Apical polarity proteins recruit the RhoGEF Cysts to promote junctional myosin assembly

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The spatio-temporal regulation of small Rho GTPases is crucial for the dynamic stability of epithelial tissues. However, RhoGTPase activity is controlled during development remains largely unknown. To explore the regulation of Rho GTPases in vivo, we analyzed the Rho GTPase guanine nucleotide exchange factor (RhoGEF) Cysts, the Drosophila orthologue of mammalian p114RhoGEF, GEF-H1, p190RhoGEF, and AKAP-13. Loss of Cysts causes a phenotype that closely resembles the mutant phenotype of the apical polarity regulator Crumbs. This phenotype can be suppressed by the loss of basolateral polarity proteins, suggesting that Cysts is an integral component of the apical polarity protein network. We demonstrate that Cysts is recruited to the apico-lateral membrane through interactions with the Crumbs complex and Bazooka/Par3. Cysts activates Rho1 at adherens junctions and stabilizes junctional myosin. Functional myosin depletion is similar in Cysts- and Crumbs-compromised embryos. Together, our findings indicate that Cysts is a downstream effector of the Crumbs complex and links apical polarity proteins to Rho1 and myosin activation at adherens junctions, supporting junctional integrity and epithelial polarity.

Introduction

Epithelial cells show pronounced apical-basal polarity and form an apical junctional complex that encircles individual cells and tightly links neighboring cells into a sheet-like tissue. Factors that regulate apical-basal polarity, and in particular apical polarity proteins such as Crumbs (Crb) or atypical protein kinase C (aPKC), play a pivotal role in the formation of the junctional belt. Consequently, compromising apical polarity protein function causes a loss of epithelial integrity similar to the loss of core junctional proteins such as E-cadherin (Tepass, 2012). How the function of apical polarity proteins supports a circumferential junctional belt is not well understood. One key mechanism that promotes junctional stability is the activity of cytoplasmic myosin II that is activated at apical junctions through the Rho-Rock pathway (Mack and Georgiou, 2014; Lecuit and Yap, 2015). Here, we ask how apical polarity proteins help to confine the activity of Rho1 to the apical junctional region to promote the formation of a junctional belt.

Rho GTPases are molecular switches that cycle between active (GTP-bound) and inactive (GDP-bound) states. Rho GTPases are used over and over again to regulate diverse molecular processes within cells including cytoskeletal dynamics, cell polarity, cell adhesion, and vesicle trafficking (Jaffe and Hall, 2005; Hall, 2012; Ridley, 2012; Ratheesh et al., 2013; Mack and Georgiou, 2014). For example, epithelial polarity in the Drosophila melanogaster embryo requires three members of the Rho protein family: Cdc42 promotes apical polarity by activation of the apical Par protein complex (Par6/aPKC; Hutterer et al., 2004; Harris and Tepass, 2008, 2010), Rac1 acts together with phosphoinositide 3-kinase as a basolateral polarity protein (Chartier et al., 2011), and Rho1 (RhoA in mammals) supports integrity of apical adherens junctions (AJs) by activating myosin II through the Rho kinase pathway (Magie et al., 1999; Fox et al., 2005; Matsuoka and Yashiro, 2014; Lecuit and Yap, 2015).

Rho GTPase-specific guanine nucleotide exchange factors (RhoGEFs) and Rho GTPase-activating proteins (RhoGAPs) promote activation and deactivation, respectively, of Rho GTPases (McCormack et al., 2013; Cook et al., 2014). The Drosophila genome encodes 26 RhoGEFs and 22 RhoGAPs that presumably regulate Rho GTPases and a wide variety of downstream effectors, many of which modulate cytoskeletal remodeling (Aspenström, 1999; Greenberg and Hatini, 2011; Hall, 2012; Cook et al., 2014). Rho1, Cdc42, and Rac (with three fly paralogs Rac1,
Rac2, and Mtl) are maternally provided to the embryo (Magie et al., 1999; Genova et al., 2000; Hakeda-Suzuki et al., 2002). These Rho GTPases play multiple essential roles in embryogenesis through their contributions to epithelial polarity, cell movements such as mesoderm invagination, germband extension, dorsal closure, and wound repair, among other processes (Harden, 2002; Mack and Georgiou, 2014; Verboon and Parkhurst, 2015). However, how RhoGTPases are regulated in development through the spatial and temporal engagement of GEFs and GAPIs still remains largely unexplored.

Here, we characterize a Drosophila RhoGEF, Cysts (Cyst). Cyst is the single orthologue of a group of four mammalian paralog GEFs characterized by the presence of a RhoGEF or Dbl homology–plekstrin homology (DH-PH) domain: p114RhoGEF, p190RhoGEF, AKAP-13, and GEF-H1 (McCormack et al., 2013; Cook et al., 2014; Ngok et al., 2014). Tissue culture studies have shown that p114RhoGEF (ARHGEF18 in humans) links the epithelial polarity machinery with the actomyosin cytoskeleton. p114RhoGEF interacts with proteins of the apical Par and Crb polarity complexes and activates Rhog in support of actomyosin 

p114RhoGEF interacts with proteins of the apical Par and Crb polarity complexes and activates Rhog in support of actomyosin organization, apical constriction, and junction assembly (Nakajima and Tanoue, 2010, 2011, 2012; Terry et al., 2011; Loie et al., 2015; Acharya et al., 2018). Our work indicates that Cyst is a key RhoGEF in the Drosophila embryo that activates Rhog and consequently myosin II at AJs. We conclude that Cyst is a crucial component of the apical polarity protein network that couples apical polarity to the junctional Rhog-myosin pathway, supporting AJ stability and epithelial integrity.

Results
Cyst is an apical epithelial polarity protein required for AJ stability during gastrulation

Maternal knockdown of CG10188 caused an embryonic lethal phenotype characterized by the formation of many small epithelial vesicles or cysts instead of a large continuous epithelial sheet. We named CG10188 therefore cysts (cyst; Fig. 1 C). These cyst RNAi embryos also develop larger patches of continuous epidermis displayed as shields of cuticle (Fig. 1 C). cyst RNAi embryos were rescued to adulthood by a CystR transgene, containing a cyst genomic sequence immune to RNAi without altering the encoded protein sequence (Fig. 1 A). We also noted that overexpression of Cyst led to embryonic lethality, with embryos displaying severe defects in head morphogenesis (Fig. 1 F). These results identify Cyst as an essential factor for maintaining epithelial integrity in the Drosophila embryo.

We generated a cyst deletion mutation (cyst) with CRISPR/Cas9 technology that removes most of the cyst coding sequence including the DH (RhoGEF) and PH domains and the entire C-terminal part of the protein (Fig. 1 A). Zygotic cyst mutant animals were not embryonic lethal and died at later stages of development. In contrast, embryos derived from cyst mutant germline clones and crossed to cyst+/+ showed two phenotypes. One half of embryos showed a prominent embryonic phenotype similar to cyst RNAi embryos (referred to as cyst(M/Z) embryos here), characterized by the presence of a large number of epithelial vesicles and shields of cuticle of various sizes (Fig. 1, D and E). The other half of animals were not embryonic lethal, suggesting that maternal depletion of Cyst can be compensated for by expression of one zygotic copy of cyst. The cyst phenotype is reminiscent of the cb1 mutant phenotype (Fig. 1 G; Tepass et al., 1990; TepAss and Knust, 1990; Tepass and Knust, 1993) or the phenotype caused by depletion of other apical polarity proteins or AJ proteins such as DE-cadherin (DEcad; Tepass et al., 1996; Uemura et al., 1996). Whereas most cb1 mutant embryos showed only cuticle vesicles, all cyst(M/Z) or cyst RNAi embryos showed cuticle shields in addition to vesicles, suggesting that the cyst-null phenotype is qualitatively similar but somewhat weaker than the cb1-null phenotype.

A cb1-like phenotype has been documented for only a small number of genes that encode apical polarity proteins such as cb1 and starburst (sdt), or genes encoding AJ proteins such as bazooka (bazo), shotgun (encoding DEcad), or armadillo (arm; Drosophila β-catenin; Tepass et al., 1990, 1996; TepAss and Knust, 1990; Tepass and Knust, 1993; Cox et al., 1996; Uemura et al., 1996; Bilder et al., 2003; Tanentzapf and Tepass, 2003). In contrast, genes encoding basolateral polarity proteins such as scribble (scrib) or yurt (yrt) have different mutant phenotypes and do not display epithelial cysts with inward-facing apical lumina. Instead, these mutants display clusters of epithelial cells with enlarged apical membranes so the cuticle appears as a series of bubbles (Bilder and Perrimon, 2000; Bilder et al., 2003; Tanentzapf and Tepass, 2003; Laprise et al., 2006, 2009). The cuticle defects observed in cyst-compromised embryos therefore strongly suggest that Cyst is a new component of the apical polarity machinery.

The development of a cb1-like phenotype was described in detail and entails the loss of polarity and AJ fragmentation during gastrulation (stages 8–11; Tepass et al., 1990; TepAss and Knust, 1990; Grawe et al., 1996; Tepass, 1996). Subsequently, these embryos show enhanced programmed cell death elicited by activation of the JNK signaling pathway (Kolahgar et al., 2011). Epithelial cells that survive form cysts that show normal epithelial polarity with an inward-facing lumen into which cuticle is secreted (Tanentzapf and Tepass, 2003). To determine whether a similar sequence of events can be found in cyst-compromised embryos, we examined the development of cyst embryos by live imaging, monitoring the AJ marker DEcad::GFP (Fig. 2) and assessing the distribution of apical (Crb and aPKC), junctional (Arm), and basolateral (Yrt) polarity markers (Figs. 3 and S1). These observations showed progressive AJ fragmentation during gastrulation until many cells, in particular in the ventral ectoderm, had lost AJs (Fig. 2, A and B). Other cells showed focal concentrations of DEcad, suggesting the beginning of cyst formation (Fig. 2 C). Formation of epithelial cysts is first seen at the end of gastrulation in stage-11 embryos and can be followed throughout the rest of development (Figs. 3 and S1). These cysts showed normal polarized distribution of Crb, aPKC, Arm, and Yrt (Figs. 3 and S1). Collectively, these findings indicate that Cyst function is closely related to apical and/or junctional epithelial polarity regulators.

A key feature of the machinery that regulates epithelial polarity is the negative feedback between apical and basolateral polarity proteins (Benton and St Johnston, 2003; Bilder et al.,
This mutual antagonism can be revealed through double-mutant analysis. For example, double mutants of crb and the basolateral polarity gene scrib or of sdt and the basolateral polarity gene lethal giant larvae (lgl) show a striking suppression of the crb or sdt mutant defects and display a scrib or lgl mutant phenotype, respectively (Bilder et al., 2003; Tanentzapf and Tepass, 2003). To reveal whether cyst behaves like crb or sdt in these tests, we generated embryos compromised for cyst and scrib or cyst and lgl (Fig. 1, H–K, M, and N). Maternal depletion of scrib with RNAi caused a strong loss-of-function phenotype characterized by epidermal cell clusters surrounded by cuticle (Fig. 1 H; Bilder et al., 2003; Tanentzapf and Tepass, 2003), whereas lgl(M/Z) embryos displayed a weaker phenotype with defects in head morphogenesis. scrib RNAi fully suppressed the cyst RNAi phenotype, and lgl(M/Z) fully suppressed the cyst(M/Z) phenotype, with double mutants showing phenotypes indistinguishable from scrib RNAi or lgl(M/Z) alone (Fig. 1, I, M, K, and N). Taken together, our analyses suggest that Cyst is a new key component...
of the apical polarity machinery that acts during gastrulation to maintain junctional and epithelial integrity in fly embryos.

Cyst regulates planar epithelial organization in the early embryo

To further assess the role of Cyst in the early embryo, we analyzed the lateral ectoderm at the onset of germ band extension, when circumferential AJs form. At this stage, DEcad, Baz, and other AJ proteins become enriched at the apico-lateral boundary but also acquire a planar polarized distribution to the dorsal and ventral edges of the apico-lateral domain (Zallen and Wieschaus, 2004). Disrupted planar polarization, with Baz hyperpolarizing as prominent, single foci along cell edges, has been observed when actin is reduced or in embryos with abnormal activity of polarity proteins including aPKC, Par1, and Crb (Harris and Peifer, 2007; Jiang et al., 2015; Vichas et al., 2015). This tissue thus provides a context to examine whether Cyst cooperates with polarity proteins in the initial formation of a normal circumferential AJ belt.

We found that cyst depletion by maternal expression of shRNA alone had minimal effects on Baz distribution. However, further depletion of cyst by maternal heterozygosity for a deletion uncovering cyst \( \text{Df}(\text{cyst}) = \text{Df}(2L)\text{BSC301} \) produced Baz hyperpolarization in contrast to control (mCherry shRNA) or \( \text{Df}(\text{cyst})/+ \) alone (Fig. 4, A and B). To test if Baz hyperpolarization was subject to regulation by aPKC, we analyzed cyst RNAi; \( \text{Df}(\text{cyst})/+ \) embryos derived from mothers heterozygous for a null aPKC allele and discovered even greater Baz hyperpolarization, whereas \( \text{Df}(\text{cyst}) \text{ apkc}/+ \) control embryos were normal (Fig. 4, A and B). As compromising the Cdc42-binding region of Par6 disrupts the cortical localization of the Par6/aPKC complex (Hutterer et al., 2004), we investigated whether the mis-regulation of Baz by cyst depletion could be explained by a loss of aPKC from the apical domain. However, cyst loss-of-function embryos displayed no detectable effects on aPKC localization when the blastoderm was fully formed and before the germ band started to extend (Fig. 4 C) or on aPKC levels around the margins of the apical domain (Fig. 4, C and D). As an alternative possibility, we examined if disruption of F-actin could explain the observed hyperpolarization of Baz. Treatment of embryos with cytochalasin D resulted in Baz hyperpolarization (Fig. 4 E), with no apparent effect on aPKC levels at the apico-lateral membrane (Fig. 4 F), mimicking the effects of cyst loss of function. These results indicate that Cyst contributes to the initial formation of a

Figure 2. Cyst is required for AJ integrity.

Z-projections (9.5 µm) taken from live control \((n = 3)\) or cyst RNAi \((n = 3)\) embryos expressing DEcad::GFP controlled by its endogenous promoter (see Videos 1 and 2). Times indicate minutes after onset of germ band extension. Scale bars, 10 µm. (A) Ventral view of the ectoderm at stage 11 showing a loss of AJs in cells adjacent to the ventral midline (dashed line) in a cyst RNAi embryo. (B) Ventral ectoderm cells at the indicated time points showing the increasing fragmentation and loss of AJs in a cyst RNAi embryo. (C) Ventral ectoderm cells at the indicated time points showing clustering of AJ material in a cyst RNAi embryo (arrows).
normal circumferential AJ belt. Cyst appears to act by regulating the actin cytoskeleton, either downstream or in parallel with an aPKC-dependent mechanism.

Cyst localizes to the apico-lateral cortex

The impact of Cyst on AJs suggests that it may localize to the apico-lateral cortex to exert its function. We examined the

Figure 3. Epithelial cysts in cyst-compromised embryos maintain epithelial polarity. (A and B) Staining of cyst RNAi (A) and cyst(M/Z) (B) embryos compared with wild-type controls for the apical markers Crb and aPKC, the junctional marker Arm, and the basolateral marker Yrt. All panels show side views of the ectoderm or epidermis at the indicated stages. Epithelial cysts in cyst-compromised embryos are evident in stage-11, -13, and -15 embryos (arrowheads) but not at stage 10. Markers show normal subcellular distributions of apical markers in cysts, facing the lumen where cuticle will be secreted (see Fig. 1, C–E). Insets show triple-labeled cells with Crb (A) or aPKC (B) shown in blue. n = number of embryos analyzed. Scale bars, 10 µm; insets, 5 µm.
distribution of an N-terminally GFP-tagged isoform of Cyst (GFP::Cystε) expressed under the cyst endogenous promoter in live embryos. GFP::Cystε was enriched at the apico-lateral cortex (Fig. 5 A). GFP::Cystε was barely detectable at stage 6, increased at the cortex from stage 8 to mid-embryogenesis (stage 14), and decreased at later stages (Fig. 5 B). These findings suggest that Cyst is enriched at AJs or their immediate vicinity from the onset of germband extension (stage 6/7) and throughout organogenesis.

The RhoGEF domain and the C-terminal region are essential for Cyst function

shRNA-resistant genomic structure–function constructs were tested for their ability to rescue the embryonic lethality and
phenotype of cyst RNAi embryos. Cyst RNAi embryos are embryonic lethal and display a loss of epithelial integrity (Figs. 1 and 3). As expected, a construct lacking the DH-PH domain of Cyst (CystRΔDH-PH::GFP) did not rescue cyst RNAi embryos, in contrast to control constructs (CystR, CystR::GFP, GFP::CystR; Fig. 6), indicating that the RhoGEF domain of Cyst is essential for epithelial integrity. Also, a construct lacking the C-terminal region of Cyst (CystRΔC::GFP) did not rescue the cyst RNAi phenotype (Fig. 6). In contrast, cyst RNAi embryos expressing GFP::CystRΔPBM, which lacks the C-terminal predicted PDZ domain binding motif (PBM), were viable, indicating that the Cyst C-terminal region, but not the PBM, is essential for function, consistent with our finding that Cyst interacts with PDZ domain–containing proteins Baz and Patj independent of its PBM (see below). Expression of a construct lacking the Cyst N-terminus (GFP::CystRΔN) rescued the lethality of most cyst shRNA embryos (Fig. 6, A and B), with dead embryos displaying a normal cuticle, indicating that the N-terminal region of Cyst is dispensable for most Cyst activity. As Cyst contains a putative aPKC phosphorylation site in its N-terminal region at S320 (Wang et al., 2012a), we asked whether aPKC phosphorylation of Cyst could potentially modify its activity. However, non-phosphorylatable and phosphomimetic isoforms of Cyst (GFP::CystRΔS320A and GFP::CystRΔS320E) fully rescued cyst shRNA embryos (Fig. 6). Taken together, our analysis shows that the RhoGEF activity and the coiled-coil domain (CC) containing C-terminal region of Cyst are essential for its function.

**Apical recruitment of Cyst requires physical interaction with the Crb complex and Baz/Par3**

Previous work indicated that mammalian p114RhoGEF forms complexes with Lulu2 (also known as EPB41L4B, one of two mammalian homologues of *Drosophila* Yrt), Patj, and Par3 (Nakajima and Tanoue, 2011). To probe for similar interactions among *Drosophila* proteins, we first asked whether the Crb complex, which includes Patj, and Baz are required for Cyst recruitment to the apico-lateral cortex. We injected GFP::Cyst-expressing embryos with double-stranded RNA (dsRNA) against *crb* or *baz*. In both cases, GFP::Cyst was lost from the cortex (Fig. 7 A).

To probe for physical interactions, we coexpressed various GFP-tagged fragments of Cyst with FLAG-tagged Baz or Patj (Baz::FLAG or Patj::FLAG) in HEK293T cells. We found that
GFP::Cyst and GFP::Cyst-C coimmunoprecipitated with Baz::FLAG or Patj::FLAG, with the interaction between Cyst-C and Patj appearing particularly robust (Fig. 7, B and D). Interestingly, GFP::CystΔPBM also formed Baz::FLAG- or Patj::FLAG-containing complexes, suggesting that the interaction between Cyst and Baz or Patj takes place in the C-terminal region of Cyst but does not require the Cyst PBM. To further assess whether Cyst and Baz can interact, we coexpressed FLAG-tagged versions of Cyst N, DH-PH, and C-terminal regions with GFP-tagged Par3 (GFP::Par3) in HeLa cells. We found that FLAG::Cyst-C and GFP::Par3 appeared to coaggregate in puncta (Fig. S2). These findings suggest that Drosophila Cyst undergoes molecular interactions with the Crb complex and Baz to support its apico-lateral localization. In contrast to findings in mammalian cells (Nakajima and Tanoue, 2011; Loie et al., 2015), we did not detect molecular interactions between Yrt and Cyst. This correlates with the observation that Yrt acts as a basolateral polarity protein in early Drosophila embryos and that the yrt mutant phenotype is different from the cyst phenotype (Laprise et al., 2006, 2009).

To further explore the function of the C-terminal region of Cyst, which is crucial for Cyst function, we coexpressed FLAG::Cyst-C with various GFP-tagged fragments of Cyst in HEK293T cells. We detected binding of GFP::Cyst, GFP::CystΔPBM, and GFP::Cyst-C to FLAG::Cyst-C (Fig. 7, C and D). These constructs all contain the Cyst CC, which facilitates oligomerization in some CC proteins (Schultz et al., 1998; Letunic et al., 2015). Taken together, our data support the view that Cyst is directed to the apico-lateral cortex through a multifaceted mechanism, potentially involving Cyst oligomerization, and redundant and/or parallel interactions between Cyst C-terminal region and Baz and the Crb complex.

Cyst targets Rho1
RhoGEF domains often show specificity for Rho, Rac, or Cdc42, although there are examples of promiscuity (McCormack et al., 2013; Ngok et al., 2014). Phylogenetic analysis suggests that Cyst is the single orthologue of a group of four mammalian RhoGEFs that target RhoA in cell culture. To ask whether Cyst targets Rho1 in vivo, we assayed the activity of two probes that are thought to preferentially bind to Rho1-GTP: Anillin-RBD::GFP (Munjal et al., 2015; Fig. 8, A and B) and PKNG58A::GFP (Simões et al., 2014; Fig. S3). The junctional localization of both probes was reduced by ~30–60% in cyst-compromised embryos compared with controls.

To further assess interactions between Cyst and Rho1, we asked whether Cyst activates Rho1 in cell culture. We coexpressed FLAG-tagged versions of Drosophila Rho1, Rac1, and Cdc42 along with the DH-PH domain of Cyst fused to GFP.
(Cyst-DH-PH::GFP) or N or C fragments (GFP::Cyst-N or GFP::Cyst-C) as controls in HEK293T cells (Fig. 8 C). Immunoprecipitation of the GTPases was followed by direct analysis of absolute levels of associated GTP and GDP by liquid chromatography/tandem mass spectrometry (LC-MS/MS). Coexpression of Cyst-DH-PH::GFP with Rho1 showed a twofold increase in Rho1-associated GTP as normalized to the levels seen with GFP::Cyst-N (Fig. 8 D). As a positive control, the DH-PH domain of LARG, a known mammalian RhoA-GEF (Cook et al., 2014), showed a level of Rho1 GTP loading comparable to that of Cyst-DH-PH::GFP (Fig. 8 E). These data are consistent with our observation that the expression of Cyst-DH-PH::GFP, but not GFP::Cyst-N or GFP::Cyst-C, produces dorsal ruffling and stress fiber formation in HeLa cells (Fig. S2), a phenotype reminiscent of RhoA activation in fibroblasts (Hall, 2012; Hanna and El-Sibai, 2013). We detected a 1.5-fold relative increase in GTP-associated Rac1. However, the level of Rac1 targeting by Cyst-DH-PH::GFP was less than that seen for an active version of the Rac-specific GEF STEFΔN (Matsuo et al., 2003), which acted as a positive control (Fig. 8, D and E). No detectable activation of Cdc42 was found.

Finally, we tested the ability of Cyst to suppress the effects of dominant-negative (DN) isoforms of Rho1 (Strutt et al., 1997), Rac1, and Cdc42 (Luo et al., 1994). Expression of any DN GTPases produced a prominent cuticle phenotype. This is presumably due to the sequestration of GEFs preventing activation of Rho, Rac, or Cdc42. We hypothesized therefore that overexpression of a GEF could rescue the effects of a DN GTPase if active levels of its target GTPase are restored. We coexpressed upstream activation sequence (UAS)-controlled CystR or the N-terminal region of Cyst (CystN-C) as a negative control, with DN versions of Rho1, Rac1, or Cdc42. Overexpression of CystN produced a cuticle that is intact, with the exception of a head defect (Fig. 1 F). We found that coexpression of CystN with DN-Rho1 partially rescued the DN-Rho1 cuticle defects (Fig. S3), whereas no rescue was observed with CystN-C. In contrast, expression of CystN did not ameliorate the phenotypes resulting from expression of the DN forms of Rac1 or Cdc42. Collectively, our genetic and biochemical data support the conclusion that Cyst acts on Rho1.

Cyst and Crb are required for normal myosin II enrichment at AJs

AJ stability is tightly connected to actomyosin dynamics (Lecuit and Yap, 2015). To ask whether Cyst plays a role in actomyosin dynamics at AJs, we examined the distribution of myosin II by following fluorescent protein–tagged regulatory light chain of nonmuscle myosin II (Spaghetti Squash; Royou et al., 1999) in live embryos derived from cyst RNAI Df(cyst)+ mothers (Fig. 9, A, B, and D). In the ectoderm, myosin II is recruited to the apico-lateral cortex during stages 6 and 7, just before the onset of germband extension. Defects in myosin II...
were noted from stage 7 onward, with junctional myosin levels reduced and less uniformly distributed around the apical cell perimeter. Interestingly, a similar reduction in junctional myosin was observed in \( \text{crb} \) RNAi embryos (Fig. 9, C and E). In contrast, medial myosin II levels behaved differently in Crb- and Cyst-depleted embryos as germband extension progressed. Whereas medial myosin II levels were enhanced in Crb-compromised embryos compared with controls, Cyst-depleted embryos showed a moderate reduction in medial myosin II (Fig. 9, D and E).

We also found that the loss of Cyst causes aberrant F-actin distribution and dynamics, as assayed by Utrophin::GFP (stage 11/12, Fig. S4 and Videos 9 and 10), including in small groups of cells that go on to form epithelial cysts. Moreover, high levels of F-actin were associated with apical protrusions, consistent with a loss of Rho activity and AJ integrity, which are known to limit protrusive activity mediated by Rac (Harris and Tepass, 2010). Taken together, these findings indicate that Cyst is required for the normal association of actomyosin with apical AJs and support a model positing that
Cyst couples the apical Crb complex to junctional Rho1 activity and AJ stability.

Discussion
Cyst links epithelial polarity, AJ stability, and actomyosin remodeling

Antagonistic interactions between apical and basolateral polarity regulators position AJs at the apico-lateral membrane to form a junctional complex. In turn, AJs are thought to maintain apical-basal polarity through the segregation of the apical and basolateral membrane domains, organization of the cytoskeleton, and direct polarity by acting as signaling centers for polarity complexes (Harris and Tepass, 2010; Laprise and Tepass, 2011; Harris, 2012; Tepass, 2012). Although a number of Drosophila RhoGEFs and RhoGAPs have been implicated in epithelial polarity and AJ stability (McCormack et al., 2013; Mack and Georgiou, 2014), no single RhoGEF or RhoGAP has been found...
to phenocopy the polarity or junctional defects that are seen in embryos compromised for factors such as Crb, aPKC, or E-cadherin (Tepaß and Knust, 1990; Tepass et al., 1996; Uemura et al., 1996; Hutterer et al., 2004). Our findings suggest that loss of the RhoGEF Cyst causes a polarity phenotype strikingly similar to the loss of core apical polarity proteins. Moreover, we find that Cyst is recruited to the apico-lateral cortex by the action of polarity proteins and, by activating Rho1, stabilizes AJ-associated actomyosin, which supports junctional and epithelial integrity.

In Cyst-compromised embryos, AJ formation is disrupted in early gastrulation, and AJs do not form a circumferential belt. These defects in AJ assembly or stability correlate with reduced and irregular myosin accumulation at the apico-lateral cortex. Given the molecular function of Cyst as a GEF for Rho1, loss of myosin activity is presumably the immediate cause for the defects in AJ formation and the subsequent loss of apicobasal polarity in many epithelial cells. crb-depleted embryos failed to recruit Cyst to apical junctions and showed a similar decline in junctional myosin. Therefore, a major function of the apical Crb polarity complex appears to be the Cyst-mediated support of junctional actomyosin (Fig. 10).

While many cells in crb or cyst mutants undergo programmed cell death, others retain or recover polarity and form small epithelial cysts, a process seen from mid-embryogenesis (post-gastrulation stages) onward. Several polarity proteins such as Crb, Sdt, and Baz are needed for normal epithelial polarization in early embryos but are not essential for polarization in post-gastrulation embryos, which explains the ability of some epithelial cells in these mutants to form epithelial cysts with normal polarization (Bilder et al., 2003; Tanentzapf and Tepass, 2003; Laprise et al., 2009). In fact, when programmed cell death is suppressed, Cyst formation is shown by all epithelial cells in crb mutants (Tanentzapf and Tepass, 2003). Formation of epithelial cysts seen in cyst mutant embryos therefore suggests that Cyst is also not essential for epithelial polarity in late embryos. This view is supported by the decline of Cyst protein accumulation at AJs seen in late embryos.

Several observations, including the genetic interaction of cyst with genes encoding basolateral polarity proteins, the dependence of the junctional localization of Cyst on the apical polarity proteins Baz and Crb, the physical interactions between Cyst and apical polarity proteins, and the function of Cyst in stabilizing AJs, indicate that Cyst is an integral part of the apical polarity machinery in early Drosophila embryos. A particularly striking finding was the complete suppression of the cyst phenotype by codepletion of the basolateral polarity proteins Scrib or lgl, seen in double-mutant embryos that showed phenotypes indistinguishable from single scrib or lgl mutants. This mimics previous observations with double mutants of crb or sdt and scrib, lgl or discs large (Bilder et al., 2003; Tanentzapf and Tepass, 2003). Moreover, we found that a reduction of aPKC enhanced Baz mislocalization in Cyst-compromised embryos, suggesting that aPKC cooperates with Cyst and acts upstream or in parallel to Cyst to organize Baz. These findings emphasize that Cyst, similar to Crb and aPKC, is a component of a negative feedback circuit between apical and basolateral regulatory networks that govern epithelial polarity. The dependence of Cyst localization on Crb and Baz suggests that Cyst acts downstream of these two proteins. Once polarized, Cyst appears to maintain polarity and junctional stability through actomyosin remodeling.

**Figure 10. Model of Cyst function in the Drosophila embryo.** The Crb complex and Baz recruit the RhoGEF Cyst to the apicolateral membrane where it activates Rho1 and myosin II, supporting junctional and epithelial integrity.
The GEF activity of Cyst targets Rho1

We found that Cyst becomes enriched at the apico-lateral cortex after the mesoderm and endoderm have invaginated and the germband starts to elongate. This localization coincides with the assembly of the apical-cortical actomyosin network. Rho–Rho kinase signaling plays a critical role in the activation of myosin II in this process (Lecuit and Lenne, 2007; Amano et al., 2010; Harris and Tepass, 2010; Lecuit and Yap, 2015). Our structure–function analysis showed that Cyst contains an essential RhoGEF domain as predicted, and the use of Rho activity probes, genetic interactions, and biochemical assays showed that Cyst preferentially targets Rho1. Although our biochemical assay also revealed stimulation of Rac1 activity by Cyst, all other data point to Rho1 as the primary target of Cyst. We propose therefore that Cyst activates Rho1 to organize actomyosin at the cortex at a time when AJs assemble into a circumferential belt (stages 6/7). Consistent with this, we found that Cyst is important for maintaining normal cortical levels of myosin II. A similar loss in junctional myosin was also observed in Crb-compromised embryos in line with our finding that Crb is required for Cyst junctional recruitment. The cyst mutant phenotype suggests that Cyst is the key RhoGEF that activates Rho1 at apical AJs. In contrast, RhoGEF2 functions in the mesoderm and ectoderm, where it becomes apico-cortically enriched and activates Rho1 to recruit myosin II to the apical-medial cortex (Padash Barmchi et al., 2005; Manning and Rogers, 2014; Kerridge et al., 2016; de Las Bayonas et al., 2019). Thus, RhoGEF and Cyst act in parallel on Rho1 to orchestrate the balance of cortical and medial actomyosin dynamics.

Cyst and its mammalian orthologue p114RhoGEF share a conserved function

Cyst is the single orthologue of a group of four mammalian RhoGEFs that target RhoA in cell culture (Cook et al., 2014). One of the mammalian orthologues (p114RhoGEF) stabilizes tight junctions and AJs through organization of the actin cytoskeleton associated with cellular junctions (Nakajima and Tanoue, 2010, 2011; Terry et al., 2011; Acharya et al., 2018). p114RhoGEF is recruited to apical junctions through a mechanism involving CRB3A, Ehm2/Lulu2, Par3, Patj (Nakajima and Tanoue, 2011; Loie et al., 2015), the heterotrimeric G protein Gα12, and the GPCR Sphingosine-1 phosphate receptor 2 (Acharya et al., 2018). p114RhoGEF requires the polarity regulator Ehm2/Lulu2 (a homologue of Drosophila Yrt) to activate RhoA (Nakajima and Tanoue, 2010, 2011). In contrast, we did not detect genetic or biochemical interactions between Cyst and Yrt in Drosophila. Recently, ARHGEF18, the human orthologue of p114RhoGEF, was identified as a gene associated with retinal degeneration (Arno et al., 2017), and a fish orthologue is required to maintain epithelial integrity of the retina (Herder et al., 2013). ARHGEF18 mutant retinal defects closely resemble those found in patients carrying mutations in the crb homologue CRBI (Arno et al., 2017). We conclude that the function of Cyst and p114RhoGEF/ARHGEF18 in coupling apical polarity proteins and GPCR signaling to junctional Rho activity and actomyosin function is conserved between flies and vertebrates and likely contributes to retinal health in humans, although some of the molecular interactions may have shifted in relative importance.

The other mammalian orthologues of Cyst, p90RhoGEF, AKAP-13, and GEF-H1 have not been implicated as regulators of epithelial polarity (Cook et al., 2014). GEF-H1 (also known as ARHGEF2 and Lfc) was shown to be inactive at mature tight junctions (Aljaz et al., 2005; Terry et al., 2011). In this case, the tight junction protein Cingulin forms a complex with GEF-H1, preventing it from activating RhoA (Aljaz et al., 2005; Terry et al., 2011). Instead, GEF-H1 is thought to promote junction disassembly and cell proliferation, presumably through an association with the mitotic spindle (Ren et al., 1998; Aljaz et al., 2005; Samarín et al., 2007; Birkenfeld et al., 2008; Terry et al., 2011; Cullis et al., 2014). GEF-H1 was also implicated in the morphogenesis of the vertebrate neural tube (Itoh et al., 2014), and in the regulation of RhoA activity during cytokinesis (Birkenfeld et al., 2007). Like GEF-H1, p90RhoGEF has been shown to associate with microtubules (Birkenfeld et al., 2008). GEF-H1 and AKAP-13 were also found to serve additional functions independent of their RhoGEF activity (Shibolet et al., 2007; Cullis et al., 2014). Whether and how Cyst might consolidate the functions of its various mammalian orthologues remains to be explored.

Materials and methods

Drosophila genetics

Flies were raised on standard media at 25°C. Cyst was depleted in the germline of females carrying mat-GAL4 (F[mata4-GAL-VP16]67; F[mata4-GAL-VP16]15; Häcker and Perrimon, 1998) and cyst RNAi (Valium20-SHO0146.N-40 inserted at attP40 on the second chromosome; Ni et al., 2011; Transgenic RNAi Project [TRIP]). Virgin females were crossed to males carrying cyst RNAi to produce cyst RNAi embryos. A second insertion of the same cyst shRNA (Valium22-SHO0146.N2) gave similar results.

The following fly lines were used: cyst FRT40A/CyO (this work); cyst RNAi (Valium20-SHO0146.N-40, Bloomington Drosophila Stock Center [BDSC] #38292); D(fly) (Df[2L];BS3C01, BDSC #23684); D(fly) (Df[2L];BS3C01) cyst RNAi (Valium22-SHO0146.N2, BDSC #41578); scrib RNAi (Valium20-SHO2077.N BDSC #35748); y w, lg4 FRT40A (gift from T. Xu, Yale University, New Haven, CT); y w, lg4 cyst FRT40A; aPKC(D)O26403 (gift of C. Doe, University of Oregon, Eugene, OR), mCherry shRNA (BDSC #35785); UAS-PKNS58A::Venus (Simões et al., 2014); UAS-Anillin-RBD::GFP (Munjal et al., 2015); and histone::GFP (gift of A. Wilde, University of Toronto, Toronto, ON, Canada).

shg–DeCad::GFP (DeCad::GFP controlled by its endogenous promoter; Huang et al., 2009) and sqh–sqh::GFP (Sqh controlled by its endogenous promoter; Royou et al., 1999) were recombined with the mat-GAL4 driver. Utrophin::GFP cyst RNAi males were crossed to the mat-GAL4 driver, and then crossed to cyst RNAi males to generate cyst RNAi embryos expressing Utrophin::GFP (eGFP fused to the actin binding domain of human Utrophin; Rauzi et al., 2010). Cyst overexpression UAS constructs were driven by da-GAL4 (Wodarz et al., 1995) or mat-GAL4. To examine genetic interactions between cyst and Rho GTPases, we crossed females expressing UAS-controlled Cyst or...
Cyst-N with the mat-GAL4 driver to P{UAS-Rho1.N19}1.3 (Strutt et al., 1997), P{UAS-Rac1.L89.6}, or P{UAS-Cdc42.N17} (Luo et al., 1994) males.

**Generation of the cyst^R** mutation and mutant germline clones

We generated a null mutation for cyst (cyst^R) using the RNA-guided CRISPR/Cas9 system (Gratz et al., 2013; GenetiVision Corp.). The second exon of cyst was targeted using gRNA1 (5’-GGTAGCAATACTAATCGCA-3’) and gRNA2 (5’-AGCTCTCTGAGCCAAGCCGC-3’) and replaced with a 3xP3-GFP cassette. Sequencing confirmed the following breakpoints: 19503155 and 19506410. To generate germline clones for cyst^R or lgl^4 cyst^R mutations, we crossed cyst^R FRT40A/CyO or w; lgl^4 cyst^R FRT40A/CyO to Ovo^lo males (y[w] P{ry[t+7.2]-hsFLP}12; 2212P[w+mc] =ovoD1-18)2La P[w+mc] =ovoD1-18)2Lb P{ry[t+7.2]=neoFRT}40A). Ovo^lo males resulted from a cross of BDSC stocks #1929 and #2121. FLP/FRT-mediated mitotic recombination in resulting females was induced by two heat shocks in a 37°C water bath for 1 h at late second and late third larval instar (Chou and Perrimon, 1992). Females were outcrossed to heterozygous cyst^R or lgl^4 cyst^R mutant males, respectively, and eggs were collected for analysis.

**Preparation of cuticle**

To prepare the cuticle of fully differentiated embryos (Wieschaus and Nüsslein-Volhard, 1986), embryos were aged for 36–48 h after egg collection at 25°C, washed, and dechorionated in a 2% bleach solution for 5 min. After washing with double-distilled H2O, eggs were transferred onto a slide into a 1:1 mixture of Hoyer’s medium and lactic acid, covered with a coverslip, and incubated overnight at 85°C. Images were taken with a Carl Zeiss Axiophot2 microscope using a phase-contrast 20× lens (NA 0.5). Pictures were recorded with a Canon Rebel XSi camera using Canon software and processed in Adobe Photoshop and Illustrator.

**Molecular biology**

**Genomic rescue constructs**

An ∼70-kb fragment (genomic region 19509164–19502181) encompassing the cyst gene was amplified from BACR27M12 (BACPAC) and recombined into pENTR221 (Invitrogen). To confer RNAi resistance on the resulting Entry Clone, silent mutations were introduced at two distinct sites corresponding to OvoD males resulted from a cross of BDSC stocks #1929 and #2121. FLP/FRT-mediated mitotic recombination in resulting females was induced by two heat shocks in a 37°C water bath for 1 h at late second and late third larval instar (Chou and Perrimon, 1992). Females were outcrossed to heterozygous cyst^R or lgl^4 cyst^R mutant males, respectively, and eggs were collected for analysis.

**Cell culture and immunoprecipitation LC-MS/MS**

HEK293T and HeLa cells were cultured in DMEM (Fujifilm Wako Chemicals) with 10% FBS (Thermo Fisher Scientific), penicillin, and streptomycin, and transfection was performed using polyethyleneimine (Polysciences Warrington). Cells were transfected in 10-cm Petri dishes and incubated with serum-free DMEM for 4 h before processing.

For the LC-MS/MS assay, HEK293T cells were cotransfected with FLAG-tagged versions of Drosophila Rho1, Rac1, or Cdc42 and either the GFP-tagged Cyst fragments shown in Fig. 8 C–E or GFP alone. For coimmunoprecipitation experiments (Fig. 7, B–D), cells were cotransfected with GFP-tagged Cyst fragments and FLAG:Cyst-C (as described above) or FLAG-tagged Drosophila Baz or Patj. Cells were rinsed once with ice-cold PBS and extracted with ice-cold lysis buffer B (10 mM Tris/HCl, pH 7.5, 300 mM NaCl, 10 mM MgCl2, 1%...
Embryos were heat-fixed (Tepass, 1996) or fixed for Drosophila Antibody staining to that of the Cyst N-terminal fragment or GFP controls. Error GTPase protein in each sample. Values were further normalized × 100, which normalizes for the amount of immunoprecipitated of GTP-bound small GTPases was expressed as GTP/(GTP + GDP) addition source in the positive ion mode. The MRM transitions of m/z 524→152.1 and m/z 444.1→152.1 were used to quantify GTP and GDP, respectively. Sample concentrations were calculated from the standard curve obtained from serial dilution of each nucleotide standard (Sigma-Aldrich, Canada). Analytical conditions were optimized using standard solutions. The percentage of GDP, respectively. Sample concentrations were calculated from the standard curve obtained from serial dilution of each nucleotide standard (Sigma-Aldrich, Canada). Analytical conditions were optimized using standard solutions. The percentage of GTP-bound small GTPases was expressed as GTP/(GTP + GDP) × 100, which normalizes for the amount of immunoprecipitated GTPase protein in each sample. Values were further normalized to that of the Cyst N-terminal fragment or GFP controls. Error bars represent the SD of four independent experiments.

Antibody staining

Drosophila embryos were heat-fixed (Tepass, 1996) or fixed for 20 min in 3.7% formaldehyde diluted in a 1:1 PBS:heptane mixture. To visualize the PKNG58A::Venus sensor, embryos were fixed in a 1:1 mixture of 3.7% formaldehyde and phosphate buffer, pH 7.4, and heptane for 40 min under agitation followed by hand devitellinization. Primary antibodies used were anti-Crb (rat polyclonal, extracellular F3; 1:1,000; Pellikka et al., 2002), anti-Yrt (guinea pig polyclonal, GP; 1:500; Laprise et al., 2006), anti-Arm (mouse monoclonal, N2-7A1; 1:50; Developmental Studies Hybridoma Bank), anti-HA (rat monoclonal, 3F10; 1:500; Roche), anti-Baz (1:5,000), anti-Baz (GP, 1:500, a gift from Jennifer Zallen, Memorial Sloan Kettering Cancer Center, New York, NY), anti-PKCζ (C-20; 1:100; rabbit polyclonal; Santa Cruz), and anti-Dlg (1:100; mouse; Developmental Studies Hybridoma Bank).

Transfected HeLa cells were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature, followed by treatment with 0.2% Triton X-100 in PBS with 2 mg/ml BSA for 10 min, and processed for immunostaining. Primary and secondary antibodies used were chicken anti-GFP (Abcam), mouse anti-FLAG M2 (Sigma-Aldrich),), Alexa Fluor 488 goat anti-chicken IgY, and Alexa Fluor 555 goat anti-mouse IgG (Thermo Fisher Scientific). F-actin was visualized with Alexa Fluor 555 phalloidin (Thermo Fisher Scientific). Samples were mounted in 50% glycerol in PBS.

Imaging and signal intensity quantification

Except for Fig. 4, imaging of live or fixed samples was done with a Leica TCS SP8 scanning confocal microscopy with 40×, 63×, and 100× objectives (HC PL APO CS2 with NAs of 1.30, 1.40, and 1.40 respectively). Time-lapse acquisitions were done in a manner similar to that previously described (Blankenship et al., 2006). Three to five live embryos were examined for each genotype. A 0.5-µm step was used to collect z-stacks. Stills and videos were assembled from maximum-intensity projections of six apical planes (ImageJ; National Institutes of Health). Supplemen- tal videos shown were downsampled in iMovie (Apple). Original videos are available upon request. Adobe Photoshop and Adobe Illustrator were used to process and arrange images. The same settings were applied to all images within an experimen- tal series. The average fluorescence intensity of junctional and medial Sqh::GFP, junctional Anillin-RBD::GFP, and PKNG58A::Venus was quantified in segmented cells using Matlab and the script SIESTA (scientific image segmentation and analysis; Fernandez-Gonzalez and Zallen, 2011). To quantify junctional Sqh::GFP, Anillin-RBD::GFP, and PKNG58A::Venus, we manually drew 3-pixel-wide lines (180 nm/pixel) for cell edges at the indicated time points to obtain the mean pixel intensity for a cell edge. The mode (cytoplasmic) intensity was subtracted for background correction. To quantify medial Sqh::GFP mean protein levels, each cell was divided into two com- partments (Fernandez-Gonzalez and Zallen, 2011). The junc- tional compartment was determined by a 3-pixel-wide (0.54 µm) dilation of the cell outline identified using watershed or LiveWire segmentation in SIESTA. The medial compartment was obtained by inverting a binary image representing the junctional compartment. Given the dynamic nature of medial Sqh::GFP, we quantified it as the mean pixel intensity in the medial compartment in 10 consecutive time frames per cell (total elapsed time of 5 min per data point), centered at the indicated time points, and subtracted the mode (cytoplasmic) intensity for background correction.

For data presented in Fig. 4, we used a spinning disk confocal microscope (Quorum Technologies) with a 63× Plan Apochromat objective (NA 1.4; Carl Zeiss), piezo top plate, and electron multiplier charge-coupled device camera (Hamamatsu Photonics). Baz puncta were quantified by dividing the number of cells with two Baz puncta by the total number of cells. aPKC levels were the average difference between the cell cortical and cytoplasmic signals of five different cells per embryo, normalized to internal GFP-expressing controls. Cytochalasin D and DMSO were diluted 2,000 times in an NaCl-octane solution before embryo incubations. To quantify apical aPKC levels, the apico- lateral section with the strongest apical signal was selected for each embryo. Within each embryo section, regions of interest were selected for the cell cortex and for the neighboring cytoplasm of five different cells. The cytoplasmic values were subtracted from the cortical values for background correction, and the five corrected values were averaged to produce one quantification of apical aPKC level per embryo. These quantifications were corrected values were averaged to produce one quantification from the cortical values for background correction, and the five corrected values were averaged to produce one quantification of apical aPKC level per embryo. These quantifications were...
normalized to the average of the quantifications for control embryos. Controls were costained and comounted for Fig. 4 D and were treated, stained, and mounted in parallel preparations for Fig. 4 F.

Data shown in Fig. S2 were acquired with a LSM700 laser scanning confocal microscope (Carl Zeiss) with a 40× C-Apochromat water-immersion objective (NA 1.2). Images were obtained using ZEN2009 software (Carl Zeiss) and processed with Adobe Photoshop.

Statistics
Statistical analysis was performed in Microsoft Excel or Prism v7 (GraphPad). We used Student’s t test for pairwise comparisons in Figs. 4 and 8 D, with data presented as mean ± SD. We used the nonparametric Kolmogorov–Smirnov test for Figs. 8 B, 9 (D and E), and S3 B, with data presented as mean ± SEM.

Online supplemental material
Figure S1 shows aPKC distribution in cyst RNAi embryos. Figure S2 shows Cyst coaggregates with Par3 and elicits formation of ruffles and stress fibers. Figure S3 shows interactions between Cyst and Rho1. Figure S4 shows that Cyst regulates actin dynamics. Video 1 shows DEcada::GFP in control embryo. Video 2 shows DEcada::GFP in cyst RNAi embryo. Video 3 shows AnillinRBDB::GFP biosensor expression in a control embryo. Video 4 shows AnillinRBDB::GFP biosensor expression in a cyst RNAi embryo. Video 5 shows myosin II dynamics in a mock-RNAi control embryo. Video 6 shows myosin II dynamics in a cyst RNAi embryo. Video 7 shows myosin II dynamics in a control RNAi embryo. Video 8 shows myosin II dynamics in a crb RNAi embryo. Video 9 shows actin dynamics in control embryo. Video 10 shows actin dynamics in a cyst RNAi embryo.

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