AIP1 acts with cofilin to control actin dynamics during epithelial morphogenesis

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SUMMARY
During epithelial morphogenesis, cells not only maintain tight adhesion for epithelial integrity but also allow dynamic intercellular movement to take place within cell sheets. How these seemingly opposing processes are coordinated is not well understood. Here, we report that the actin disassembly factors AIP1 and cofilin are required for remodeling of adherens junctions (AJs) during ommatidial precluster formation in Drosophila eye epithelium, a highly stereotyped cell rearrangement process which we describe in detail in our live imaging study. AIP1 is enriched together with F-actin in the apical region of preclusters, whereas cofilin displays a diffuse and uniform localization pattern. Cofilin overexpression completely rescues AJ remodeling defects caused by AIP1 loss of function, and cofilin physically interacts with AIP1. Pharmacological reduction of actin turnover results in similar AJ remodeling defects and decreased turnover of E-cadherin, which also results from AIP1 deficiency, whereas an F-actin-destabilizing drug affects AJ maintenance and epithelial integrity. Together with other data on actin polymerization, our results suggest that AIP1 enhances cofilin-mediated actin disassembly in the apical region of precluster cells to promote remodeling of AJs and thus intercellular movement, but also that robust actin polymerization promotes AJ general adhesion and integrity during the remodeling process.

KEY WORDS: AIP1 (Flare), Actin dynamics, Adherens junction remodeling, Epithelial morphogenesis, Cofilin (Twinstar)

INTRODUCTION
Adherens junction (AJ) remodeling is crucial for the dynamic rearrangement of cells within cell sheets during epithelial morphogenesis, which occurs widely during developmental and physiological processes (Nishimura and Takeichi, 2009). Cadherins, such as E-cadherin (E-cad), together with β-catenin and α-catenin comprise the core of the AJ. The actin cytoskeleton is thought to provide the mechanical support for AJs to join neighboring cells together as a tight layer, but recent studies have increasingly shown that the nature of the actin network underlying AJs is more dynamic than previously thought (Harris and Tepass, 2010). During epithelial remodeling in Drosophila embryos, the actin network turns over rapidly and is proposed to promote the lateral mobility of microdomains of homophilic E-cad clusters (sites of strong intercellular adhesion), allowing AJ remodeling to take place (Cavey et al., 2008).

Biochemical studies have established cofilin as a major factor in promoting actin dynamics, which is achieved through its dual function of filament severing and monomeric actin (G-actin) dissociation from the pointed ends (Bamburg, 1999). The depolymerizing function of cofilin was reported to promote a high rate of actin treadmilling, which occurs when G-actin depolymerized from the pointed ends of the filaments is continuously polymerized onto their barbed ends, allowing continuous and robust actin polymerization to take place in dynamic cellular regions including the lamellipodia, growth cones and sites of endocytosis (Bamburg, 1999; Wang et al., 2007). AIP1 (Actin interacting protein 1) was identified as a major co-factor that collaborates with cofilin to disassemble F-actin. AIP1 can bind to the cofilin–F-actin complex and strongly enhance the severing activity of cofilin on actin filaments by capping the barbed ends of the severed filaments, resulting in acceleration of actin depolymerization (Ono, 2003). Evidence from microscopy indicates that AIP1 can actively disassemble cofilin-bound actin filaments (Ono, 2003).

Both cofilin (17 kDa) and AIP1 (67 kDa) are highly conserved across all eukaryotes, from yeasts and plants to mammals. Our previous studies showed that cofilin is genetically required for cell migration in the Drosophila ovary and that it promotes lamellipodial protrusions (Chen et al., 2001; Zhang et al., 2011). Other genetic studies have shown that cofilin is essential for a variety of cellular and developmental processes requiring strong actin dynamics, including cytokinesis, axon growth and endocytosis (Bamburg, 1999; Ono, 2003). Functional analyses of AIP1 in model organisms has revealed its roles in actin-based processes including body wall muscle formation in C. elegans, neutrophil response in mouse, cytokinesis, phagocytosis and cell motility in Dictostyelium (Kile et al., 2007; Ono, 2003). However, whether AIP1 or cofilin is essential for AJ remodeling in the epithelia is unclear. Recently, point mutations in flare (flr), the gene that encodes Drosophila AIP1, have been reported to disrupt planar cell polarity (PCP) and the morphology of wing hairs (Ren et al., 2007), but no specific AJ defects were directly attributed to disruption of AIP1 function in the wing disc epithelium.

In this study, we show that AIP1 and cofilin are essential for AJ remodeling during morphogenesis of eye epithelia in Drosophila larvae. Treatment with drugs that reduce actin disassembly reveals the important role of actin turnover in epithelial morphogenesis, including the process of AJ remodeling during ommatidial precluster formation. Here, we also present a live imaging study that describes in detail the process of AJ remodeling in precluster formation, which is affected in the flr mutant. Furthermore, enrichment of AIP1 together with F-actin in the apical region of
these preclusters suggests that AIP1 specifically promotes an environment of strong actin dynamics to allow fast AJ remodeling to take place during eye epithelial morphogenesis.

MATERIALS AND METHODS

Drosophila genetics
Fly strains are described in supplementary material Table S1. Flies were raised on standard cornmeal and sucrose media, and all crosses were performed at 25°C except for temperature shift experiments.

Immunohistochemistry and microscopy
Eye discs of third instar larvae were dissected in 1×PBS, fixed in 7.4% formaldehyde for 30 minutes, blocked for 30 minutes in PBT (0.3% Triton X-100 in PBS) containing 10% normal goat serum, and incubated overnight at 4°C in primary antibodies as follows: rat anti-Elav [1:100, Developmental Studies Hybridaoma Bank (DSHB)], mouse anti-Prospero (1:50, DSHB), mouse anti-Armadillo (1:100, DSHB), mouse anti-Dlg1 (1:100, DSHB), rabbit anti-PKCCζ (1:500, Santa Cruz), rabbit anti-β-galactosidase (1:2000, Rockland), rabbit anti-cofilin (1:10,000; gift of James Bambarburg; Shaw et al., 2004), rat anti-E-cad (DCADZ2, 1:50, DSHB), rat anti-α-catenin (DCAT-1, 1:100, DSHB), mouse anti-α-spectrin (3A9, 1:50, DSHB), goat anti-Arp2 [sc-11968, 1:10, Santa Cruz (Rogers et al., 2003)] and mouse anti-profilin (CHI 1J, 1:10, DSHB). Cy3- and Cy5-conjugated secondary antibodies (1:250, Jackson) were used. F-actin was labeled by Rhodamine phalloidin (1:400, Sigma) for 30 minutes. Single confocal sections and z-stacks were acquired using a Leica TCS SL or Olympus FV1000 confocal microscope; cross-section reconstructions were performed using Leica or Olympus software.

Western blotting and immunoprecipitation
Second instar larvae of various flr mutations were collected for western analysis using standard protocols. Protein extracts were analyzed by immunoblotting with the following primary antibodies: rabbit anti-AIP1 (1:5000, raised against full-length Drosophila AIP1 protein), mouse anti-actin (1:10,000, Millipore), mouse anti-GFP (1:1000, Roche) and rabbit anti-cofilin (1:10,000; Shaw et al., 2004). Secondary antibodies were HRP conjugated (Jackson). The intensity of protein bands was quantified using ImageJ (NIH). For the immunoprecipitation assay, protein extracts from third instar larvae were incubated with 20 μl anti-GFP a-gorse beads (MBL, Japan) for 8 hours at 4°C; lysates and immunoprecipitates were then immunoblotted.

Time-lapse imaging and fluorescence recovery after photobleaching (FRAP)
Third instar larval eye discs were dissected with optic lobes attached in Schneider’s medium cocktail (Prasad et al., 2007) and mounted on a Lumox dish (Sigma) for live imaging with an Olympus FV1000 confocal microscope.

For the FRAP experiment, eye discs were dissected in Schneider’s medium cocktail (without insulin), incubated in the medium containing 10 μM Jasp (dissolved in DMSO, Invitrogen) or an equivalent volume of DMSO for 2 hours, and then fixed as described above.

RESULTS

AIP1 is required for eye development
The crystalline lattice pattern of 750-800 ommatidia in the Drosophila adult compound eye is laid down at the late larval (third instar) stage, when equally spaced columns of ommatidial rudiments are formed in the wake of the morphogenetic furrow (MF) passing from the posterior end of the eye disc epithelium to the anterior end. Anterior to the MF, cells remain undifferentiated and unpatterned. Posterior to the MF, active morphogenesis and cell fate specification take place to form columns of equally spaced clusters of photoreceptor progenitors, namely ommatidial preclusters immediately posterior to the MF and the more differentiated ommatidial clusters further posterior to the MF (Wolf and Ready, 1991). This early phase of eye morphogenesis is crucial for the development and patterning of the adult eye, as its disruption can cause perturbation in cluster morphology and spacing, resulting in a ‘rough’ adult eye. To address whether AIP1 is required for epithelial morphogenesis during eye development, we performed genetic mosaic experiments using the FRT-FLP system (Chen et al., 2005; Xu and Rubin, 1993), and showed that lethal mutations in flr induced by P-elements resulted in a strong rough phenotype in the mosaic eyes of adult flies (Fig. 1A; supplementary material Fig. S1). The three P-element alleles tested were hypomorphic and gave a lethal phenotype at 25°C except for flrBG, which produced homozygous escapers at 18°C and also displayed rough eyes (Fig. 1A’). A genomic fragment encompassing the flr locus was able to rescue both the lethality and rough eye phenotype for all three alleles (Fig. 1D; supplementary material Fig. S1), indicating that they were bona fide flr mutations. Lastly, expressing flr RNAi in the eye disc in flip-out clones or by ey-Gal4 produced similar adult and larval eye defects (see Fig. 7F’; supplementary material Fig. S1D).

To determine the cause of the rough phenotype, we first examined the organization of ommatidial clusters in the mosaic larval eye epithelium. Immunostaining for Elav, a nuclear marker for photoreceptor (R) cell fate, showed that cells in flr mutant clones did acquire R fates but were disorganized in their arrangement within each ommatidial cluster, as compared with adjacent heterozygous (wild-type) tissues (Fig. 1B’). Consequently, spacing between mutant clusters was more irregular than between wild-type clusters. Labeling with Syp-β-gal (the R3, R4, R1, R6 marker) and antibody against Prospero (marking R7 and the 4 cone cells) further confirmed that most of the mutant clusters displayed the proper cell fates but were disorganized (Fig. 1B’). The disorganization was not caused by excessive apoptosis (supplementary material Fig. S2H). Lastly, the nuclear Elav staining in the mutant R cells appeared less intense than in neighboring wild-type R cells (Fig. 1B’), which was likely to be due to the more basal positioning of mutant nuclei (supplementary...
Disrupting AIP1 function affects actin cytoskeleton and AJ in preclusters and clusters

To reveal the underlying cause of cluster disorganization, we examined the actin cytoskeleton and epithelial morphology. Immunostaining using antibodies against Armadillo (Arm; β-catenin), E-cad (Shotgun – FlyBase) and α-catenin showed that AJ were significantly disrupted in the flr mutant clusters (Fig. 2A-B; supplementary material Fig. S2A-F). In wild type, AJ within a typical mature eight-cell (R1-8) cluster usually display six or seven tightly connected Arm-stained lines in the apical region, which is highly constricted (Fig. 2B'). In flr mutant clusters, these lines of strong staining were dramatically disrupted. Arm or α-catenin staining appeared disjointed, diffuse and sometimes punctate, and the apical region was no longer constricted (Fig. 2B',E'; supplementary material Fig. S2C',F'). To determine whether the flr mutation primarily affects AJ and not other junctional regions, we examined the distribution of the apical marker aPKC (part of the aPKC-Par3-Par6 complex) and the lateral (septate) junction marker Dlg1. In flr mutant clones, a cross-section reconstructed from a confocal z-stack showed that Dlg1 was distributed along the apical-basal axis normally (Fig. 2D'). Within mutant clusters, aPKC was localized at a region more apical than basally than in wild type (Fig. 2E-E'). Furthermore, a single confocal section taken in the apical plane showed that in both mutant and wild-type clusters aPKC staining appeared as a cap over the AJ of each cluster (Fig. 2E'), with the reduced staining intensity in the mutant clusters a likely result of the expanded apical surface. Together, these results indicate that flr mutation primarily affects AJ morphogenesis in the ommatidial clusters.

To determine whether the dramatic AJ disorganization in the ommatidial clusters arose from earlier AJ defects, we examined the process of precluster formation, as occurs in and immediately posterior to the MF. Phalloidin staining showed that F-actin levels were increased across the mutant tissue posterior to the MF, with the increase more prominent in preclusters and clusters (Fig. 2C',F; supplementary material Fig. S2B'). This is consistent with the proposed role of AIP1 as a co-factor of coflin in

![Image](https://example.com/figure1.png)

**Fig. 1.** flr mutations result in defects in the adult eye and larval eye disc. (A-A') Scanning electron micrographs of adult eyes from wild-type (w1118), homozygous flrHG and flrGF mosaic Drosophila. (B-B') Confocal micrographs of an flrGF mosaic larval eye disc showing Elav and Prospero staining. Clones are marked by the absence of GFP, and clones of significant size are outlined (white dotted line). (C-C') Magnified view of a mutant clone showing the presence of R3, R4, R1 and R6 in disorganized ommatidial clusters, as revealed by the merge of Elav and Svp-β-gal stainings. White box outlines a wild-type cluster and yellow box outlines a mutant cluster. (D) Structure of the flr locus showing three P-element insertions in the 5' UTR region and a GFP trap insertion in the first intron. A black line indicates the region encompassed by the genomic rescue fragment G2A, and red blocks indicate the exonic sequence used in the RNAi construct. (E) A western blot stained with antibody against Drosophila AIP1 shows significant reduction of AIP1 levels in the larvae of flrEP1, flrEP2 and flrHG, as compared with the wild type (w1118). (F) Quantification of data in E. (G) Western blot analysis of endogenous AIP1 and AIP1-GFP in w1118 and flrGFP larvae. (H) w1118 and flrGFP larval extracts were immunoprecipitated with anti-GFP-bound agarose beads and then immunoblotted with anti-cofilin. Scale bars: 100 μm in A-A'; 20 μm in B-C'.
disassembling F-actin. A cross-section showed that the increase in F-actin staining was largely confined to the apical and sup-apical region that includes AJs (Fig. 2C’), suggesting that AIP1 acts in this region of preclusters and clusters to regulate actin dynamics. By contrast, F-actin levels and cell shape remained unaffected in mutant clones anterior to the MF (Fig. 2C; supplementary material Fig. S2I’), suggesting that AIP1 function is required in regions of the eye epithelium that are undergoing more active morphogenesis (i.e. posterior to the MF). However, since the flr alleles used were all hypomorphic, we cannot rule out the possibility that AIP1 might still be required for morphogenesis of anterior tissue.

Upon closer examination of the MF-proximal area, we found clear F-actin defects in three to four columns of preclusters at various stages of formation (Fig. 3A-C’). Arm staining revealed that these preclusters exhibited disorganization in the AJ pattern compared with wild type (Fig. 2A-A’, Fig. 3B-F’; supplementary material Fig. S2E-E’). AJs within a typical wild-type precluster (containing the core of R8, the R2 R5 pair and the R3 R4 pair) usually appeared as four strongly stained lines between the five cells with future R fates, along with lines of lesser intensity between non-R cells (Fig. 2A’, Fig. 3B’). By contrast, in flr mutant preclusters, marked by the significantly increased cortical F-actin staining, these four Arm-stained lines between the five R cells had lost some of their staining intensity and became indistinguishable from AJs between other non-R cells (Fig. 2A’, Fig. 3C-C’).

Together, these data show that the actin disassembly function of AIP1 is required in epithelial cells undergoing morphogenesis posterior to the MF and that its activity is required to remodel AJs within the preclusters so as to achieve a polarized AJ distribution between the five R cells.

**Live imaging of eye discs reveals dynamic remodeling of AJs in and posterior to the MF**

Previous work by Wolff and Ready described various stages of precluster formation in the MF based on fixed samples (Fig. 3H) (Wolff and Ready, 1991). Recently, Escudero and co-workers performed live imaging analysis on the dynamic distribution of myosin II in the MF and in the preclusters, and they have traced precluster rotation (after preclusters have formed) in real time using Arm-GFP (Escudero et al., 2007). To our knowledge, no study has yet been done to specifically describe in detail how AJs are remodeled in real time during precluster formation. To better understand this dynamic morphogenetic event and the AJ remodeling defects resulting from flr mutation, we performed live imaging of eye discs using an Arm-GFP transgene to track the dynamic changes in AJs that occur during normal precluster formation.

Late third instar larval eye discs can be cultured in medium for 3 hours without obvious morphological defects, and Arm-GFP signals were indistinguishable from Arm staining in fixed tissues (Fig. 3B’,G). Live imaging revealed that a precluster comprising five R cells formed through a stereotyped process (Fig. 3I,I’; supplementary material Movie 1). The first visible sign of precluster formation was in the form of a large ‘rosette’ comprising an outer ring of ~13-16 cells and an inner core of about four to six cells, with the progenitors of five R cells always at the posterior end of the ring. At this initial stage, AJs between any two adjacent cells within the rosette displayed a similarly strong Arm-GFP signal, except that the AJs at the posterior end showed a slightly more intense signal, suggesting that the five R progenitor cells might have begun their specification at this stage. Sixty minutes later, the rosette was transformed into a
more elongated cluster by a series of cell shape changes accompanied by AJ remodeling, resulting in a distinct arc of about six cells (including five R cells) at the posterior end. Notably, the Arm-GFP signal within the arc was significantly stronger than outside the arc. After 90-150 minutes, the arc underwent extensive intercellular movement so that cells at either end of the arc that previously did not contact each other could now form AJs between them. As shown in Fig. 3I (at 60 minutes), the R3 and R4 precursors were originally far apart at either end of the arc. But by 150 minutes, R3 and R4 had established an AJ between them, such that the five R precursor cells could form a distinct and compact cluster with the trio of R8, R2 and R5 at the posterior and the R3 and R4 pair at the anterior. We refer to such a cluster at the end of precluster formation as a five-cell precluster.

In \( \text{flr}^{E_{2}P2} \) mutant clones, cells at the posterior position of a rosette often failed to remodel their AJs to first form a distinct arc and then a compact five-cell precluster (Fig. 3C-F). Interestingly, the arc in the wild-type cluster always proceeded through a transitory ‘six-cell rosette’ form (16 clusters examined; Fig. 3I, 120 minutes), in which the non-adjacent R3 and R4 remodeled their AJs with a common non-R cell, called the M cell (the ‘mystery’ cell, from older literature) (Wolff and Ready, 1991), to bring themselves into contact with each other, resulting in six cells (five R cells and one M cell) sharing a common AJ ‘vertex’. In mutant cells, such an intermediate form with strong Arm staining in the vertex was rarely observed (Fig. 3E, F). Furthermore, the general AJ organization in mutant cells, as shown by the continuous E-cad or Arm staining outlining each cell (Fig. 3F-F', supplementary material Fig. S2E-E'), appeared to be...
normal, unlike the highly disjointed AJs observed in the more mature ommatidial clusters (Fig. 2B’). Finally, live imaging of mosaic eye discs containing flr/EY-P2 mutant clones clearly showed defects in AJ (visualized with E-cad-GFP) remodeling in mutant preclusters, leading to a failure to form the six-cell rosette and five-cell precluster (Fig. 4; supplementary material Movie 2). There was inadequate intercellular movement to bring R3, R4 and M cells together as a six-cell rosette in the mutant (Fig. 4B’) in contrast to the wild type (Fig. 4A’). Taken together, these data indicate that disruption of AIP1 function affects remodeling but not general maintenance of AJs during precluster formation.

**Disrupting actin turnover affects AJ remodeling posterior to the MF**

Because AIP1 was thought to promote actin turnover and its loss of function results in elevated levels of F-actin in both preclusters and clusters as shown above, drugs that block turnover of F-actin could in theory phenocopy the AJ remodeling defects in precluster formation seen in the mutant tissues. To test this, we treated wild-type eye discs with jasplakinolide (Jasp), a drug that binds to and prevents F-actin from depolymerizing, thus acting as an inhibitor of actin turnover (Bubb et al., 1994). Eye discs cultured in control medium for 2 hours displayed normal MF, precluster and cluster morphologies, as shown by the E-cad-GFP signal (Fig. 5A,A’). By contrast, eye discs cultured in 2 μM Jasp for 2 hours exhibited strong AJ remodeling defects within and posterior to the MF, similar to those observed in the flr mutant clones (Fig. 5C,C’). In a control eye disc, different stages of precluster formation were clearly seen in four columns of equally spaced clusters behind the MF as rosettes, arcs or five-cell preclusters (Fig. 5A’). But in a Jasp-treated eye disc, these distinct forms were absent and few preclusters displayed the strong and asymmetric E-cad-GFP distribution pattern characteristic of a developing wild-type precluster (Fig. 5C’). Interestingly, both Jasp treatment and flr mutation caused a significant increase in apical surface area in the five R cells of preclusters as compared with the control (supplementary material Fig. S3K). Together, these data indicate that blocking actin turnover affects various AJ remodeling processes, including that during precluster formation, similar to the flr mutant phenotype (Fig. 2A’), further confirming that the AIP1 actin turnover function plays essential roles in AJ remodeling.

Treatment with the F-actin-destabilizing drug latrunculin A (Lat-A), which sequesters G-actin and thus induces rapid disassembly of F-actin, resulted in severe disruption of AJs within and posterior to the MF (Fig. 5B,B’). Eye discs cultured in 2 μM Lat-A for 2 hours exhibited a strong defect in the general organization of AJs, E-cad-GFP signals appeared to be mostly diffuse and disorganized and this effect was more dramatic in the seven to eight columns behind the MF (Fig. 5B), often accompanied by severe disruption of epithelial integrity around the MF region (supplementary material Fig. S3A). Together, these data suggest that Lat-A leads to disruption of cell adhesion and AJ integrity, whereas reduction of actin turnover from Jasp treatment results in insufficient AJ remodeling and thus a lack of intercellular movement within the epithelial sheet.

To further understand the dynamic nature of the actin cytoskeleton underlying AJs in the eye disc, we utilized a previously reported actin dynamics assay to label newly polymerized F-actin (Lee et al., 2009). Following incubation of eye discs in a high concentration (10 μM) of Jasp for 10 minutes to mask existing F-actin, immediate staining with Rhodamine phalloidin, which competes with Jasp for F-actin binding, would normally detect no signal (supplementary material Fig. S3A-A’). If Jasp-treated eye discs were incubated in drug-free medium to recover for a period of time, any newly polymerized F-actin could be labeled by staining with Rhodamine phalloidin at different time points. We found that after 90 minutes of recovery time, the four to eight columns behind the MF comprised the first region in the eye disc to show phalloidin staining, and the staining was most intense in the developing preclusters (supplementary material Fig. S3B-B’).
S3C-C′). After longer periods of recovery, additional phalloidin labeling could be detected in more posterior columns of ommatidial clusters (data not shown). These data suggest that actin polymerization is most robust in the region within or immediately posterior to the MF, particularly within individual preclusters undergoing morphogenesis. Lastly, when the same Jasp recovery assay was performed on mosaic eye discs containing flrEXP2 clones, we found that newly polymerized F-actin levels were increased within the mutant clones as compared with adjacent wild-type tissues (supplementary material Fig. S3D′). This result suggests that AIP1 functions to depolymerize newly polymerized F-actin, and the accumulation of newly polymerized F-actin underscores the importance of depolymerization during rapid actin treadmilling in the developing preclusters and clusters.

**Reduced actin turnover leads to decreased E-cad turnover within AJs of preclusters**

A previous study of *Drosophila* embryo morphogenesis demonstrated that homophilic E-cad clusters within the AJ underwent lateral mobility to allow AJ remodeling to take place (Cavey et al., 2008). Therefore, reducing actin turnover might affect AJ remodeling in the preclusters by restricting the lateral mobility of E-cad within AJs. To test this, we performed FRAP analyses on the effects of *flr* mutation and Jasp treatment on E-cad turnover in precluster AJs (Fig. 5G-J). AJ dynamics can also be regulated by clathrin-dependent endocytosis, as shown by previous reports, and cofilin is required for endocytosis in yeast (Bamburg, 1999; Nishimura and Takeichi, 2009). To address whether the AJ remodeling defects that result from AIP1 deficiency or Jasp treatment are due to a defect in endocytosis, we treated the eye disc with monodansylcadaverine (MDC), a drug that blocks clathrin-dependent endocytosis (Schütze et al., 1999). MDC treatment resulted in uniform elevation of E-cad levels throughout the eye disc (supplementary material Fig. S5B,B′), which is consistent with its endocytosis blocking effect, but this was never observed in *flr* or Jasp-treated eye discs. In MDC-treated discs, all stages of precluster formation were still clearly discernible except that the shapes of some preclusters appeared slightly different from those of wild type, whereas Jasp treatment and *flr* mutation both caused much more severe AJ remodeling defects including loss of distinct arc, six-cell rosette and five-cell precluster forms in the first four to five columns (Fig. 5D-F′, Fig. 5C′). Lastly, MDC did not result in any significant apical surface increase, in contrast to *flr* or Jasp-treated eye discs (supplementary material Fig. S3G). These results suggest that the AJ remodeling defects caused by AIP1 loss of function or Jasp treatment are unlikely to be due to a block in E-cad endocytosis.

**Inhibition of endocytosis results in different AJ phenotypes than AIP1 loss of function**

AJ dynamics and adherens junction remodeling

Inhibition of endocytosis results in different AJ phenotypes than AIP1 loss of function. AJ dynamics can also be regulated by clathrin-dependent endocytosis, as shown by previous reports, and cofilin is required for endocytosis in yeast (Bamburg, 1999; Nishimura and Takeichi, 2009). AIP1 is enriched in the apical region of preclusters whereas cofilin displays a diffuse localization. To further address the role of AIP1 in eye epithelial morphogenesis, we examined its distribution pattern. We obtained a protein-trap insertion allele, resulting in an N-terminal fusion of GFP to the AIP1 protein. This *flr*GFP allele was homozygous viable with no adult eye defects and generated only the AIP1-GFP fusion (94 kDa) and no endogenous AIP1 (67 kDa), as shown in a western blot using antibodies generated against full-length *Drosophila* AIP1 (Fig. 1G).
These data indicate that AIP1-GFP can fully replace the function of endogenous AIP1 in the homozygous flr<sup>GFP</sup> animals and can thus be used as a reliable marker for AIP1. Indeed, immunostaining with AIP1 antibody showed an almost identical staining pattern to that of AIP1-GFP (supplementary material Fig. S4B-B’); thus, we used flr<sup>GFP</sup> for all the distribution analyses below.

We found that anterior to the MF, AIP1 was cytoplasmic and diffusely localized, whereas posterior to the MF its levels were upregulated and AIP1 was enriched together with F-actin in the apical region of individual preclusters and clusters (Fig. 6; supplementary material Fig. S5), confirming the idea that AIP1 acts in the apical/AJ region of precluster cells to promote actin dynamics and AJ remodeling. Surprisingly, cofilin did not display enrichment in the AJ region of preclusters and clusters, and its distribution pattern posterior to the MF was much more uniform than that of AIP1 (Fig. 6C-C’). This difference in distribution patterns suggests a specific functional role of AIP1 in promoting F-actin dynamics underlying precluster or cluster AJs. Lastly, we found that AIP1-GFP was enriched in a variety of developing epithelial tissues including embryonic ectoderm undergoing gastrulation, follicle epithelium, pupal eye epithelia undergoing rhabdomere morphogenesis, larval wing disc epithelium and Malpighian tubule (supplementary material Fig. S4C-G). These data suggest that AIP1 could be an actin dynamics promoter that specifically regulates AJ function during epithelial morphogenesis.

**Cofilin is required for AJ remodeling and its overexpression rescues AIP1-deficient AJ defects**

To further determine whether cofilin plays a role in AJ remodeling in ommatidial preclusters and clusters, we examined the eye disc phenotype of mutants in *twinstar (tsr)*, the gene that encodes *Drosophila* cofilin. Since *tsr<sup>null</sup>* results in cell lethality and precludes the use of mosaic clones, we utilized two previously reported temperature-sensitive alleles of *tsr* for functional analysis (Blair et al., 2006). At the non-permissive temperature at 29° C, the *tsr<sup>V27Q</sup>*/*tsr<sup>I842</sup>* allelic combination produced similar ectopic F-actin and AJ remodeling defects (30.6% of third instar eye discs displayed defects, n=36) to those resulting from flr loss of function (Fig. 7C-D’). Furthermore, we generated GFP-labeled clones expressing *tsr* RNAi to knockdown *tsr* function using a flip-out method, and the resulting F-actin and AJ phenotypes were similar to those of *tsr* mutants. In the *tsr* mutant or knockdown eye discs, the various stages of precluster formation (including rosettes, arcs and five-cell preclusters) were not discernible in their distinct forms within and immediately posterior to the MF (Fig. 7C; supplementary material Fig. S6A-A’), and the more mature ommatidial clusters displayed highly disorganized AJs and a lack of apical constriction (Fig. 7D; supplementary material Fig. S6B-B’), similar to the *flr* mutant. Furthermore, F-actin levels were strongly increased in both preclusters and clusters, and a cross-sectional view showed that the strong F-actin accumulation occurred both apically and basally, which differs from the apical accumulation found in the *flr* mutant (Fig. 7C’-D’; supplementary material Fig. S6A’-B’). These results suggest that, like AIP1, the actin disassembly function of cofilin is also required for AJ remodeling. Interestingly, mutation in *slingshot (ssh)*, which encodes a cofilin phosphatase and activator) or *capt* (act-up), which encodes a cofilin activator that recycles cofilin and actin for depolymerization and polymerization, respectively) each caused AJ defects and F-actin accumulation in preclusters and clusters (supplementary material Fig. S6C-F’), consistent with previous reports (Benlali et al., 2000; Corrigall et al., 2007; Rogers et al., 2005).

To test whether *tsr* genetically interacts with *flr* in precluster and cluster formations, we overexpressed the *tsr<sup>+</sup>* transgene to rescue the *flr* mutant defects. Using flip-out, we generated clones expressing *UAS-flr* RNAi (together with *UAS-lacZ*), and the resulting phenotypes of increased F-actin level and disorganized AJs in precluster and cluster cells were the same as those of *flr* mutant clones (Fig. 7E-F’). Strikingly, overexpressing the *tsr<sup>+</sup>* transgene in these *flr* knockdown clones almost completely rescued the ectopic F-actin and AJ defects (Fig. 7G-H’). In fact, the previously increased F-actin level in the apical plane was suppressed below the wild-type level present in the adjacent tissues, while the morphology of preclusters and clusters appeared normal (Fig. 7G-G’), suggesting that an increase in cofilin-mediated actin dynamics is not detrimental to AJ remodeling. To determine whether cofilin physically interacts with AIP1 in vivo, an immunoprecipitation assay was performed using tissue extracts from third instar *flr<sup>GFP</sup>* larvae. The result showed that GFP-tagged AIP1 could be immunoprecipitated with cofilin in the extract (Fig. 1H), indicating a physical interaction between *Drosophila* cofilin and AIP1.

Interestingly, we observed a twofold (2.0±0.1, n=6) increase of cofilin levels in the apical region within the *flr* RNAi clones posterior to the MF (Fig. 7E-I; supplementary material Fig. S6H’), and this increase was not uniform in the clones and was more prominent within clusters in the more posterior region. However, no cofilin or F-actin increase or AJ defects were detected in *flr* RNAi clones anterior to the MF or in the larval wing disc epithelium (Fig. 7J-K’), where much less intercellular movement and AJ remodeling were apparent. These results suggest that differentiating epithelial cells undergoing robust
morphogenesis and AJ remodeling require a high level of actin dynamics and have a mechanism to sense ectopic F-actin (lack of actin dynamics), leading to elevation of the cofilin level in the AJ region to depolymerize the ectopic and less dynamic actin filaments present there. This twofold increase of cofilin activity was however insufficient to make up for the loss of AIP1 function. The complete rescue of AJ defects and F-actin increase achieved by a tenfold increase in cofilin levels (10.3±0.6, n=5; Fig. 7G–I) due to tsr transgene overexpression suggests that AIP1 can strongly enhance the actin disassembly activity of cofilin in the AJ region.

**DISCUSSION**

Here, we demonstrate an essential role for the actin disassembly factors AIP1 and cofilin in promoting the actin cytoskeleton dynamics underlying AJs, which is crucial for AJ remodeling during epithelial morphogenesis. Based on data from the distribution patterns and the genetic and physical interaction experiments, AIP1 appears to specifically act in the apical region to enhance cofilin-mediated actin dynamics. Such a high level of actin dynamics is crucial for AJ remodeling, as demonstrated by Jasp treatment, which phenocopied the flr or tsr AJ defects within and posterior to the MF. However, the distribution of cofilin is
diffuse and it seems to provide a basal level of actin dynamics, which might be generally required for multiple cellular processes but is not sufficient for AJ remodeling. Recent studies have shown that myosin II and Shh are enriched in the apical region of developing preclusters and clusters, similar to the AIP1 pattern (Escudero et al., 2007; Rogers et al., 2005). Thus, evidence suggests that the apical region of preclusters and clusters constitutes a distinct zone that contains activators of cofilin to effect a much enhanced rate of actin disassembly. Indeed, we and others have found that strong AJ defects in preclusters and clusters are caused by mutations in ssh or capt (Benlali et al., 2000; Corrigall et al., 2007; Rogers et al., 2005) (supplementary material Fig. S7), each of which encodes a cofilin activator.

A fundamental question in the field of epithelial morphogenesis and AJ remodeling is how cells both maintain general cell adhesion essential for their tight association and overall epithelial integrity and allow intercellular movement to take place within cell sheets. Results from our study support a model in which enhanced actin depolymerizing function is essential to allow these two seemingly opposing processes to take place simultaneously. First, active polymerization in the apical region would ensure a constant supply and a certain level of F-actin to be always available underlying AJs. Such a level of F-actin is essential for the general cell adhesion mediated by the E-cad–β-catenin–α-catenin complex connecting to the actin network, as blocking endogenous actin polymerization with Lat-A caused severe disruption of E-cad-GFP localization in the AJs within the MF and preclusters, often accompanied by collapse of epithelial integrity around this region. Indeed, the Jasp recovery assay demonstrates that actin polymerization appeared to be most robust in this MF-proximal region, and we also found that the actin polymerization factor Arp2/3 complex is upregulated posterior to the MF and is enriched in the apical/AJ plane, displaying a similar distribution pattern to that of AIP1 (supplementary material Fig. S5A–A’). Arp2/3 has been implicated to affect AJ remodeling through its direct interaction with α-catenin and the underlying actin network, or through its promotion of E-cad endocytosis (Georgiou et al., 2008; Harris and Tepass, 2010). However, what role Arp2/3 plays during AJ remodeling in the eye epithelium remains to be elucidated.

Second, AIP1, and probably other cofilin activators, provide much enhanced actin depolymerization underlying AJs to make the highly polymerized F-actin more dynamic or treadmilling at a faster pace. A rapidly turning over actin network has been shown to be essential for lateral mobility of E-cad clusters in AJs of embryonic epithelium, and excessive polymerization restricts the mobility (Cavey et al., 2008). Consistently, we showed that Jasp and flr mutation resulted in a significant reduction of the E-cad-GFP turnover rate in the precluster AJs and in a failure of precluster progenitor cells to undergo cell rearrangement. It is interesting to note that the AJs of preclusters within the four to five columns posterior to the MF displayed the most robust actin polymerization and were sensitive to both Jasp and Lat-A, suggesting that both actin polymerization and depolymerization need to be at high levels (i.e. fast actin treadmilling) to effect fast AJ remodeling in this morphogenetically active region of the eye disc.

Our live imaging study shows that precluster formation involves a series of stereotypical steps: from a large rosette of ~20 cells to an arc of about six or seven cells and finally a five-cell precluster, which is largely consistent with the description of the stages of precluster formation inferred from staining of fixed samples (Wolff and Ready, 1991). However, live imaging revealed new details of the dynamics of AJ remodeling. We found that this patterned morphogenetic program always involved a transient intermediate stage of a six-cell rosette, with the five R cells and one M cell sharing a common AJ vertex. Such a rosette is crucial for R3 and R4 to establish AJ contact with each other and for the M cell to be excluded from contact with R8, R2 and R5. In the flr mutant, these six-cell rosettes were rarely observed during precluster formation. Interestingly, similar kinds of multicellular rosette structures with a common vertex have been described in various epithelial morphogenetic systems including Drosophila embryonic ectoderm and in gastrulating and neurulating chick embryos undergoing primitive streak formation and neural plate invagination, respectively (Blankenship et al., 2006; Nishimura and Takeichi, 2008; Wagstaff et al., 2008), and blocking the function of the actomyosin network can prevent the formation of rosettes in these systems. Thus, our findings support the notion that the rosette-like structure could be a highly conserved intermediate form that is crucial for epithelial morphogenesis in general.

Acknowledgements

We thank Frank Laski for providing fly strains and critical comments on the manuscript; James Barnburg for antibody provision; and the Bloomington Drosophila Stock Center, Vienna Drosophila RNAi Center and Drosophila Genetic Resource Center for fly strains.

Funding

This work is supported by grants from the National Natural Sciences Foundation of China [90608018] and Ministry of Science and Technology [2007CB947101, 2006CB943503] to J.C.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.079491/-DC1

References


DEVELOPMENT


