Actin capping protein α maintains vestigial-expressing cells within the Drosophila wing disc epithelium

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Tissue patterning must be translated into morphogenesis through cell shape changes mediated by remodeling of the actin cytoskeleton. We have found that Capping protein α (Cpa) and Capping protein β (Cpb), which prevent extension of the barbed ends of actin filaments, are specifically required in the wing blade primordium of the Drosophila wing disc. cpa or cpb mutant cells in this region, but not in the remainder of the wing disc, are extruded from the epithelium and undergo apoptosis. Excessive actin filament polymerization is not sufficient to explain this phenotype, as loss of Cofilin or Cyclase-associated protein does not cause cell extrusion or death. Misexpression of Vestigial, the transcription factor that specifies the wing blade, both increases cpa transcription and makes cells dependent on cpa for their maintenance in the epithelium. Our results suggest that Vestigial specifies the cytoskeletal changes that lead to morphogenesis of the adult wing.

KEY WORDS: Actin, Cytoskeleton, Capping protein, Wing disc, Vestigial, Epithelium, Drosophila

INTRODUCTION

The specification of organ primordia by intercellular signaling has been well described, but it is not yet clear how such tissue patterning events are translated into organ morphogenesis through cytoskeletal reorganization (Braga and Yap, 2005; Jamora and Fuchs, 2002). The actin cytoskeleton controls cell morphology and polarity, endocytosis, intracellular trafficking, contractility and cell division. Filamentous (F) actin is assembled from monomeric (G) actin subunits. Polymerization occurs predominantly by extension of the fast-growing barbed ends of the filaments, while filaments are disassembled by loss of monomers from the slow-growing pointed ends. Actin filament growth, stability and disassembly are controlled by a plethora of actin-binding proteins. Profilin, formins and Enabled/VASP (Ena/VASP) family proteins promote actin polymerization, while Cofilin, Cyclase-associated protein (CAP) and capping proteins (CPs) restrict actin polymerization by different mechanisms (dos Remedios et al., 2003; Wear and Cooper, 2004; Winder and Ayscough, 2005). Cofilin severs filaments and enhances dissociation of actin monomers from the pointed end (Bamburg, 1999); CAP sequesters actin monomers, preventing their incorporation into filaments (Amberg et al., 1995; Gottwald et al., 1996); and CPs restrict accessibility of the barbed end, inhibiting addition or loss of actin monomers (Schafer et al., 1995).

Functional CPs are a highly conserved αβ heterodimer that bind the barbed ends of actin filaments through the C-terminal regions of both subunits (Amatruda et al., 1992; Schafer et al., 1992; Wear and Cooper, 2004). CPs and the Arp2/3 complex, which promotes filament branching, favor formation of the short highly branched actin filaments required to generate protrusive force at the leading edge of migrating cells. Ena/VASP proteins have the opposite activity, promoting formation of long unbranched parallel bundles of actin filaments (Bear et al., 2002; Pantaloni et al., 2000; Pollard and Borisy, 2003). In mouse or Dictyostelium cells, depletion of CPs can cause extensive formation of filopodia and increase the length and bundling of actin filaments, reducing cell motility (Hug et al., 1995; Mejillano et al., 2004). Another function of CPs is to cap a short filament of the actin-related protein Arp1 in the Dynactin complex, which is required for Dynein-mediated transport along microtubules (Schafer et al., 1994).

Drosophila imaginal discs are bilayered epithelial tissues consisting of a columnar monolayer epithelium covered by a squamous peripodial epithelium. The columnar epithelial cells are polarized along the apicobasal axis. Adherens junctions (AJ) composed of E-cadherin and α- and β-catenin link the actin cytoskeleton of neighboring cells, forming an adhesive belt. In Drosophila, two complexes important for polarity are present in the subapical region just apical to the AJ: the Bazooka/Par-6/aPKC complex, which is required for Dynein-mediated transport along microtubules (Fristrom, 1988). The wing disc has a concentrically organized proximodistal (PD) axis; the primordium of the wing blade is in the center, surrounded by the wing hinge primordium, with the notum and pleura at the periphery. During the second larval instar, an antagonistic relationship between epidermal growth factor (EGF) and Wingless (Wg) signaling divides the disc into a dorsal region that gives rise to the notum, and a ventral region that forms the wing. The wing is further subdivided by expression of the selector genes vestigial (vg) and scalloped (sd) in the wing blade and homothorax (hth) in the wing hinge (Klein, 2001). Vg and Sd encode subunits of a heterodimeric transcription factor that controls wing identity (de Celis, 1999); vg is required for wing formation (Williams et al., 1991) and its misexpression can induce ectopic wing tissue (Kim et al., 1996).

In a mosaic genetic screen for genes required during early eye differentiation (Janody et al., 2004), we identified loss-of-function mutations in the genes encoding Capping protein α (Cpa), the CAP
homolog Capulet (Capt) and the Cofilin homolog Twinstar (Tsr). Here, we show that Cpa, as well as the previously identified Capping protein β (Cpb), prevent extrusion and death of cells in the wing blade epithelium, but are not required for this function in other regions of the wing disc. Although cpa, capt and tsr mutations all increase actin filament polymerization, only cpa is required to maintain vg-expressing cells within the epithelium. Furthermore, Vg enhances transcription of cpa in the wing blade region. These results provide a link between pattern formation controlled by Vg and morphogenesis of the wing blade through cytoskeletal regulation mediated by actin capping proteins.

MATERIALS AND METHODS

Fly strains and genetics

The mosaic screen for mutations affecting early eye development has been described in detail (Janody et al., 2004). Five lethal alleles of cpa (cpa36P, cpa376, cpa66A, cpa396 and cpa4267) and four lethal alleles of tsr (tsr80, tsr96, tsr108 and tsr108M) were recovered from a screen of 45,000 flies on the right arm of the second chromosome. Recombination mapping relative to P(w+) elements localized the cpa gene between 57A8-9 and 57D11-12, 1.5 cM proximal to P element l(2)K02206 and 0.9 cM distal to P element domK05108 (FlyBase). Our alleles failed to complement the lethal P element element CG10540/K005264 (FlyBase), which is inserted in the cpa gene. Recombination mapping relative to P(w+) elements localized the tsr alleles to 0.42 cM distal to P element elf6(K13214) (FlyBase) and identified a lethal P element in the tsr gene, 1(2)K05633/5657 (FlyBase), which failed to complement our tsr alleles. Other fly stocks used were cpbM141, khs13314, bs2 (FlyBase), capBP86, capP550 (Benali et al., 2000), da-Gal4 (Wodarz et al., 1995), tub-Gal4 (Lee and Luo, 1999), puc-lacZ (Martin-Blanco et al., 1998), UAS-th (Ryoo et al., 2002), UAS-vg23 (Kim et al., 1996), UAS-A-rmo (Doherty et al., 1996), UAS-bks106 (Adachi-Yamada et al., 1999b), UAS-Mal-D-ΔN and UAS-dia1E (Somogyi and Rorth, 2004). To generate clones marked by the absence of GFP in the wing disc, y; w, FRT42D, cpa or tsr/CyO P(y+) or y; w, FRT40, cpa/S6M-TM6B males were crossed to y, w, hsFLP122; FRT42D or 40, ubi-GFP females. To generate clones positively labeled by GFP, y; w, FRT42D, cpa or tsr/CyO P(y+) or y; w, FRT40, cpa or cpb/CyO P(y+) males were crossed to y, w, hsFLP122, UAS-GFP; FRT42D or FRT40, tub-GAL80; tub-GAL4/TM6B, puc-lacZ, UAS-th, UAS-vg or UAS-N lines transgenes were combined with the FRT42D or FRT42D, cpa chromosomes. The offspring were heat-shocked for 1 hour at 37°C at both 24 and 48 hours after a 24 hour egg collection, corresponding to the first and second larval instar. To visualize cpa or tsr mutant clones at different times after induction, larvae were heat-shocked for 1 hour at 37°C at 60 hours after a 24 hour egg collection. Wing discs were dissected from crawling late third instar larvae either 36 or 60 hours after the heat shock; these had clones induced at early third instar or at second instar, respectively. All experiments were performed at 25°C. To rescue survival of cpa mutants, da-GAL4 or tubGAL4; cpa/S6M-TM6B flies were crossed to UAS-HA-cpa, cpa/S6M-TM6B. To rescue cpa mutant clones in the wing imaginal disc, y, hsFLP122, UAS-GFP; FRT42D, tub-GAL80; tub-GAL4/TM6B flies were crossed to FRT42D, cpa, UAS-HA-cpa/S6M-TM6B.

Immunohistochemistry and in situ hybridization

Third instar larval imaginal discs were stained with antibodies as described (Lee and Treisman, 2001). Antibodies used were guinea pig anti-DirG (1:300; gift from P. J. Bryant), mouse anti-Arm [N2 7A1, 1:10; Developmental Studies Hybridoma Bank (DSHB)], rabbit anti-Caspase 3 (1:500; BD Bioscience), mouse anti-β-galactosidase (1:200; Promega), rabbit anti-Vg (1:20); (Williams et al., 1993), mouse anti-HA (1/5000; Covance). Secondary antibodies were from Jackson Immunoresearch, used at 1:200 or Molecular Probes, used at 1:500, conjugated to FITC, Texas Red or Cy5. Rhodamine-conjugated phalloidin (Sigma) was used at a concentration of 0.3 μM. Fluorescence images were obtained on a Leica TCS NT confocal microscope or on a LSM 510 Zeiss confocal microscope. For in situ hybridization, antisense or sense RNA probes, labeled with digoxigenin-UTP (Roche), and encompassing the entire cpa cDNA were used. In situ hybridization was performed as described (Mauret-Zaffran and Treisman,

RESULTS

Clones lacking CPs, but not Tsr or Capt, are extruded from the wing blade epithelium

In a mosaic genetic screen for genes required during early eye differentiation (Janody et al., 2004), we identified lethal alleles of cpa, tsr and capt. Capping protein, Cofilin and CAP all restrict actin filament polymerization (Bamburg, 1999; Gottwald et al., 1996; Schafer et al., 1995), and loss of any of these genes results in a severe degenerative phenotype in the adult eye (Delalle et al., 2005) (and data not shown).

Surprisingly, we found a region-specific requirement for cpa in the wing disc: cpa mutant clones induced at the first or second larval instar could not be recovered in the wing blade epithelium, although mutant clones could develop in the remainder of the wing disc (Fig. 1A). By contrast, clones mutant for tsr or capt survived equally well in all regions of the disc (Fig. 1B,C). Optical cross-sections through the wing disc showed that in the wing blade region, positively labeled cpa mutant cells were found on the basal surface of the disc rather than within the epithelium (Fig. 1D). However, tsr and capt mutant clones were maintained within the epithelium in all regions of the wing disc (Fig. 1F,G). Cpa functions as a heterodimer with its partner Capping protein β (Cpb) (Amatruda et al., 1992; Schafer et al., 1992), and the stability of each subunit depends on its association with the other (Casella and Torres, 1994; Mejillano et al., 2004). As expected, we found that cpb mutant cells were also extruded on the basal surface in the wing blade region, but not in the remainder of the wing disc (Fig. 1E). Thus, cpa and cpb are both required to maintain epithelial integrity specifically in the wing blade primordium, suggesting that this subregion of the epithelium has a distinct cellular organization.

The cpa alleles exhibiting this phenotype included likely null alleles that would truncate the protein before the actin-binding domain. To confirm that the loss of cells from the wing pouch epithelium was due to mutations in the cpa gene, we generated transgenic fly lines expressing the full-length cpa transcript under the control of UAS sequences (Brand and Perrimon, 1993). Expression of this transcript rescued the cell extrusion phenotype of cpa mutant clones in the wing blade epithelium (Fig. 1H). Driving expression of this transcript ubiquitously throughout development using daughterless (da)-GAL4 or tubulin (tub)-GAL4 was also sufficient to rescue the lethality of cpa mutants, although some of the rescued adults exhibited wing, eye or bristle defects (Table 1; data not shown).
cpa is required for cell survival in the wing blade epithelium

Our failure to recover cpa mutant clones in the adult wing suggested that extruded mutant cells might be eliminated by apoptosis. Indeed, we found that most cpa mutant cells on the basal surface of the wing blade epithelium contained activated Caspase 3 (Fig. 2A-B). Extruded cpa mutant clones also expressed puckered-lacZ (puc-lacZ; Fig. 2C), a transcriptional target of the proapoptotic Jun N terminal Kinase (JNK) pathway (Adachi-Yamada et al., 1999a), indicating that JNK signaling is activated.

Vertebrate epithelial cells destined for death are surrounded by a contractile ring of actin and extruded basally (Rosenblatt et al., 2001). To determine whether extrusion of cpa mutant cells from the wing blade epithelium reflects a preliminary stage of apoptosis, we used the MARCM system to express the Drosophila Inhibitor of Apoptosis Protein (DIAP1; th – FlyBase) to prevent apoptosis of cpa mutant cells. Expression of th in wild-type clones had no visible effect (Fig. 2E-H). Survival of cpa mutant cells was greatly enhanced when th was overexpressed (compare Fig. 2D with 2F); however, mutant cells still lost contact with the apical epithelial surface and were extruded basally (Fig. 2I). The same result was obtained using the caspase inhibitor P35 to block cell death (data not shown). This suggests that extrusion of cpa mutant cells is independent of programmed cell death.

cpa is required to localize adherens junction components in cells in the wing blade

In epithelial cells, the actin cytoskeleton is connected to adherens junctions (AJ) and septate junctions (SJ), which are necessary for stable cell-cell adhesion (Gibson and Perrimon, 2003). Disruption of cortical actin assembly and apicobasal polarity can induce cell

Fig. 1. cpa or cpb mutant clones are extruded from the wing blade epithelium. All panels show third instar wing discs. (A-C) Standard confocal sections. (A) cpa^{107E}, (B) tsr^{110M} and (C) capt^{E630} mutant clones are marked by the absence of GFP (green). (D-H) Optical cross-sections through the wing disc epithelium. Mutant clones are positively labeled with GFP (green) and sections are stained with anti-Dlg (red) and anti-Arm (blue) to outline apical cell membranes. (D) cpa^{107E}, (E) cpa^{107H}, (F) tsr^{110M}, (G) capt^{E630}, (H) cpa^{107E} mutant clones overexpressing full-length cpa. cpa or cpa mutant clones are extruded basally in the wing blade primordium (red arrows), but not the notum; this extrusion is rescued by full-length cpa. tsr and capt mutant clones are not extruded. The white arrows define the wing blade region. Dorsal is towards the left on optical cross-sections in this and subsequent figures.

Fig. 2. Extrusion of cpa mutant cells is independent of programmed cell death. All panels show third instar wing imaginal discs. (A-C) Optical cross-sections of discs stained with anti-Dlg (blue) to outline apical cell membranes and anti-Caspase 3 (red in A, B, C) or anti-β-Galactosidase to reveal puc-lacZ expression (red in C). Anti-Caspase 3 antibody gives a non-specific background staining seen at the apical surface of the discs. (A) T155-Gal4; UAS-flp induced cpa^{107E} mutant clones marked by the absence of GFP (green). (B-C) hs-flp induced cpa^{107E} (B) or cpa^{107E} (C) mutant clones, positively labeled with GFP (green). (A, B, C) The overlay of all three channels. cpa mutant clones express Caspase 3 and puc-lacZ cell autonomously. Cell death is seen when FLP is induced either by heat shock or by the epithelial driver T155-GAL4, and is therefore not due to stressed conditions induced by heat shock, as described for clones mutant for the Dpp receptor thickveins (tkv) (Gibson and Perrimon, 2005). (D-I) Standard confocal sections (D-F) or optical cross sections (G-I) of clones positively labelled with GFP (green) and stained with anti-Dlg (red) and anti-Arm (blue). (D, G) cpa^{107E} mutant clones; (E, H) clones overexpressing th; (F, I) cpa^{107E} mutant clones overexpressing th. th overexpression promotes survival of cpa mutant cells, but fails to prevent their extrusion. The white arrows define the wing blade region.
extrusion in the wing disc, as in moesin mutants (Speck et al., 2003). By contrast, tkv mutant cells are extruded as cysts that maintain apical junctions with one another (Gibson and Perrimon, 2005; Shen and Dahmann, 2005). Interestingly, we found that an HA-tagged form of Cpa, which could rescue extrusion of cpa mutant cells, accumulated at the apical cell membrane in all regions of the wing disc (Fig. 3A). In the wing blade primordium, HA-Cpa partly colocalized with components of epithelial junctions, including Armadillo (Arm) (Fig. 3B-B”) or anti-Dlg (red in C,D,E,F or white in B,B”). (A,B) Magnification of the blade primordium. HA-cpa accumulates at the apical membrane, partly co-localizes with Arm in all regions of the wing disc and rescues extrusion of cpa mutant clones in the wing blade primordium. (C,D,F) cpa69E mutant clones positively labeled with GFP (blue in C,D,F) in the wing blade (C,D) or notum (F) primordium, induced both at second or early third instar and dissected at the late third instar stage either 60 hours (C) or 36 hours (D,F) after clone induction. Although we could recover mutant clones within the disc epithelium 36 hours after clone induction, all mutant cells were extruded by 60 hours. (E) cpa69E mutant clones in the blade primordium, dissected 36 hours after clone induction and positively labeled with GFP (blue in E). Arm is mislocalized to basolateral regions in extruding cpa mutant cells and in tsr mutant cells that are maintained within the epithelium (red arrows in D’ and E”). Following extrusion of cpa mutant cells, expression of both Arm and Dlg are lost (C). The white arrows in A define the wing blade region.

Table 1. Rescue of lethality of cpa mutant combinations by full-length Cpa

<table>
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<tr>
<th>cpa rescue</th>
<th>% rescue to pupal stage</th>
<th>Number</th>
</tr>
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<tbody>
<tr>
<td>cpa1D; tub-GAL4/SM6.TM6B × cpa69E/SM6.TM6B</td>
<td>0</td>
<td>134</td>
</tr>
<tr>
<td>cpa1D;SM6.TM6B × cpa1D; UAS-HA-cpa1B/SM6.TM6B</td>
<td>0</td>
<td>215</td>
</tr>
<tr>
<td>cpa1D;SM6.TM6B × cpa69E; UAS-HA-cpa1D/SM6.TM6B</td>
<td>0</td>
<td>201</td>
</tr>
<tr>
<td>cpa1D;SM6.TM6B × cpa69E; UAS-HA-cpa1D/SM6.TM6B</td>
<td>0</td>
<td>137</td>
</tr>
<tr>
<td>cpa1D; tub-GAL4/SM6.TM6B × cpa69E; UAS-HA-cpa1B/SM6.TM6B</td>
<td>31</td>
<td>79</td>
</tr>
<tr>
<td>cpa1D; tub-GAL4/SM6.TM6B × cpa69E; UAS-HA-cpa1D/SM6.TM6B</td>
<td>95</td>
<td>238</td>
</tr>
<tr>
<td>cpa1D; tub-GAL4/SM6.TM6B × cpa1D; UAS-HA-cpa2A/SM6.TM6B</td>
<td>29</td>
<td>52</td>
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For each cross, the percentage rescue to the pupal stage is given, based on comparing the number of non-balancer pupae with the total number of pupae. The SM6.TM6B balancer is marked with Tubby.

cpa has cell type-dependent effects on actin filament accumulation

CPs tightly cap the fast-growing barbed ends of actin filaments, inhibiting the addition of actin monomers to the filament (Schafer et al., 1995). Decreased expression of cpa or cpb in the Drosophila eye imaginal disc causes actin filament accumulation (Delalle et al., 2005). To confirm that cpa also restricts growth of actin filaments in the wing disc epithelium, we stained wing imaginal discs with...
phalloidin 36 hours after clone induction, allowing visualization of mutant clones before cell extrusion. As expected, cpa mutant clones accumulated actin filaments in all regions of the wing disc (Fig. 4A-A’). Optical cross-sections through the notum showed that, like capt mutant cells (Fig. 4E-E’) (Baum and Perrimon, 2001), cpa mutant cells accumulated actin filaments near the apical cell membrane (Fig. 4C-C’). However, cpa mutant cells in the wing blade accumulated actin filaments around the entire cell cortex (Fig. 4B-B’), resembling tsr mutant cells (Fig. 4D-D’). As Cpa is concentrated at the apical cell membrane (Fig. 3A-B’), the presence of excess actin filaments in basolateral regions may be due to the effect of cpa on apicobasal polarity. Indeed, some of these actin filaments colocalized with Arm displaced from apical adherens junctions (Fig. 4B-B’).

**Vestigial controls the requirement for cpa in the wing blade epithelium**

We observed extrusion of cpa mutant clones only in the wing pouch, indicating that either the position or identity of these cells makes them dependent on cpa. Signaling pathways activated by Wg, Decapentaplegic (Dpp) and Notch (N) can influence growth and/or survival in the wing blade primordium (Klein, 2001). Target genes of these pathways, including Vg (Klein and Arias, 1999), were still expressed in cpa mutant clones (Fig. 5A-A’ and data not shown), indicating that cpa is not required for the reception of these signals. Vg is a nuclear protein that confers a wing blade fate on cells in which it is expressed (Williams et al., 1991). We wondered whether transforming hinge or notum cells to wing blade identity by misexpressing Vg would induce extrusion of cpa mutant cells. When we misexpressed vg in cpa mutant clones, we found that clones in all regions of the wing disc were extruded from the epithelium and contained activated Caspase 3 (compare Fig. 5B,D with Fig. 5E,G). Misexpressing vg in clones that were not mutant for cpa had neither of these effects (Fig. 5C,F). Thus, cpa is autonomously required for the survival and maintenance of vg-expressing cells in the wing disc epithelium, independently of the identity of the surrounding cells.

**cpa is a Vg target gene**

The requirement for cpa to maintain vg-expressing cells in the wing pouch epithelium suggests that capping actin filaments is crucial for Vg-induced wing blade morphogenesis. We wondered whether cpa expression might itself be induced by Vg. Indeed, in situ hybridization showed that cpa mRNA was more strongly expressed in cpa mutant clones (Fig. 5A-A’). Target genes of these pathways, including Vg (Klein and Arias, 1999), were still expressed in cpa mutant clones (Fig. 5A-A’ and data not shown), indicating that cpa is not required for the reception of these signals. Vg is a nuclear protein that confers a wing blade fate on cells in which it is expressed (Williams et al., 1991). We wondered whether transforming hinge or notum cells to wing blade identity by misexpressing Vg would induce extrusion of cpa mutant cells. When we misexpressed vg in cpa mutant clones, we found that clones in all regions of the wing disc were extruded from the epithelium and contained activated Caspase 3 (compare Fig. 5B,D with Fig. 5E,G). Misexpressing vg in clones that were not mutant for cpa had neither of these effects (Fig. 5C,F). Thus, cpa is autonomously required for the survival and maintenance of vg-expressing cells in the wing disc epithelium, independently of the identity of the surrounding cells.

**cpa prevents extrusion of wing pouch cells**

We observed extrusion of cpa mutant clones only in the wing pouch, indicating that either the position or identity of these cells makes them dependent on cpa. Signaling pathways activated by Wg, Decapentaplegic (Dpp) and Notch (N) can influence growth and/or survival in the wing blade primordium (Klein, 2001). Target genes of these pathways, including Vg (Klein and Arias, 1999), were still expressed in cpa mutant clones (Fig. 5A-A’ and data not shown), indicating that cpa is not required for the reception of these signals. Vg is a nuclear protein that confers a wing blade fate on cells in which it is expressed (Williams et al., 1991). We wondered whether transforming hinge or notum cells to wing blade identity by misexpressing Vg would induce extrusion of cpa mutant cells. When we misexpressed vg in cpa mutant clones, we found that clones in all regions of the wing disc were extruded from the epithelium and contained activated Caspase 3 (compare Fig. 5B,D with Fig. 5E,G). Misexpressing vg in clones that were not mutant for cpa had neither of these effects (Fig. 5C,F). Thus, cpa is autonomously required for the survival and maintenance of vg-expressing cells in the wing disc epithelium, independently of the identity of the surrounding cells.
expressed in the wing pouch than in the hinge primordium or the notum regions, accumulating primarily at the basal surface of the epithelium (Fig. 6A,C; Fig. 6B indicates the background level of the cpa sense probe). As vg is required for the survival of cells in the wing blade, we could only test its effect on cpa transcription in gain-of-function experiments. Interestingly, misexpressing vg in the notum increased cpa transcript levels (Fig. 6D-D'). The same effect was observed in clones expressing the constitutively active intracellular domain of N (N\textsuperscript{intr}) (Fig. 6E-E'), which induces misexpression of vg (Fig. 6F-F'). These results suggest that Vg alters the cytoskeletal structure of cells fated to form the wing blade in part by upregulating the expression of actin capping proteins.

**DISCUSSION**

**Actin capping proteins maintain epithelial integrity**

Wing blade cells lacking either cpa or cpb are extruded from the epithelium and subsequently die. A number of possible mechanisms might account for this loss of CP mutant cells. As extrusion of cpa mutant cells still occurs in the presence of the apoptotic inhibitors p35 or th, apoptosis is likely to be a secondary consequence of extrusion; extruded cells might undergo apoptosis because they are deprived of anti-apoptotic signals present in their normal niche. In addition, JNK activity is not essential for extrusion, as cpa mutant clones were not rescued by expression of a dominant-negative form of basket (bsk), which encodes JNK (see Fig. S1E in the supplementary material). However, we cannot exclude the possibility that the p35 or Th inhibitors block apoptosis too late to prevent release of an extrusion signal, as inhibition of caspases with z-VADfmk does not block extrusion of apoptotic MDCK cells (Rosenblatt et al., 2001).

The function of CPs in organelle or vesicle transport is unlikely to explain the extrusion phenotype. CPs are thought to stabilize the barbed end of the Arp1 microfilament in the Dynactin complex, which is required for transport along microtubules (Schäfer et al., 1994). cpa and cpb, like other Dynactin complex subunits, are required to maintain the position of nuclei in *Drosophila* photoreceptor neurons (Whited et al., 2004) (and data not shown). However, removal of kinesin heavy chain (khc), which counteracts Dynein/Dynactin-based transport, failed to rescue extrusion of cpa mutant cells in the wing disc (see Fig. S1C in the supplementary material).

We considered the possibility that the cpa phenotype was due to its effect on monomeric G-actin levels rather than on the filamentous actin cytoskeleton. G-actin has been shown to negatively regulate the nuclear localization and activity of Mal, a transcriptional co-factor for SRF (Miralles et al., 2003), and overexpression of Mal or of its activator diaphanous (Somogyi and Rorth, 2004) can cause extrusion and death of wing epithelial cells (see Fig. S1F,G in the supplementary material). However, overactivity of the MAL/dSRF pathway is unlikely to be responsible for extrusion of cpa mutant cells in the wing blade, as clones mutant for both cpa and bldestered (bs), which encodes *Drosophila* SRF, were still extruded from the wing epithelium (see Fig. S1H in the supplementary material).

Extrusion of cpa or cpb mutant cells might be a direct result of defects in the actin cytoskeleton. Consistent with the requirement for CPs to inhibit addition of actin monomers to the fast-growing end of actin filaments (Schäfer et al., 1995), we observed a strong accumulation of actin filaments in cpa mutant clones. However, tsr and capt mutations also induce excessive actin filament polymerization (Fig. 4) (Baum and Perrimon, 2001) but do not cause cell extrusion. The major function of Tsr (Cofilin) is to promote dissociation of ADP-actin from the pointed end of the filament, while Cpa prevents elongation of the barbed end of each branch and Capt sequesters actin monomers. The phenotypic differences between cpa, tsr and capt might therefore be due to different degrees of branching of the actin network formed in mutant cells (Fig. 7A). Possibly long unbranched filaments do not provide a framework of sufficient strength to withstand forces that place tension on the cell within the epithelium.

Extrusion is associated with dispersion of the adherens junction components Arm and DE-Cad along the lateral membranes. However, this defect is also observed in tsr mutant clones, and mislocalization of adherens junction components caused by overexpression of a dominant form of the polarity gene crumbs...
Loss of cpb displaces actin bundles from the cell membrane in *Drosophila* bristles by increasing the concentration of non-bundle actin snarls (Hopmann et al., 1996; Frank et al., 2006) and CPs may specify actin filament position in the sarcomere (Schafer et al., 1995). In the *Drosophila* wing blade epithelium, loss of CPs might disrupt attachment of the actin cytoskeleton to the adherens junctions, breaking the connection between cells and inducing cell extrusion (Fig. 7C). The localization of HA-Cpa to apical junctions and the mislocalization of actin filaments throughout *cpa* mutant cells in the wing blade are consistent with this possibility. Such a role would be restricted to the wing blade, as *cpa* mutant cells within the notum epithelium accumulate actin filaments only at the apical cell membrane.

**Selector proteins regulate the actin cytoskeleton to pattern the wing imaginal disc**

Surprisingly, we found that *cpa* and *cpb* are required to prevent cell extrusion and death only in the region of the wing disc giving rise to the wing blade, but not in the primordia of the hinge or notum, or in the eye or leg discs (Delalle et al., 2005) (and data not shown). The requirement for *cpa* depends on the wing blade selector gene Vg, as expression of Vg in notum cells is sufficient to induce their extrusion in the absence of *cpa*. Vg also enhances the transcription of *cpa* in the wing blade primordium. Taken together, these results imply that patterning genes regulate cytoskeletal properties in order to achieve distinct morphological outcomes (Fig. 7B,C).

The molecular mechanism that makes Vg-expressing cells dependent on CPs for their maintenance in the epithelium is unknown, although our data support a cell-autonomous target of Vg. One possibility is that Vg promotes the expression or recruitment of an actin filament polymerizing factor. The role of CPs might be to restrict its activity at barbed ends, preventing the formation of a specific actin-based structure that induces loss of cell-cell contacts and extrusion. For example, Vg activates the expression of the type II transmembrane protein Four jointed (Fj), which regulates the activity of the cadherin Fat (Cho and Irvine, 2004). Mammalian Fat1 can recruit Ena/VASP proteins, which promote actin polymerization at cell-cell contacts by antagonizing CPs (Moeller et al., 2004; Tanoue and Takeichi, 2004). However, misexpression of *fj* does not induce extrusion of either wild-type or *cpa* mutant cells in the notum (Cho and Irvine, 2004) (and data not shown). DE-cadherin levels are also higher in the wing pouch (Jaiswal et al., 2006), but increasing them in the hinge or notum by activating Wg signaling does not cause extrusion of *cpa* mutant cells (data not shown). Alternatively, Vg might control the expression of factors that promote the remodeling of cell junctions required for morphogenesis of the wing. Cpa could be required to maintain the connection between cells in the epithelium during these morphogenetic movements.

The non-uniform distribution of and requirement for *cpa* suggests that cytoskeletal organization varies in different regions of the wing disc. Gibson and Perrimon (Gibson and Perrimon, 2005) observed that lateral wing disc cells had moderately reduced levels of basolateral cortical F-actin. In addition, filopodial extensions called cytonemes are oriented towards the AP and/or DV boundary within the wing pouch, while hinge cells do not extend cytonemes and notum cells radiate short cytonemes in all directions (Hsiung et al., 2005). Changes in cytoskeletal organization have been shown to establish cell affinity boundaries (Major and Irvine, 2005), to control the subcellular localization of transcription factors (Miralles et al., 2003) and to modulate the transport of signaling molecules (Benlali et al., 2000; Hsiung et al., 2005). Investigating the control of *cpa* by...
Vg may help us to understand how and why patterning genes regulate cell architecture. In addition, identifying additional target genes of Vg may illuminate how actin dynamics and changes in intercellular adhesion control the formation of the wing blade.

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