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The Drosophila CD2AP/CIN85 orthologue Cindr regulates junctions and cytoskeleton dynamics during tissue patterning

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D eveloping tissues require cells to undergo intricate processes to shift into appropriate niches. This requires a functional connection between adhesion-mediating events at the cell surface and a cytoskeletal reorganization to permit directed movement. A small number of proteins are proposed to link these processes. Here, we identify one candidate, Cindr, the sole Drosophila melanogaster member of the CD2AP/CIN85 family (this family has been previously implicated in a variety of processes). Using D. melanogaster retina, we demonstrate that Cindr links cell surface junctions (E-cadherin) and adhesion (Roughest) with multiple components of the actin cytoskeleton. Reducing cindr activity leads to defects in local cell movement and, consequently, tissue patterning and cell death. Cindr activity is required for normal localization of Drosophila E-cadherin and Roughest, and we show additional physical and functional links to multiple components of the actin cytoskeleton, including the actin-capping proteins capping protein alpha and capping protein beta. Together, these data demonstrate that Cindr is involved in dynamic cell rearrangement in an emerging epithelium.

Introduction

By recruiting proteins into complexes, adaptor proteins create nodes of regulation and activity. The founding member of the CD2AP/CIN85 family was initially isolated in a yeast interaction screen as a binding partner of the T cell receptor CD2 (Dustin et al., 1998), independently from the kidney (MET-1; Lehtonen et al., 2000) and as a ligand for p130Cas (Kirsch et al., 1999). The homologue CIN85 was identified as a partner of the E3 ubiquitin ligase Cbl (Take et al., 2000) and separately as SETA and Ruk (Bogler et al., 2000; Gout et al., 2000). Many roles have been ascribed to the CD2AP/CIN85 family but its function in situ remains poorly understood. Here, we examine the sole Drosophila melanogaster CD2AP/CIN85 orthologue, cindr.

The phenotype of CD2AP knockout mice is chiefly one of tissue degeneration: cardiac hypertrophy, splenic and thymic atrophy, glomerular sclerosis, and a loss of podocyte foot processes (Shih et al., 1999). The CD2AP/CIN85 family is primarily proposed to function in endocytosis to down-regulate receptor tyrosine kinase activity (Dikic, 2003). This model arises from coimmunoprecipitation and interaction experiments that have identified a wealth of CD2AP/CIN85 interactors, colocalization studies performed in culture or tissue, and in vitro assays. CIN85 constitutively associates with endophilin and, on growth factor stimulation, complexes with Cbl to mediate receptor down-regulation. Furthermore, interactions between CD2AP/CIN85 and other trafficking proteins have been described including AP-2, Dab2, Rab4, PAK2, ALIX, and ESCRT-1 (Chen et al., 2000; Brett et al., 2002; Cormont et al., 2003; Kowanetz et al., 2003; Schmidt et al., 2003; Schiwek et al., 2004; Gaidos et al., 2007; Gauthier et al., 2007). CD2AP has also been reported to bind the actin-capping protein CPα/β.
dimer and inhibit its function in vitro (Hutchings et al., 2003; Bruck et al., 2006) and anillin at the actin-rich cleavage furrow (Monzo et al., 2005). CD2AP activity is required for migration of rat gastric mucosal cells (Mustonen et al., 2005) and polarization of the cytoskeleton during T cell receptor activation (Dustin et al., 1998). The role and mechanism by which CD2AP/CIN85 regulates cytoskeletal dynamics within an epithelium in situ remains unclear.

Furthermore, the CD2AP/CIN85 family has also been reported to bind the adhesion molecules E-cadherin and nephrin (Shih et al., 1999, 2001; Palmen et al., 2002; Lehtonen et al., 2004; Mustonen et al., 2005). Nephrin and NEPH-1 form the backbone of the slit diaphragm, a specialized junction that traverses podocyte foot processes in the mammalian kidney (Huber and Benzing, 2005; Patari-Sampo et al., 2006). Direct interactions between CD2AP, nephrin, and podocin and between CD2AP and the podocyte-specific actin-bundling protein synaptopodin are essential for slit diaphragm integrity. In addition, a protein complex containing nephrin, cadherin, p120-catenin, ZO-1, and CD2AP has been isolated from Madin-Darby canine kidney cells and mouse glomerular lysates (Lehtonen et al., 2004). Collectively, these data suggest that CD2AP may have a role in anchoring junctions to the cytoskeleton or in regulating actin dynamics at this important intersection.

The challenge remains to understand how different roles of CD2AP/CIN85 are integrated in the organism, which interactors are recruited into CD2AP/CIN85 complexes, and how these are regulated. Here, we show that targeted reduction of cindr in the pupal fly eye resulted in defects in overall patterning due to aberrant local cell movements. We link these defects to misregulation of actin dynamics and mislocalization of Drosophila E-Cadherin (DE-Cad) and the fly NEPH-1 orthologue Roughest (Rst) which, with its binding partner Hibris (Hbs; a D. melanogaster Nephrin orthologue), is a central mediator of cell-cell adhesion in the pupal retina. We link Cindr functionally and physically to orthologues of capping protein alpha (Cpa) and capping protein beta (Cpb) and further explore the role of Cindr in modulating the actin cytoskeleton during eye maturation. Our data support a primary role for cindr in linking junction and actin regulation and help account for many of the phenotypes ascribed to mutations in mammalian CD2AP/CIN85.

Results

Expression and localization of cindr

The developing D. melanogaster pupal eye is one of the most thoroughly characterized developing epitheliums, and it provides a useful model to explore the role of CD2AP/CIN85 proteins. Emergence of the pupal eye is shown in Fig. 1 (A–D). Ommatidia emerge in the larval eye as photoreceptor neurons and (glial-like) “cone cells” form the ommatidial core. Between the ommatidia lies an initially undifferentiated pool of “inter-ommatidial precursor cells” (IPCs). From the IPCs emerge 1°, 2°, and 3° (primary, secondary, and tertiary) pigment cells, which are support cells that will eventually optically insulate each ommatidium. These pigment cells find their niche through cell rearrangements, and we focus on their movements in this work.

During early pupariation, each ommatidial core is enveloped by two 1° pigment cells. Shortly afterward, 2° and 3° pigment cells, along with sensory bristles, rearrange into an interweaving hexagonal lattice that organizes the ommatidia into a precise honeycomb (Fig. 1). These patterning events occur 18–42 h after pupariation formation (APF).

These precise movements require the junction-associated protein DE-Cad and the adhesion protein Rst, raising the question as to how these surface proteins can influence cell movements. In our search for new factors that regulate this patterning process, we identified CG31012 in silico as the single D. melanogaster orthologue of vertebrate CD2AP and CIN85 and christened the locus cindr (CIN85 and CD2AP orthologue). The annotation of this locus predicts four isoforms (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200706108/DC1). We identified at least two isoforms using a rabbit polyclonal antibody generated against the common central region. Antibody specificity was confirmed by Western analysis of a deficiency that overlaps cindr (Fig. S1 B) and by detection of ectopic or reduced Cindr levels in the third larval instar wing disc and pupal eye (Figs. S2 and S3).

In tissue, Cindr transcript and protein was expressed ubiquitously at all stages examined (Fig. 1, E and F). The protein itself was found primarily at the apical surface and in vesicles near the surface. For example, undifferentiated embryonic and larval tissue contained puncta of Cindr in close association with the adherens junctions (AJs; Fig. S2 and not depicted). As cells emerged from the IPC pool, the concentration of puncta increased in the emerging 2° and 3° cells 18–41 h APF but remained low in emerging 1° cells (Figs. 1 E, S3 C, and S5, B–H’), available at http://www.jcb.org/cgi/content/full/jcb.200706108/DC1). Cindr levels were high in the photoreceptor neurons and cone cells as well as the sensory bristles (Figs. 1 and S3). Low levels were also observed near the fenestrated basement membrane at the base of the retina (Fig. S3 E).

Reducing Cindr activity leads to junctional instability

The available D. melanogaster cindr P insertion–mediated alleles did not sufficiently perturb the locus to generate significant phenotypes. We therefore targeted the locus using RNAi. We generated multiple transgenic fly lines with inverted repeat (IR) transgenes (Fig. S2 A) that specifically targeted the long (PC, cindr-IR3), long plus medium (PC and PD, cindr-IR4), or all three transcripts (cindr-IR5) when expressed using the GAL4/UAS system (Brand and Perrimon, 1993). Based on quantitative real-time PCR (qrt-PCR; Table I), immunocytochemistry (Fig. S2), and phenotype (see Fig. 1, I–M), cindr-IR4 was the most effective at reducing transcript and protein levels. Ubiquitous expression of cindr-IR5 and cindr-IR4 led to embryonic and early larval lethality, respectively; cindr-IR2 generated no observable adult phenotype.

When both cindr-IR3 and cindr-IR1 transgenes (glass multimer reporter [GMR] > cindr-IR2/+) were driven together in the eye for at least 24 h, virtually no Cindr was detected as the animal progressed to pupal stages (Fig. S3 B’). GMR > cindr-IR4/+ directed less decrease in Cindr protein during larval development
and no developmental defects were observed, which suggests that the protein can show significance perdurance (unpublished data). Therefore, we cannot rule out a role for Cindr during the larval stages of eye development.

In wild-type pupal eyes, the process of patterning IPCs into a precise arrangement of 1°, 2°, and 3° pigment cells is mostly complete by 28 h APF (Fig. 1 B). This process was delayed or lost when cindr activity was reduced in GMR>cindr-IR2 and GMR>cindr-IR3 eyes; GMR>cindr-IR1 eyes exhibited a lower level of defects (Fig. 1, H–M). Cells were misplaced, pattern elements within the ommatidial field were misoriented, and the number of pigment cells was variable.

Quantitation of these defects at 41 h APF, when pigment cell patterning is completed, emphasized the broad nature of the patterning defects. For example, an additional one to two pigment cells surrounded 23% of GMR>cindr-IR2 ommatidia.
Table I. qrt-PCR analysis of relative reduction of cindr transcripts using RNAi

<table>
<thead>
<tr>
<th>Genotype</th>
<th>B</th>
<th>D</th>
<th>K</th>
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<tbody>
<tr>
<td>da&gt;cindr-IR1</td>
<td>d</td>
<td>0.45 (0.05)</td>
<td>0.71 (0.04)</td>
</tr>
<tr>
<td>da&gt;cindr-IR2</td>
<td>0.31 (0.15)</td>
<td>Not done2</td>
<td>Not done</td>
</tr>
<tr>
<td>da&gt;cindr-IR3</td>
<td>0.76 (0.25)</td>
<td>Not done2</td>
<td>0.68 (0.31)</td>
</tr>
</tbody>
</table>

aCompared to control cDNA isolated from da>GFP tissue.
bn = 3 per two independent cDNA preparations.
cda, daughterless-GAL4.
dPrimer set recognizes cindr-IR transgene.

(n = 159) and 46% of GMR>cindr-IR2 ommatidia; 16% of GMR>cindr-IR1 ommatidia were surrounded by an additional three to five cells (n = 204). The most posterior fifth of GMR>cindr-IR2 retinas showed still more severe defects: 81% of ommatidia had a shortfall of one to five surrounding IPCs (n = 86; compare Figs. 1 L and 5 A). To further quantitate these phenotypes, we developed a mean “ommatidial mispattering score” (OMS) system that accounted for errors in patterning, cell number, and rotation (Fig. 2). Focusing on ommatidia at the center of the eye for consistency, we found that reducing cindr activity (GMR>cindr-IR2+) led to defects in each of these aspects (Fig. 2). Control GMR>lacZ eyes were scored with an OMS of 0.9 errors per ommatidial cluster (n = 75); the OMS for cindr-IR2+1 ommatidia was 9.7 errors per ommatidium (n = 75) and the OMS for cindr-IR2 was 6.4 (n = 1,310). This analysis highlights the requirement by retinal cells for normal cindr activity during their maturation into a functional neuroepithelium.

Reducing cindr activity led to instability of cells within the pupal eye

Our analysis with fixed tissue indicated a requirement for Cindr as pigment cells moved into their proper niches. However, the patterning process in the pupal eye is rapid, progressive, and highly dynamic. Unlike cell culture, exploring the role of factors such as Cindr during epithelial maturation is difficult to achieve in situ, especially dynamically and with subcellular resolution. For example, the cindr patterning defects we observed could be accounted for by cell movements that are reduced, increased, unstable, or misdirected. An important advantage to using the fly eye as a model for exploring Cindr function is the recent establishment of live imaging to observe events in situ (Larson et al., 2008), and we took advantage of this technology to answer several questions about the role of Cindr in the patterning process. We used fluorescently labeled α-catenin–GFP, a protein associated with DE-Cad, to visualize junctions in situ.

As expected, in live imaging movies of control eyes, we observed two emerging 1° cells enwrapping each ommatidium as each 1° cell formed a stable junction with its 1° partner. The result was a stereotypical “collar” of two 1° cells surrounding each ommatidium’s central cone cell quartet (Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200706108/DC1).

Initial recruitment of 1° cells in GMR>cindr-IR2+1 eyes commenced normally and many, though not all, fledgling 1° cells successfully encircled a central cone cell quartet (Video 2). However, these emerging 1° cells commonly failed to maintain their niches: 1° cells often lost contact with their partners, a loss typically coupled with dissolution of their shared junction. As a result, one proto-1° cell was often replaced by another cell to generate a new 1° cell. Remarkably, this instability of the 1°–1° interface continued throughout the stages of IPC patterning, hours after 1° cells have normally completed their movements (Fig. 3, A and B; and Video 3). Similar junctional instability and 1° cell replacements were rarely observed in control eyes. These results are consistent with a model in which loss of cindr activity permits cell movement but prevents cells from maintaining their niche through stable junctions. We note that cells that are not undergoing cell movements, such as cone cells, do not show similarly destabilized junctions.

A failure to maintain proper position and stable junctions was also observed in the emerging arrays of 2° and 3° pigment cells. The movement of the IPCs in these older cindr-IR2+1–expressing retinas was similarly stochastic: excessive switching of cell–cell contacts, severe alterations in apical cell profiles, and a failure of IPCs to stably rearrange stepwise into single file (Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200706108/DC1). The result was a failure to achieve coherent patterning. In contrast, in control eyes, IPCs moved smoothly and with small local movements to gradually establish the hexagonal array of 2° and 3° cells (Video 4). These abnormal cell movements and contacts were again suggestive of unstable and transient junctions in cindr-IR2+1 retinas and also indicate a failure of proper morphogenesis.

We observed further evidence of unstable junctions by examining small clonal patches of cells expressing cindr-IR2+1. Isolated cindr-IR2+1 1° cells were consistently smaller than their wild-type neighbors (Fig. 3, C, D, and G). Paired cindr-IR2+1 1° cells frequently retracted, permitting a neighboring wild-type IPC direct contact with the central cone cells (Fig. 3, E–G) and often leading to an ectopic third 1° cell.

Cindr is functionally linked to DE-Cad

Our results indicate a role for Cindr in mediating surface events. A subset of Cindr protein was found associated with DE-Cad (Figs. 1 E, S3, and S5), a central component of the AJ, and loss of cindr activity led to unstable junctions and abnormal cell movements. What is the relationship between Cindr and DE-Cad? Modifying a phenotype by removing one genomic copy of a gene is a standard and powerful technique to demonstrate that two loci function together in a common process. DE-Cad is encoded by the shotgun (shg) locus: the GMR>cindr-IR2 mispatterning phenotype (OMS = 7.2) was enhanced in a heterozygous shgRa61a background (OMS = 10.8; Figs. 4 D and 2). In addition, large patches of black tissue were observed in the eyes of 68.3% (n = 97) of these adult flies (Fig. 4 G); this was a significant enhancement of the penetration and extent of degenerative tissue that was observed in GMR>cindr-IR2 alone (12.6%, n = 254; Fig. 4 E). This blackened eye phenotype is suggestive of late epithelial cell death.
restored after cell patterning is completed and cell movements end (Fig. 5 F). This progressive loss of DE-Cad between 2° and 3° cells was disrupted when cindr activity was decreased (GMR > cindr-IR2 and GMR > cindr-IR2+3): abnormally weakly stained and discontinuous DE-Cad was observed in all cell types and all time points examined (Figs. 1 I and 5, A, C, and E). Later, at 41 h APF, mildly discontinuous DE-Cad was still observed in over 1/2 the retina, most often in posterior regions corresponding with the most severe patterning defects and indicates that the relationship between Cindr and DE-Cad extends to later stages of development.

In wild-type pupal eye tissue, DE-Cad protein is found at high continuous levels between all apical cell profiles. An exception is between emerging 2° and 3° cells when the level of DE-Cad present at AJs decreases during their rearrangement into a hexagonal lattice (Fig. 5, B and D; Bao and Cagan, 2005). This decrease may represent a necessary step to allow for local cell rearrangements; higher levels of DE-Cad are normally restored after cell patterning is completed and cell movements end (Fig. 5 F’). This progressive loss of DE-Cad between 2° and 3° cells was disrupted when cindr activity was decreased (GMR>cindr-IR2 and GMR>cindr-IR2+3) abnormally weakly stained and discontinuous DE-Cad was observed in all cell types and all time points examined (Figs. 1 I and 5, A, C, and D). Later, at 41 h APF, mildly discontinuous DE-Cad was still observed in over ~25% of the retina, most often in posterior regions corresponding with the most severe patterning defects.
were localized normally when similarly expressed (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200706108/DC1, and not depicted). This suggests that Cindr’s SH3 domains are required specifically for membrane localization. Further consistent with this view, a complimentary N-terminal construct (Cindr-H9004PB) that includes the three SH3 domains was normally localized including to the AJs (Fig. S4 and not depicted).

Although this data suggests that Cindr may interact directly with DE-Cad through its SH3 domains, we failed to detect a Cindr–DE-Cad interaction in conditions that permitted binding to other proteins (see Materials and methods).

Two additional observations are notable regarding the relationship between Cindr and DE-Cad. First, in addition to rescuing the GMR>cindr 2 phenotype, ectopic DE-Cad accumulated at the apical membrane, which became elaborated (Fig. 4 B). The mammalian orthologue CIN85 has been implicated in regulating endocytosis of receptors such as those for EGF and PDGF (Dikic and Giordano, 2003). The membrane elaboration of ectopic DE-Cad may reflect a role for Cindr in its endocytosis; however, this model would not account for the discontinuous localization of DE-Cad was enhanced in a heterozygous shg background (Fig. 4 D and not depicted), most markedly at the “tricellular junctions” where three cells interface.

Our localization studies indicate that Cindr acts to both permit dynamic regulation of DE-Cad during cell movement and to stabilize its presence at the cell surface. If this view is correct, expressing ectopic DE-Cad should rescue cindr-IR patterning defects. Importantly, ectopic DE-Cad strongly rescued the GMR>cindr 2 mispatterning phenotype and reduced the cindr-IR 2 OMS from 7.2 to 5.6 (Figs. 2 and 4 B). This provides further evidence that a central role for Cindr in situ is to mediate events at the AJs.

Previous work reported that CD2AP can interact in vitro with E-cadherin via its SH3 domains (Lehtonen et al., 2004; Mustonen et al., 2005). We found that ubiquitous expression of a Cindr-PB construct, which retained only the C-terminal half of the full-length protein but lacked SH3 domains, was distributed in a striking perimembrane pattern but was apparently excluded from the AJ; full-length Cindr-PC or Cindr-PD isoforms
loss of endogenous DE-Cad at the surface when cindr activity was reduced (Fig. 5). Second, Western analysis did not detect any changes in overall levels of DE-Cad (or Rst, see following section) in GMR>cindr-IR2; pupal eyes (unpublished data), again supporting the view that CinDr acts primarily on DE-Cad’s localization and not its stability. Together, our data indicate that CinDr acts in situ in part by regulating dynamic junctional remodeling and DE-Cad function during a period of dynamic cell movement.

Cindr is functionally linked to the adhesion protein Rst
In addition to regulating AJs we found a functional link between CinDr and Rst, a transmembrane protein and NEPH-1 orthologue that mediates cell–cell adhesion during IPC patterning (Schneider et al., 1995). Rst acts with its binding partner Hbs to play a central role in directing the cell movements required for 2/3° patterning (Bao and Cagan, 2005). Initially expressed at all IPC–IPC boundaries (Fig. 5 B') by 22 h APF, Rst protein becomes expressed primarily in 2° and 3° cells as the 2/3° hexagonal lattice emerges (Reiter et al., 1996). Rst protein is localized predominantly to AJs and, to a greater extent than DE-Cad, becomes progressively capped to the interface between 2/3° cells and 1° cells (Fig. 5, D' and F''; and Fig. S3 C) due to its physical interaction with Hbs, which is expressed in 1° cells (Bao and Cagan, 2005).

Cindr protein colocalized with Rst at the AJ (Fig. S3 C). Reducing CinDr blocked the dynamic redistribution of Rst protein: In GMR>cindr-IR2 pupal eyes, Rst remained at steady diffuse levels at the junctions of most IPC–IPC contacts through the staging of hexagonal lattice emergence (Fig. 5, C' and E'). We also observed abnormally high levels of diffuse cytoplasmic Rst. Similar to shg, Western analysis indicated that Rst protein was present at normal levels and that reducing the gene dosage of rst or hbs did not restore proper Rst protein localization (unpublished data). This indicates that levels of Rst protein are not responsible for the observed cindr-IR defects in Rst localization.

Our genetic modifier data also supported a functional interaction between CinDr and Rst/Hbs. Pupae heterozygous for hbs459/ or rstCT exhibited mild but consistent suppression of the GMR>cindr-IR2 phenotype (Fig. 4, H, I, and J; and not depicted). The prevalence of cone and 1° cell defects was decreased (Fig. 2) and the number of 2° and 3° cells was reduced to slightly below normal.

Together, these data provide a functional link between CinDr and the surface proteins DE-Cad and Rst during the stages of dynamic AJ and adhesion remodeling and cell movement.
Cindr acts with regulators of the actin cytoskeleton

In the course of searching for binding partners for Cindr that are active in the eye, we identified components of the actin cytoskeleton. Of note, the patches of dead tissue we observed in eyes of GMR>cindr-IR2+3 adults (Fig. 4 E) phenocopied those reported for alleles of the actin-related loci cpa and cbp (Delalle et al., 2005). We also note a similarity between the twisted, thickened, short, and bent thoracic macrochaetae observed when cindr activity was reduced (see Fig. S1) and similar defects found for actin regulatory loci such as cbp (Hopmann et al., 1996; Hopmann and Miller, 2003; Frank et al., 2006).

CPα/CPβ heterodimers reversibly bind barbed, fast-growing ends of actin filaments to prevent further elongation or depolymerization (Isenberg et al., 1980; Hopmann et al., 1996). Demonstrating a functional link between cindr and the D. melanogaster orthologues cpa/cpb, we found that multiple alleles of either cpa or cbp strongly and dominantly enhanced the patterning defects of GMR>cindr-IR2 (Figs. 2 and 5, B, C, F, and G). Similarly, we observed an enhancement of the cindr-IR phenotype when ectopic cpa or cbp was concurrently expressed (Fig. 6 H and not depicted); neither protein gave rise to a detectable phenotype when misexpressed alone (not depicted). The ability of both increased and decreased cpa/cpb activity to enhance cindr-dependent phenotypes indicates that the balance of Cindr and Cpa/Cpb is important. Cpb protein was expressed in all cell types within the pupal eye (Fig. 6 D) and the distribution and levels of Cpb were not detectably altered in a cindr-IR context (not depicted).

Further, mass spectrometry analysis of embryonic protein precipitates identified Cpa and Cpb as physically interacting with a tandem affinity purification (TAP)-tagged isoform of Cindr (Fig. 6 E). This is consistent with recent work indicating that mammalian CP1/H9251 and CP1/H9252 binds CD2AP (Hutchings et al., 2003; Bruck et al., 2006) and, indeed, a potential CP1/H9251/CP1/H9252 binding site has been partly conserved in Cindr (Fig. S1 C).

These results strongly link Cindr function to actin remodeling, and genetic tests with other components of the actin remodeling machinery support this view. We examined Wiskott-Aldrich syndrome protein (WASp), suppressor of cAR (SCAR), profilin (Chickadee [Chic], required for the addition of actin monomers to filaments), coflin (Twinstar [Tsr], promotes F-actin depolymerization), and the tyrosine kinase Abelson (Siripala and Welch, 2007a, b). The cindr-IR2 phenotype was enhanced in retinae heterozygous for mutations in each of these loci (Figs. 2 and 7, F and G). Mutations in each of these seven loci increased the mean IPC number and enhanced the number of cone and/or 1° cell errors in the presence of GMR>cindr-IR2 and mildly but detectably increased the OMS value.
been previously described in the pupal eye. We therefore used phalloidin to explore the role of actin dynamics in patterning and its regulation by Cindr (Figs. 8 and S5). During early development stages (18–22 h APF), filamentous F-actin was found predominantly at cell membranes in pre-1° cells and IPCs (Fig. S5A/H11032); as development progressed, the levels of membrane- and AJ-associated F-actin increased (Fig. S5, B–G/H11032), reaching a peak 27–30 h APF (Fig. 8B). Importantly, Cindr foci were observed to colocalize with enriched phalloidin staining at the AJ (Fig. S5). Concurrently, numerous bundles of F-actin formed within the IPCs and eventually the 1° cells, radiating from the AJs to densely fill the apical cytoplasm (Figs. 8E and S5, B’–H’). Membrane-associated actin decreased after 30 h APF, and by 41 h APF, little or no membrane-associated F-actin remained between IPCs and 1° cells, though it remained at AJs of other cell types (Fig. 8E).

In summary, actin showed stereotyped and dynamic polymerization and AJ localization during the period of maximal IPC.

Conversely, ectopic expression of the actin-related protein (Arp) 2/3 component arp66B led to strong rescue of GMR>cindr-IR2; interestingly, errant localization of Rst to IPC–IPC membrane interfaces was not corrected in this genetic context despite its phenotypic rescue (Figs. 2 and 7, H and H’). Expression of arp66B alone did not disrupt patterning (unpublished data). This result further emphasizes that reducing cindr activity leads to reduction of actin regulation. In mammalian systems, the interaction between CD2AP and the Arp2/3 complex is indirect and mediated by association with Cortactin (Cort; Lynch et al., 2003) but the cindr-IR retinal phenotype was unmodified in a cortMT or cortDT heterozygous background (unpublished data).

Cindr regulates actin dynamics
In addition to regulating events at the surface, our genetic and biochemical data strongly indicated that Cindr is used by retinal cells to regulate actin cytoskeleton remodeling during the patterning process. The dynamics of actin remodeling have not been previously described in the pupal eye. We therefore used phalloidin to explore the role of actin dynamics in patterning and its regulation by Cindr (Figs. 8 and S5). During early development stages (18–22 h APF), filamentous F-actin was found predominantly at cell membranes in pre-1° cells and IPCs (Fig. S5 A’’); as development progressed, the levels of membrane- and AJ-associated F-actin increased (Fig. S5, B’’–G’’), reaching a peak 27–30 h APF (Fig. 8B). Importantly, Cindr foci were observed to colocalize with enriched phalloidin staining at the AJ (Fig. S5). Concurrently, numerous bundles of F-actin formed within the IPCs and eventually the 1° cells, radiating from the AJs to densely fill the apical cytoplasm (Figs. 8 E and S5, B’’–H’’). Membrane-associated actin decreased after 30 h APF, and by 41 h APF, little or no membrane-associated F-actin remained between IPCs and 1° cells, though it remained at AJs of other cell types (Fig. 8E).

In summary, actin showed stereotyped and dynamic polymerization and AJ localization during the period of maximal IPC.
unstable cell junctions, and severe mispatterning observed when Cindr activity was reduced. To further assess this model, we compared IPCs’ requirement for Cindr with their requirement for capping proteins, which have been previously demonstrated to regulate F-actin dynamics. We examined discrete clones of tissue that were homozygous mutants for null alleles of \textit{cpa} or \textit{cpb}.

Abnormally high levels of F-actin were observed to radiate from the membranes of mutant cells, extending with apparent continuity into neighboring wild-type cells (Fig. 8 J, and not depicted). When generated early, clones showed errors in photoreceptor and cone cell organization, reduced cell viability, and pools of undifferentiated cells (unpublished data). When generated later to circumvent differentiation defects, DE-Cad was localized discontinuously around the cell circumference (Fig. 8 J and unpublished data). These phenotypes were similar to, though more severe than, those observed in cells with a partial reduction of cindr activity (e.g., Figs. 5 and 8 C) and confirm that a disorganized cytoskeleton can disrupt both the AJ and IPC patterning.

**Discussion**

Our evidence indicates that Cindr provides a functional link between dynamically regulated surface adhesion and the cytoskeletal changes required for normal pupal eye patterning. Loss of cindr activity led to misplacement of retinal support cells, which adopted shapes uncharacteristic for their niche in the retinal field. The reasons for this became apparent when we examined movement, and the dynamics of Cindr localization roughly mirrored F-actin throughout this period. This coevolving of F-actin and Cindr localization was also observed in other cell types including the cone cells and sensory bristles (Figs. 8 and S5). The emerging bristles were especially instructive: as the tip of each bristle shaft emerged, F-actin bundles are generated from successive short linear actin modules that form at the growing tip of each bristle and elongate to graft together (Guild et al., 2003).

Cindr puncta colocalized along the length of these elongating F-actin shafts but particularly at newer distal ends, which suggests a role for Cindr in active actin polymerization (Fig. 8 G) and was reflected in malformed thoracic macrochaetae when Cindr was reduced during their development (Fig. 8 I).

Strongly reducing cindr activity (\textit{GMR}\textgreater\textit{cindr-IR}^{2+3}) had a marked effect on F-actin during IPC patterning: at 28 h APF, the density of F-actin fibrils and spikes filling the cytoplasm of cindr-IR IPCs was markedly increased (Fig. 8 C). At later stages of patterning, a more dense and disorganized cytoskeletal network was observed in IPCs and the radiating arrangement of actin fibrils in 1° cells was absent at 41 h APF (Fig. 8 F). Within cone cells, F-actin levels were mildly elevated at 28 h APF, and distinct F-actin spikes failed to radiate from cell membranes (Fig. 8 C). These data directly support a role for Cindr in regulating dynamic actin organization rather than regulating actin polymerization per se.

This failure of proper dynamic changes to the actin cytoskeleton presumably accounts for the abnormal cell movements, unstable cell junctions, and severe mispatterning observed when Cindr activity was reduced. To further assess this model, we compared IPCs’ requirement for Cindr with their requirement for capping proteins, which have been previously demonstrated to regulate F-actin dynamics. We examined discrete clones of tissue that were homozygous mutants for null alleles of \textit{cpa} or \textit{cpb}.

Abnormally high levels of F-actin were observed to radiate from the membranes of mutant cells, extending with apparent continuity into neighboring wild-type cells (Fig. 8 J, and not depicted). When generated early, clones showed errors in photoreceptor and cone cell organization, reduced cell viability, and pools of undifferentiated cells (unpublished data). When generated later to circumvent differentiation defects, DE-Cad was localized discontinuously around the cell circumference (Fig. 8 J and unpublished data). These phenotypes were similar to, though more severe than, those observed in cells with a partial reduction of cindr activity (e.g., Figs. 5 and 8 C) and confirm that a disorganized cytoskeleton can disrupt both the AJ and IPC patterning.
cell behavior in live tissue. Reducing cindr prevented 1° cells from maintaining enwrappment of the cone cells; instead, cindr 1° cells were unable to firmly establish this niche and frequently retracted, allowing neighboring IPCs to have direct contact with the cone cells. Similar instability was observed in the remaining IPCs fated to establish the 2°/3° hexagonal lattice. Histology demonstrated further changes both to AJ components and the actin cytoskeleton. Although we cannot rule out additional roles for Cindr such as regulation of endocytosis, we did not observe evidence for such a role during cells’ rearrangements.

In wild-type tissue, Hbs and Rst are localized exclusively to 1°–IPC interfaces during IPC patterning; heterophilic interactions between these molecules are thought to direct the rearrangement of IPCs into single rows around each ommatidium (Reiter et al., 1996; Bao and Cagan, 2005; Grzeschik and Knust, 2005). In cindr-IR tissue, we observed a mislocalization of Rst to the entire IPC circumference. Such mislocalization would impede the generation of a preferential adhesive force, disrupting the direction or flow of cell movement and subsequent patterning. We also detected irregularities in the localization of DE-Cad around the circumference of retinal cells when Cindr was reduced. This presumably resulted in uneven or unreliable junctional stability that further destabilized the dynamic switching of cell positions. Genetic interactions with the loci for rst, hbs, and shg confirmed that these loci cooperate with cindr during patterning.

We also demonstrate that the actin cytoskeleton is dynamically remodeled during pupal eye patterning and that reducing cindr activity led to a change in the details of cytoskeletal dynamics. In wild-type tissue, polymerized actin was initially detected almost exclusively at AJs of pre-1° cells and IPCs. These actin rings intensified as cells became rearranged and then, remarkably,
Once patterning was established, membrane-associated F-actin strongly diminished. The functional significance of these changes is likely linked to concurrent modification of Rst- and AJ-mediated adhesion. For example, the levels of both DE-Cad and Armadillo (β-catenin) decrease between IPCs as they are rearranged, which would serve to facilitate Rst-mediated IPC movements toward 1° cells (Fig. 9; middle; Bao and Cagan, 2005). We show data indicating that the actin cytoskeleton is coordinately reinforced at AJs as adhesion is weakened. This may serve to maintain the surface integrity of the retinal cells while junctional strength decreases and the tissue is remodeled. Once patterning is achieved, the BMP receptor Thickvein then acts to reestablish AJs (Cordero et al., 2007); the reduction of F-actin may reflect the reduced need for a dense actin ring. Throughout this process, Cindr acts as a pivotal regulator to coordinate AJ modification and actin polymerization.

Several data presented in this paper suggest that Cindr acts directly to regulate actin. First, the intensity and distribution of cytoplasmic Cindr puncta in retinal cells tracks that of F-actin in IPCs during development. Second, the striking dynamics of F-actin polymerization are lost, presumably helping F-actin in IPCs during development. Second, the striking dynamics of F-actin polymerization are lost, presumably helping F-actin in IPCs during development. Third, strong genetic interactions were observed between cindr-IR and multiple components of the actin regulatory machinery. Fourth, we coimmunoprecipitated the two capping protein subunits Cpa and Cpb together with Cindr from D. melanogaster embryos. And, finally, several phenotypes are shared between mutant for cindr-IR and tissue mutant for cpa or cpb: pupal eye mispatterning, gaps in the distribution of DE-Cad around the circumference of retinal cells, bristle malformation, and tissue degeneration.

Our results also emphasize the important role of the actin cytoskeleton in regulating or maintaining AJ integrity (Mege et al., 2006). However, our data also argue that Cindr regulates the localization of transmembrane adhesion proteins at least in part independently of the cytoskeleton (Fig. 7); reducing the genetic activity of actin regulators enhanced the patterning defects of cindr-IR but not the disruption to DE-Cad, and ectopic Arp66B rescued cindr-IR mispatterning but did not rescue aberrant localization of Rst. In the absence of Cindr, miscoordination of the actin machinery together with aberrant localization of junctional complexes is likely the underlying cause of tissue mispatterning during development. Similarly, deregulation of actin and junction instability is apparently cell lethal in more mature tissue, eventually leading to degeneration of mutant tissue. This may be analogous to the degeneration of mammalian podocytes that has been associated with mutations in CD2AP (Schiffer et al., 2004; Peters et al., 2006; Woroniecki et al., 2006). How Cindr itself is regulated during development remains an open question.

**Materials and methods**

*D. melanogaster* stocks

The following stocks were used (described in Flybase, http://flybase.bio.indiana.edu/): abelson; ap66B (gifts from F. Janody, Instituto Gulbenkian, Oeiras, Portugal); cpa210, cpa22, and cpa20 (gifts from I. Harihara, University of California, Berkeley, Berkeley, CA); cpa211 and USAS-cpb (gifts from P. Garrity, Brandeis University, Waltham, MA); cpb2; Df(3R)8194; hbs; hbs1; UAS-hbs-R, UAS-hbs-R, and UAS-rst (gifts from S. Bao, Mount Sinai School of Medicine, New York, NY); rac1;2, rac2, Rho1;2, ras1;2, ras2; ras3; SCAr;3; SCP3; SCP4; SCP5; SCP6; SCP7; SCP8; SCP9 to generate D. melanogaster embryos.

We show data indicating that the actin cytoskeleton is coordinated cell movement. The ligands for Rst (Hbs) and Thickvein (Dpp) are not depicted.
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Western analysis of embryos
50 embryos homozygous for Dr[3R]Exel1914 (a deletion encompassing cindr) and 50 heterozygous embryos were gathered 6–8 h after egg laying, homogenized in sample buffer, and separated on a NuPage 4–12% gradient SDS-polyacrylamide gel (Invitrogen). Rabbit anti-Cindr was used at 1:1,000 and HRP-conjugated secondary antibodies were used at 1:5,000 (Cell Signaling Technology).

Communoprecipitation and mass spectrometry

cindr-PCaTAP [or for controls, TAP only] was expressed using tubulin-GAL4 and isolated from embryos 2–24 h after egg laying. Dechorionated embryo extracts were washed, lysed in a modified Tris buffer (Veraksa et al., 2005), and incubated for 20 min on ice. The lysate was then cleared by a 3-min centrifugation at 5,500 rpm and processed as described previously (Rigaut et al., 1999). Protein isolated from 2.5 ml of embryos was sufficient for LC-MS mass spectrometry (Siteman Cancer Center Proteomics Core Facility, Washington University). Communoprecipitation of Cpb/Cpa with Cindr was confirmed by Western analysis of the final eluant; rat anti-Cpb-IR was used at 1:50 (Hopmann et al., 1996).

In situ hybridization, immunofluorescence, and live imaging
Pupae were collected at 0 h APF and maintained at 25°C until dissection as described previously (Bao and Cagan, 2005). For in situ hybridization, digoxin-labeled RNA probes were synthesized from the entire cindr-coding region from RH08284. For immunofluorescence, primary antibodies were rabbit anti-Cindr (1:100), rat anti-DE-Cad2 (1:20, DSHB), mouse anti-Armadillo N2 7A1 (1:10; Developmental Studies Hybridoma Bank), mouse anti-Rat Mab24A5.1 (1:50; Schneider et al., 1995), and rat anti-Cbp (1:50). To visualize polymerized actin, rhodamine-phalloidin was included in primary antibody incubations (1:100; Invitrogen). Secondary antibodies were conjugated Alexa 488 and 568 (Invitrogen) and Cy5 (Jackson Immunoresearch Laboratories). The mounting media was Vectashield (Vector Laboratories). Samples were analyzed at RT with either an objective microscope (Axioplan2; Carl Zeiss, Inc.) using a Plan-Apochromat 63× 1.40 NA oil objective (Carl Zeiss, Inc.) or using a confocal system (TCS SP2; Leica) with confitriangular objectives and Plan-Apochromat 63× 1.32 NA or Plan-Apochromat 100× 1.40 NA oil objective lenses (Leica). Results were processed for publication using Photoshop (Adobe) with minimal adjustment of brightness or contrast applied to the whole image and analyzed for mis-matching errors as described in Fig. 2. Live imaging was performed as described previously (Larson et al., 2008) for GMR-GAL4/cinder-R2; UAS-a-catenin-GFP/cindr-IR (experimental) and GMR-GAL4/UAS-a-catenin-GFP (control) pupae. Images were gathered every 15 min at multiple focal planes using an Axioimager microscope (Carl Zeiss Inc.) and composite images for each time point were assembled in Photoshop, from which QuickTime (Apple) movies were generated.

Online supplemental material

Fig. S1 shows predicted Cindr isoforms, sequence, and Western analysis of embryos. Fig. S2 illustrates the cinder-IR transgenes and qPCR primer sets and shows verification of the Cindr antibody and IR transgenes in the wing. Fig. S3 shows detection of Cindr in GMR-cindr-IR2 retinae and at the apical, basal, and rhabdomere planes in a 41-h APF Canton-S ommatidium. Fig. S4 shows localization of GFP-tagged Cindr isoforms. Fig. S5 shows F-actin over a time course of development. Videos 1 and 4 are time lapse studies of control retina from 18–22 and 23–27 h APF, respectively. Videos 2 and 3 are of experimental cinder-IR retinae from 18–22 and 24–28 h APF, respectively. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200706108/DC1.

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