lateral polarity factor. This leads to a revised network of polarity protein interactions in which RhoGAP19D functions as the third lateral factor that antagonizes the activity of apical factors, alongside Lgl, which inhibits apkc, and Par-1, which excludes Baz/Par-3 (Fig. 7 A; Wirtz-Peitz et al., 2008; Benton and St Johnston, 2003).

The function of RhoGAP19D is very similar to that of its ortholog, PAC-1, which inhibits Cdc42 at sites of cell contact in early C. elegans blastomeres to generate distinct apical and basolateral domains (Anderson et al., 2008). Both RhoGAP19D and PAC-1 are recruited to the lateral domain by E-cadherin complexes, although the exact mechanism is slightly different. RhoGAP19D recruitment is strictly dependent on α-catenin, which links it through β-catenin to the E-cadherin cytoplasmic tail, whereas α-catenin (HMP-1) and p120-catenin (JAC-1) play partially redundant roles in recruiting PAC-1 to E-cadherin (HMR-1) in the worm (Klopmstra et al., 2015). Nevertheless, in both cases, the recruitment of the Cdc42GAP translates the spatial cue provided by the localization of cadherin to sites of cell-cell contact into a polarity signal that distinguishes the...
lateral from the apical domain. Classic work on the establishment of polarity MDCK cells grown in suspension has revealed that the recruitment of cadherin (uvomorulin) to sites of cell–cell contact is the primary cue that drives the segregation of apical proteins from basolateral proteins (Wang et al., 1990). Furthermore, the expression of E-cadherin in unpolarized mesenchymal cells is sufficient to induce this segregation, although the mechanisms behind this process are only partially understood (Wang et al., 1990; McNeill et al., 1990; Watabe et al., 1994; Nejsum and Nelson, 2007). Our observation that RhoGAP19D directly links cadherin adhesion to the polarity system in epithelial cells extends the results of Klompstra et al. (2015) in early blastomeres, strongly suggesting that PAC-1/RhoGAP19D plays an important role in the first steps in epithelial polarization.

Although PAC-1 and RhoGAP19D perform equivalent functions in early blastomeres and epithelial cells, there is one important difference between their mutant phenotypes. In pac-1 mutants, Par-6 and aPKC are mislocalized to the contacting surfaces of C. elegans blastomeres where Cdc42 is ectopically active (Anderson et al., 2008). By contrast, Par-6 and aPKC are not mislocalized laterally in rhogap19d mutant Drosophila epithelial cells, even though lateral Cdc42-GTP does recruit two other Cdc42 effectors, N-WASP and Gek. Thus, lateral Cdc42 activity is sufficient to recruit Par-6/aPKC to the lateral domain in early blastomeres, but not in epithelial cells. Instead, we observed that lateral Cdc42 activity in rhogap19d mutant follicle cells acts at a distance to expand the size of the apical domain. A likely explanation for this difference is the presence of Crumbs in epithelial cells. The interaction between Cdc42-GTP and Par-6 alters the conformation of Par-6 so that it can bind to Crumbs, which anchors the Par-6–aPKC complex to the apical membrane and activates aPKC’s kinase activity (Peterson et al., 2004; Whitney et al., 2016; Dong et al., 2020). Although Par-6 presumably binds to Cdc42 laterally in rhogap19d mutants and undergoes the conformational change, it cannot be anchored laterally in the absence of Crumbs. This activated Par-6–aPKC complex can then diffuse until it is captured by Crumbs in the apical domain, thereby increasing apical aPKC activity, providing an explanation for why the apical domain expands in rhogap19d mutant cells (Fig. 7 B).

C. elegans has three Crumbs orthologues, but removal of all three simultaneously has no effect on viability or polarity (Waaijers et al., 2015). Thus, in contrast to Drosophila epithelial cells, C. elegans Crumbs proteins are not required for Par-6/aPKC localization and activation, suggesting that some other mechanism, such as Cdc42 binding, is sufficient to activate aPKC.

If the failure of active Cdc42 to recruit aPKC laterally in rhogap19d mutant cells is due to the absence of Crumbs in this
region, there must be a mechanism to exclude Crumbs from the lateral domain. One proposed mechanism depends on Yurt (Moe and EPB41L5 in vertebrates), which is restricted to the lateral domain by aPKC and binds to Crumbs to antagonize its activity (Laprise et al., 2006). However, we did not observe any lateral recruitment of aPKC in \textit{rhogap19d; yurt} double-mutant cells. Thus, there must be some parallel mechanism that excludes Crumbs, Par-6, and/or aPKC from the lateral domain.

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**Figure 4.** \textit{rhogap19D} mutant cells are taller than wild-type cells and have an enlarged apical domain. (A–E) Regions of the stage 7 follicle cell epithelium stained with DAPI (blue) containing clones of \textit{rhogap19d} mutant cells marked by the loss of RFP (magenta). (A) Cell mask staining (green) of the plasma membranes reveals that mutant cells are taller than wild-type cells and have domed apical surfaces. The arrows indicate the apical surfaces of the mutant cells. (B) The adherens junctions marked by endogenously tagged E-cadherin (ECad)-GFP (green) form at the same level in \textit{rhogap19d} mutant and wild-type cells (phalloidin; white). (C) In \textit{rhogap19d} mutant cells, GFP-aPKC localizes all around the apical domain above the adherens junctions (marked by Cno staining; white; indicated by the white arrow). (D) Crb-GFP marks an enlarged subapical region in \textit{rhogap19d} cells. (E) \textit{rhogap19d} mutant cells have slightly shorter lateral domains than wild-type cells, as shown by Lgl-GFP localization (green; white arrow). (F) A graph showing the mean cell height in wild-type and \textit{rhogap19d} mutant cells. (G) A graph showing the mean cell width in wild-type and \textit{rhogap19d} mutant cells. (H) A graph showing the mean apical domain length in wild-type and \textit{rhogap19d} mutant cells. (I) A graph showing the mean lateral domain length in wild-type and \textit{rhogap19d} mutant cells. The error bars represent SEM; ****, P < 0.0001; **, P < 0.002. Scale bars, 10 µm.

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RhoGAP19D restricts Cdc42 to the apical membrane

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Although loss of RhoGAP19D only leads to a partial disruption of polarity, it causes the follicular epithelium to invade the adjacent germline tissue with 40% penetrance. This invasive behavior is not driven by an epithelial-to-mesenchymal transition, because the cells retain their apical adherens junctions and epithelial organization. Instead, the deformation of the epithelium seems to be driven by the combination of an increase in lateral contractility and an expansion of the apical domain, because...

Figure 5. rhogap19d mutant cells are more motile than wild-type cells and contract laterally. (A) Single frames and a temporal projection of a time-lapse movie of a stage 7 egg chamber containing a large rhogap19d mutant clone (marked by the loss of RFP, magenta) and expressing GFP-aPKC (green). The blurred apical surfaces of the mutant cells in the temporal projection indicate that they are moving between frames. (B) Magnification of the boxed areas in A, showing that rhogap19d mutant cells (bottom panels) are more motile (white arrows) than wild-type cells (top panels). Temporal projections after 1, 2, and 4 min. (C) A single frame from a movie showing a cluster of rhogap19d mutant cells (white arrow; marked by the loss of RFP; magenta) beginning to invade the germline. The cells in the cluster appear to have detached from the basement membrane (cell mask; white). The lower panel shows a magnification of the boxed area. (D) rhogap19d mutant cells have lateral foci of NMYII foci (Zipper-GFP; green) and reduced levels at the apical side compared with wild-type cells (white arrows; DAPI, blue). This phenotype was observed in 154 of 157 mutant cells. (E and F) Gek (green) localizes apically in wild-type follicle cells (E) but extends along the lateral domain of all rhogap19d mutant cells (F; DAPI, blue). Scale bars, 10 µm. n = 11 homozygous mutant egg chambers.
reducing the dosage of Gek, which activates myosin II to drive the contractility, significantly reduces the frequency of this phenotype, as does halving the dosage of any of the apical polarity factors. The expansion of the apical domain makes the domain too long for the cells to adopt the lowest-energy conformation, giving them a tendency to become wedge shaped, which could drive the evagination. It is also possible that buckling of the epithelium contributes to invasion. Recent work has shown that epithelial monolayers under compressive stress and constrained by a rigid external scaffold have a tendency to buckle inward (Trushko et al., 2020). The follicular cell layer is surrounded by an ECM that constrains the shape of the egg chamber and that should therefore resist expansion (Haigo and Bilder, 2011). In addition, the pulses of lateral contractility are likely to generate compressive stress because transiently reducing cell height while maintaining a constant volume will increase the cells’ cross-sectional area, thereby exerting a pushing force on the neighboring cells. This compression coupled to the tendency to become wedge shaped due to apical expansion could therefore trigger the rare buckling events that initiate invasion. In support of this view, lateral contractility has been shown to drive the folding of the imaginal wing disc between the prospective hinge region and the pouch (Sui et al., 2018). This phenotype provides an example of how a partial disruption of polarity can induce cell shape changes that lead to major alterations in tissue morphogenesis (St Johnston and Sanson, 2011).

The rhogap19d phenotype resembles the defects earliest observed in the development of ductal carcinoma in situ (Halaoui et al., 2017). In flat epithelial atypia (FEA), the ductal cells are still organized into an epithelial layer, but they display apical protrusions that are strongly labeled by the apical polarity factor Par-6. This suggests that the apical domain has expanded and bulges out of the cell, just as we observed in the rhogap19d mutant follicular cells. In the next stage, atypical ductal hyperplasia (ADH), the ductal cells start to invade the lumen of the duct while retaining aspects of normal apical–basal polarity (Fig. S4 A). This again resembles the invasive phenotype of rhogap19d mutants, although overproliferation of the ductal cells probably also contributes to invasion in this case. Thus, these abnormalities, which can sometimes progress to ductal carcinoma in situ and breast cancer, mirror the effects of lateral Cdc42 activation. The RhoGAP19D human orthologues, ARHGAP21 and ARHGAP23, have been shown to bind directly to α-catenin and localize to cell–cell junctions (Sousa et al., 2005; Van Itallie et al., 2014).

**Figure 6. Genetic interactions between rhogap19d and other polarity factors.** A histogram showing the penetrance of the germline invasion phenotype of large rhogap19d mutant clones in combination with other polarity mutants. Removing one copy of scrib, lgl, or Pak1 strongly enhances the penetrance of the invasion phenotype. rhogap19d/fasII double-mutant clones and rhogap19d clones in which nrg has been depleted by RNAi also show a highly penetrant invasive phenotype. Loss of one copy of apKC, gek, crb, or par-1 strongly reduces the frequency of invasions. The error bars represent SEM; *, P < 0.05; **, P < 0.002; ****, P < 0.0001. All numerical data are presented in the table.
Furthermore, low expression of ARHGAP21 or ARHGAP23 correlates with reduced survival rates in several cancers of epithelial origin (Fig. S4, C and D; Győrffy et al., 2010). It would therefore be interesting to determine whether these orthologues perform the same functions in epithelial polarity as RhoGAP19D and if their loss contributes to tumor development.

Materials and methods

Predicting Cdc42–GAP interactions

We identified and aligned putative Drosophila GAPs by searching the Pfam (Finn et al., 2016) hidden Markov model profile PF00620.26 (RhoGAP) against the UniProt Drosophila melanogaster proteome using HMMsearch (Eddy, 2009). We aligned significantly scoring sequences together with human ARHGAP1 (from the 3D structure Research Collaboratory for Structural Bioinformatics [RCSB] Protein Data Bank accession no. 1GRN) using HMMalign (Eddy, 2009).

We scored the potential interaction of each Drosophila GAP/Cdc42 pair using the structure of human ARHGAP1/CDC42 (RCSB Protein Data Bank entry 1GRN) via InterPReTS (Aloy and Russell, 2002), which assesses the effect of evolutionary changes at the interface structure using empirical pair potentials (Betts et al., 2015).

We modeled the Cdc42–RhoGAP19D 3D complex using Swiss-Model (Waterhouse et al., 2018) and rendered the interaction interface using the PyMOL Molecular Graphics System version 2.3.0 (Schrödinger, LLC).

Drosophila mutant stocks and transgenic lines

We used the following mutant alleles and transgenic constructs: cdc42^2 (Fehon et al., 1997; Bloomington Drosophila Stock Center [BDSC] 9105), pI20^m308 (Myster et al., 2003; BDSC 81638), shg^629 (BDSC 58471), Igl^4 (Gateff, 1978; BDSC 36289), scrib^2 (Bilder and Perrimon, 2000), fastG0338 (Mao and Freeman, 2009; Kyoto Stock Center 111871), par-3^622 (Shulman et al., 2000), aPKC^HC (Chen et al., 2018), cebp^F035 (Tepass et al., 1990), gek^nblo80 (Gontang et al., 2011), Pak^22 (Newsome et al., 2000); a gift from the Dickson laboratory, Janelia Research Campus, Ashburn, VA; CadN^6328 (Prakash et al., 2005), a-catenin RNAi (BDSC 33430), nrg RNAi (BDSC 38215), fastI RNAi (BDSC 34084), fastII RNAi (BDSC 77396), scrib RNAi (BDSC 35748), cora RNAi (Vienna Drosophila Resource Center 9788), rhogap19d RNAi (P{TRiP.HMS0352}attP2; BDSC 32361), e-cadherin–EGFP (BDSC 60584; Huang et al., 2009), Lgl–EGFP (Tian and Deng, 2008), mNeonGreen-NWasp (a gift from Jenny Gallop, Gurdon Institute, Cambridge, UK), mCherry-GrabFP-Baz (Harmansa et al., 2017), aPKC–EGFP (Chen et al., 2018), Zipper-EGFP (Lowe et al., 2014), UASP–GFP RhoGAP19D (BDSC 66167), y^w; P{GawB}NP1624 (Traffic Jam-Gal4; Brand and Perrimon, 1993), and nanos-GAL4 (a gift from Ruth Lehmann, The Whitehead Institute, Massachusetts Institute of Technology, Cambridge, MA). The following stocks were used to generate mitotic clones: ubiRFP-nls, hsflp, FRT20A^4 (PBac{WH}f01417; Exelixis), ubiRFP-nls (BDSC 34050), FRT82B, ubiRFP-nls (BDSC 30555), and FRT82B ubiGFP (BDSC 5188), y w hs-FLP; Act5C–CD2–Gal4, UAS:mRFPnls (Pignon and Zipursky, 1997; BDSC 30558).

Figure 7. A revised network of inhibitory interactions between polarity factors. (A) A diagram showing how the recruitment of RhoGAP19D to the lateral membrane by E-cadherin adhesion complexes restricts Cdc42 activity to the apical domain. This adds a new inhibitory interaction to the network of interactions between apical and lateral polarity factors. (B) A model of the changes in rhogap19d mutant cells that lead to the invasive phenotype. In the absence of RhoGAP19D, active Cdc42 localizes along the lateral domain as well as the apical domain and activates Gek to induce lateral actomyosin contractions. Lateral Cdc42–GTP also alters the conformation of Par-6/aPKC so that it is competent to bind to Crumbs. This primed Par-6/aPKC then diffuses until it binds to apical Crumbs, which activates aPKC’s kinase activity, resulting in expansion of the apical domain and a dome-shaped apical surface.

Generation of endogenously tagged RhoGAP19D and N-WASP

The mNeonGreen tag (Shaner et al., 2013) was fused to the N-terminus of RhoGAP19D by CRISPR-mediated homologous recombination. In vitro synthesized gRNA to a CRISPR target (target sequence 5’-GTTGGCCGACTCCGGCAGGCGGGG-3’, CRISPR; located...
25,985 bp from the 5’ end of RhoGAP19D and a plasmid donor containing the ORF of mNeonGreen as well as appropriate homology arms (1.5 kb upstream and downstream) were co-injected into nos-Cas9–expressing embryos (BDSC 54591). Single F0 flies were mated to y w flies and allowed to produce larvae before the parents were analyzed by PCR. Progeny from F0 flies in which a recombination event occurred (as verified by PCR) were crossed and sequenced to confirm correct integration. Several independent mNeonGreen–RhoGAP19D lines were isolated. Recombinants carry the mNeonGreen coding sequence inserted immediately downstream of the endogenous start codon with a short linker (Gly-Ser-Gly-Ser) between the coding sequence of mNeonGreen and the coding sequence of RhoGAP19D. Homozygous flies are viable and healthy.

The left arm of RhoGAP19D was amplified with the following primers: forward: 5’-TACGACTCACTATAAGGGCGAATTGGTACGGGCCCCCCTCTGTGGTTGGGTAATTACATGTGCTT-3’; reverse: 5’-TCTCTCCGCCCCTGCCTGCACCATTTTTGTGGGCGGATACTGTGGT-3’.

The right arm of RhoGAP19D was amplified with the following primers: forward: 5’-TGTCATGATTGCCAGCTTTTGACAACAGG-3’; reverse: 5’-GGCCGGTCTGAAACATGTGATTTTCAGATGGTGAGCAAGGG-3’.

The following primers: forward: 5’-TGTCATGATTGCCAGCTTTTGACAACAGG-3’; reverse: 5’-GGCCGGTCTGAAACATGTGATTTTCAGATGGTGAGCAAGGG-3’.

To discriminate between the signal of mNeonGreen–RhoGAP19D in the germline and in the somatic follicular cells, UAS-RhoGAP19D RNAi was expressed in the germline under the control of nos-Gal4. The knockdown was efficient, which allowed visualization Neo-RhoGAP19D expression in the follicular cells only.

mNeonGreen–N-WASP was generated by CRISPR-mediated insertion of mNeonGreen before exon 2 of N-WASP in order to target all N-WASP isoforms as isoform C lacks exon 1. A linker sequence of four serine residues was added such that the fusion protein junction corresponds to LYKSSSSTLN. Two guide RNA sites were chosen that flanked the insertion site, with the PAM motifs being separated by 17 nucleotides. The donor plasmid pTv-[w+] mNeonGreen N-WASP constructed by In-Fusion cloning of PCR generated 5’ and 3’ homology arms, mNeonGreen and HindIII/XhoI cut pTv-[w+] vector. The 940 bp 5’ homology arm was amplified from genomic DNA with the forward primer 5’-GAATCTCGAGCTGACGCTGACTGTTCGATTGGACAGAGATTTAAAGGGGTAATTACATGTGCTT-3’ and the reverse primer 5’-CTCACCATCTGCAAGTGGGACAGAGATTTAAAGGGGTAATTACATGTGCTT-3’.

The following primary antibodies were used: anti-Armadillo (N2 7A1 1:100 dilution; Developmental Studies Hybridoma Bank), anti-Dlg (4F3, 1:100 dilution; Developmental Studies Hybridoma Bank), anti-αPKC (C-20, sc-216-G, goat polyclonal IgG, 1:500; Santa Cruz Biotechnology), anti-CN (1:1,000 dilution; Takahashi et al., 1998; a gift from M. Peifer, University of North Carolina, Chapel Hill, NC) anti-Gek (1:25 dilution; Gontang et al., 2011; a gift from the Clandinin laboratory, Stanford University, Stanford, CA), and anti-PH3 (9701S, 1:500 dilution; Cell Signaling Technology).
The following secondary antibodies were used: Alexa Fluor secondary antibodies (Invitrogen) at a dilution of 1:1,000, Alexa Fluor 488 goat anti-mouse (A11029), Alexa Fluor 488 goat anti-rabbit (A11034), Alexa Fluor 647 goat anti-mouse (A21236), Alexa Fluor 647 goat anti-rabbit (A21245).

F-actin was stained with phalloidin conjugated to rhodamine (R415, 1:500 dilution; Invitrogen). The cell membranes were labeled with CellMask Orange Plasma Membrane Stain or CellMask Deep Red Plasma Membrane Stain (Thermo Fisher Scientific).

Immunostaining

Ovaries from fattened adult females, salivary glands from L3 instar larvae, gut from L3 instar larvae, and accessory glands from virgin or mated males were dissected in PBS and fixed with rotation for 20 min in 4% paraformaldehyde and 0.2% Tween 20 in PBS. After a few washes with PBS with 0.2% Tween, tissues were then incubated in 10% BSA in PBS to block for at least 1 h at room temperature. Incubations with primary antibodies were performed at 4°C overnight in PBS, 0.2% Tween 20, and 1% BSA. This step was followed by four washes with PBS with 0.2% Tween, and samples were then incubated for 3–4 h with secondary antibody at room temperature. Specimens were then washed several times in washing buffer and mounted in Vectashield containing DAPI (Vector Laboratories).

Embryos were fixed using the formaldehyde/heptane fixation method, followed by methanol extraction.

For staining with the anti-Gek antibody, ovaries were heat fixed as described by Chen et al. (2018).

Imaging

Fixed samples and live imaging were performed using an Olympus IX81 (40×/1.3 UPlan FLN oil objective or 60×/1.35 UPlanSapo oil objective) or a Leica SP8 white laser (63×/1.4 HC Olymups IX81 (40×/1.3 UPlan FLN oil objective or 60×/1.35 UPlan Sapo oil objective) or a Leica SP8 white laser microscope. For live observations, ovaries were dissected in 10S Voltalef oil (VWR Chemicals) at room temperature. Images were processed with Fiji (Schindelin et al., 2012) or Leica analysis software.

Drosophila genetics

Standard procedures were used for Drosophila maintenance and experiments. Flies were grown on standard fly food supplemented with live yeast at 25°C. Follicular cell clones were induced by incubating larvae or pupae at 37°C for 2 h every 12 h over a period of at least 3 d. Adult females were dissected at least 2 d after the last heat shock. In some experiments, adult flies were heat shocked for at least 3 d and dissected 1 d after the last heat shock.

We used the Flipout technique with Actin5c>Cr2>Gal4 to generate marked clones of cells expressing RNAi constructs (Fig. S3). Flp recombination was induced by incubating larvae or pupae at 37°C for 2 h every 12 h over a period of at least 3 d.

Genetic interactions

To test for genetic interactions between rhogap19d and adhesion molecules or polarity factors, we analyzed the frequency of follicle cell invasions at stages 7 and 8 in large anterior rhogap19d clones that covered at least 25% of the follicular epithelium in each genetic background.

An unpaired, two-tailed Student’s t test with Welch’s correction was used to determine whether any differences between the penetration of the invasion phenotype in rhogap19d alone and in combination with each mutant or RNAi knockdown were significant.

Quantifications of the total number of follicle cells per egg chamber in rhogap19d mutants

Confocal z-stacks of whole egg chambers were collected on a Leica SP8 white laser microscope. Each egg chamber was divided in three regions. Nuclei were counted twice per region.

Reproducibility of experiments

All experiments were repeated multiple times as listed below. For each figure, the first number indicates the number of times that the experiment was repeated, and the second indicates the number of egg chambers or clones analyzed. The number of independent experiments performed were as follows: Fig. 1 A (3, 28); Fig. 2 C (5, 89); Fig. 2 E (3, 34); Fig. 2 F (2, 20); Fig. 2 H (7, 56); Fig. 2 I (4, 34); Fig. 2 J and K (3, 6); Fig. 2 L (2, 9); Fig. 2 M (3, 12); Fig. 2 N (2, 7); Fig. 3 A (4, 24); Fig. 3 B (3, 46); Fig. 3 D and E (3, 67, and 45); Fig. 4 A (7, 67); Fig. 4 B (3, 78); Fig. 4 C (5, 98); Fig. 4 D (2, 16); Fig. 4 E (3, 56); Fig. 5 A (7, 56); Fig. 5 C (5, 47); Fig. 5 D (3, 98); Fig. 5 E (3, 18); and Fig. 6: rhogap19d (8, 301), scrb/+ (3, 194), lgl/+ (3, 94), fus2 (3, 185), nrg (3, 114), part1/+ (3, 155), apKC/+ (4, 98), crb (2, 129), gek (2, 78), Pakt (3, 228).

Online supplemental material

Fig. S1 shows that RhoGAP92B, RhoGAP68F, CdGAPr, RacGAP84C, Conu, and RhoGAP93B are not required for follicle cell polarity. Fig. S2 shows that RhoGAP91D localizes laterally in multiple epithelia. Fig. S3 shows that loss of RhoGAP91D causes apical domain expansion in several epithelia. Fig. S4 shows that the rhogap19d phenotype resembles the early steps in breast cancer. Video 1 is a time-lapse movie of a stage 7 egg chamber containing a large rhogap19d mutant clone (marked by the loss of RFP) and expressing GFP-aPKC. Table S1 lists CRISPR-mediated mutations in candidate Cdc42 GAPS.

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Figure S1. RhoGAP92B, RhoGAP68F, CdGAPr, RacGAP84C, Conu, and RhoGAP93B are not required for follicle cell polarity. (A) Egg chambers containing rhoGAP92B mutant cells marked by loss of RFP. Loss of RhoGAP92B does not affect aPKC localization (A; n = 28) or Lgl localization (not shown; n = 24). Dashed lines indicate RFP-negative mutant cells. (B–F) Egg chambers from flies homozygous for rhoGAP68F (B; n = 109), cdGAPr (C; n = 12), racGAP84C (D; n = 104), conu (E; n = 90), and rhoGAP93 (F; n = 119) mutants show normal localization of aPKC (B–F) and Lgl (not shown) and normal organization of the follicular epithelium. Scale bars, 10 µm.
Figure S2. RhoGAP localizes laterally in multiple epithelia. (A and B) A top view (A) and a cross-section (B) through a salivary gland from an L3 larva, showing mNeonGreen-RhoGAP19D (green) localization to the lateral domain. DAPI (blue), n = 8 larvae. (C and D) A top view (C) and a cross-section (D) through an accessory gland from a mated male. mNeonGreen-RhoGAP19D (green) localizes laterally. DAPI (blue), n = 10 males. (E and F) A top view (E) and a cross-section (F) through a live 3-h-old embryo. mNenonGreen-RhoGAP19D (green) localizes laterally, *Autofluorescence of the vitelline membrane. n = 11 embryos. (G) Lateral mNeonGreen-RhoGAP19D (green) localization in the anterior midgut of an L3 larva; n = 3 guts. (H–J) Wild-type localization of mNeonGreen-RhoGAP19D at the lateral cortex of stage 7 follicle cells in which fasII (H; n = 21), fasIII (I; n = 19), and nrg (J; n = 25) have been knocked down using the UAS-RNAi Flp out system (cells that express RFP coexpress the RNAi constructs). Each experiment was performed three times. (K) A stage 8 egg chamber with a clone of cells mutant for N-cadherin1 and N-cadherin2 (marked by the loss of RFP). mNeonGreen-RhoGAP19D localizes normally in the mutant cells (n = 15). The experiment was performed twice. (L) mNeonGreen-RhoGAP19D localizes to the cortex in aPKC<sup>HC</sup> mutant clones (marked by the loss of RFP) at stage 8. n = 16; experiment performed twice. (M and N) mNeonGreen-RhoGAP19D is correctly localized in cells treated with RNAi against Scrib (n = 23; M) or Coracle (N; n = 16). Stage 8; experiments performed twice. Scale bars, 10 µm.
Figure S3. **rhogap19d mutant cells expand apically in different epithelia.** (A and B) Accessory glands from virgin wild-type (A) and **rhogap19d** mutant males (A) stained for Lgl (green), F-actin (phalloidin; red), and DNA (DAPI; blue). The apical surfaces of the mutant cells protrude into domes, and the cells are much taller than in wild type (n = 11 males). Scale bars, 10 µm. (C and D) Cellular blastoderm embryos (3 h after fertilization) from wild-type (C) and **rhogap19d** mutant mothers, stained for Dlg (green) and DNA (DAPI; blue). The cells in the embryos laid by homozygous mutant females bulge apically. The vertical arrow and arrowhead in A and C indicate the smooth apical surface in wild type, and the diagonal arrows mark the domed apical surfaces in **rhogap19d** mutant cells. n = 8 embryos in each background. Scale bars, 10 µm.
Figure S4. The rhogap19d phenotype resembles the early steps in breast cancer. (A) Images of breast tissue samples reproduced from Halaoui et al. (2017; Fig. S3), reprinted with permission from Genes & Development, showing examples of FEA and ADH. Samples were immunostained for Par-6 (red), E-cadherin (E-Cad; green), and DAPI (blue). The white arrows show polarized cells invading into the lumen. The yellow arrows show epithelial bridges that split the primary lumen. (B) rhogap19d mutant cells (marked by the absence of RFP), stained for E-cadherin–GFP (Cad-GFP; green) and DAPI (blue), show similar apical bulges and invasions to FEA and ADH. Cells first bulge apically (white arrows in the left panel), then start to collectively invade the germline (white arrows in the middle panel), to finally form big clusters inside the egg chamber (white arrows in the right panel). Stage 8 egg chamber. Experiment repeated five times. Scale bars, 10 µm. (C) Kaplan-Meier survival plot for ARHGAP21 expression (high; top quartile versus low; bottom quartile) in bladder carcinoma. Survival data were retrieved from the kmplot resource (kmplot.com) described in Györffy et al. (2010). (D) Kaplan-Meier survival plot for ARHGAP23 expression in lung adenocarcinoma. HR, hazard ratio, with 95% confidence limits in parentheses.
Video 1. A time-lapse movie of a stage 7 egg chamber containing a large rhogap19d mutant clone (marked by the loss of RFP; magenta) and expressing GFP-aPKC (green). Frames were captured every 15 s. Elapsed time, 11 min; playback time, 3 s.

Table S1 is provided online and lists CRISPR-mediated mutations in candidate Cdc42 GAPs.