AIP1 and coflin ensure a resistance to tissue tension and promote directional cell rearrangement

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In order to understand how tissue mechanics shapes animal body, it is critical to clarify how cells respond to and resist tissue stress when undergoing morphogenetic processes, such as cell rearrangement. Here, we address the question in the Drosophila wing epithelium, where anisotropic tissue tension orients cell rearrangements. We found that anisotropic tissue tension localizes actin interacting protein 1 (AIP1), a cofactor of coflin, on the remodeling junction via cooperative binding of coflin to F-actin. AIP1 and coflin promote actin turnover and locally regulate the Canoe-mediated linkage between actomyosin and the junction. This mechanism is essential for cells to resist the mechanical load imposed on the remodeling junction perpendicular to the direction of tissue stretching. Thus, the present study delineates how AIP1 and coflin achieve an optimal balance between resistance to tissue tension and morphogenesis.

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The global patterns of forces in a tissue (e.g., tissue tension/compression) control many aspects of development including cell proliferation, cell rearrangement, and cell polarity. Such control relies on the ability of cells to sense the distribution of forces and tune morphogenetic signaling pathways in response to the mechanical inputs. Moreover, cells must resist or release tension/compression when deforming, proliferating, and moving during development. While an understanding of molecular mechanisms for stress generation has evolved in the past decade, much less is known on how cells respond to and resist such stresses at the molecular level during morphogenesis.

The actin cytoskeleton is capable of sensing and resisting applied forces both at the network and filament levels. For example, mechanical strain on the actin network alters the structure of filament A, which crosslinks the orthogonal filaments, thus inhibiting the binding between filament A and a downstream signaling molecule. Single actin filaments decrease their helical pitch when mechanically relaxed, and such structural changes are amplified through positive feedback between F-actin twisting and coflin binding. The actin network increases its elasticity or reorients the stress direction to resist applied forces by changing filament dynamics and/or network architecture. Whether and how these force-responsive properties of the actin cytoskeleton and actin-binding proteins (ABPs) are involved in the development of multi-cellular tissue is largely unknown.

During morphogenesis, cells change their relative positions along the tissue axis by remodeling cell contact surfaces. This process, called directional cell rearrangement, shapes a tissue and develops its multi-cellular pattern. The Drosophila pupal wing epithelium provides an excellent model system to study the mechanism through which tissue tension controls directional cell rearrangement. Starting ~15 h after puparium formation (h APF), forces generated in the hinge stretch the wing along the proximal-distal (PD) axis (Supplementary Figure 1a-d). The resulting anisotropic tissue tension acts as a mechanical cue to specify the axis of cell rearrangement. Wing cells relocalize myosin-II (myo-II) at the adherens junction (AJ) that runs along the PD axis (PD junction) to resist tissue tension, and the balance between extrinsic stretching force and intrinsic cell junction tension favors PD cell rearrangement, thereby accelerating relaxation into a hexagonal cell pattern (hereafter called hexagonal cell packing; Supplementary Figure 1c, d). This relaxation may be primarily driven through interface mechanics, consistent with the observation of shear-induced reconnection of interfaces and hexagonal lattice formation in foam, non-biological soft matter. However, in biological tissues such as the wing epithelium, interface mechanics must be orchestrated with molecular regulators of cytoskeleton and cell adhesion (e.g., force-responsive ABPs) responsible for responding to and resisting tissue tension. Answering the question in the wing should provide a general mechanism of epithelial development, as all cell rearrangements are associated with sensation and resistance to forces from the surrounding cells.

Here, we show that actin regulation mediated through actin interacting protein 1 (AIP1) and coflin is responsible for supporting tissue tension-driven cell rearrangement and hexagonal cell packing in the Drosophila pupal wing. AIP1 is evolutionarily conserved from yeast to humans. In vitro studies have shown that AIP1 binds coflin and F-actin and promotes F-actin severing via coflin. In vivo, AIP1 and coflin control F-actin disassembly and remodeling during development. We show that AIP1 is localized on the remodeling anterior–posterior (AP) junctions of wing cells, and tissue stretch is necessary for the biased redistribution of AIP1. Inhibition of actin turnover by AIP1 or coflin loss-of-function (l-o-f) results in the detachment of myo-II from the AP junctions, which hampers the stabilization of newly formed PD junctions. Interestingly, the disorder of junctional actomyosin is rescued by releasing tissue tension. Together, our data illustrate that actin turnover ensures a resistance to anisotropic tissue tension and promotes directional cell rearrangement by reinforcing the structural stability of remodeling junctions. This proposed mechanism is likely to be relevant to development of other epithelial tissues in which tissue tension coordinates with morphogenetic signaling pathways in individual cells.

Results

AIP1 is localized on remodeling AP junctions. To investigate the molecular mechanisms through which cells respond to and resist tissue tension during directional cell rearrangements in the Drosophila wing, we screened candidate ABPs (Supplementary Methods). First, we examined the subcellular distribution of 19 ABPs at 24 h APF. ABPs demonstrating interesting localization patterns were assayed for l-o-f phenotypes. Since the polygonal distribution of cells is much easier to measure than the dynamics of cell rearrangement, we used the fraction of hexagonal cells at 32 h APF, when the hexagonal cell packing process involving directional cell rearrangement is nearly complete, as a proxy for cell rearrangement defects. As described below, screening identified AIP1, which promotes F-actin severing via coflin, as a potential key regulator of cell rearrangement in the wing.

During the screening process, we detected a strong signal for GFP-tagged endogenous AIP1 along a subset of AP junctions at 24 h APF (Fig. 1a, b; Supplementary Figure 1e; see Supplementary Note 1 and Supplementary Figure 2a, c for characterization of flare (flr) (Drosophila aip1 gene) protein trap line). The directional bias in the AIP1 distribution became weaker at 28 h APF, when tissue tension is thought to contribute less to directional cell rearrangement (Supplementary Figure 1f, g). Time-lapse analysis confirmed that AIP1 accumulated on AP junctions during junctional remodeling, and AIP1 gradually disappeared from newly formed PD junctions (Fig. 1c; Supplementary Movie 1). In contrast, AIP1-GFP signal intensity remains low at stable AP junctions (Fig. 1g,h; Supplementary Movie 2), indicating that AIP1 specifically localizes to remodeling AP junctions.

Fluorescence recovery after photobleaching (FRAP) of utrABD-GFP showed a smaller fraction of stable F-actin at the AP junctions than at the PD junction, which is in agreement with the AIP1 subcellular localization (Fig. 1i–l; Welch’s t-test, P < 0.01). Next, we examined the localization of AIP1 and its cofactor coflin along the apico-basal axis. We confirmed that AIP1 is enriched at the A1 plane, whereas coflin is diffusely distributed, as has been reported in the eye disc (Fig. 1c, d and Supplementary Figure 3f–h; see Supplementary Note 1 and Supplementary Figure 2b, c for characterization of twinstar (tsr) (Drosophila coflin gene) protein trap line). In the flr RNAi wing, hexagonal cell packing was disrupted, consistent with a defect in directional cell rearrangement (Fig. 2a, b, d; Welch’s t-test, P < 0.001). These results prompted us to further investigate a role for AIP1 in tissue tension-driven cell rearrangement.

Tissue tension-dependent localization of AIP1. To characterize the mechanism by which tissue tension acts on AIP1, we examined whether tissue tension is required to bias the subcellular distribution of AIP1. To this end, we relaxed the tissue stretch by detaching the wing from the hinge. AIP1 was more evenly distributed along junctions in the mechanically relaxed wing (Fig. 3a–d). WT wing cells accumulate F-actin along the PD rather than AP junctions, and such F-actin localization becomes non-polarized by relaxing the tissue stretch (Fig. 3e, f). Therefore, our data argue against the possibility that the loss of accumulation of AIP1 along the AP junctions in Fig. 3c, d is simply a result of...
the change in F-actin localization. For comparison, the same experiment was performed with another ABP, Enabled (Ena), which promotes the elongation of actin filaments. As shown in Fig. 3g, h, Ena retained its localization at junctions and vertices in the absence of an extrinsic stretching force. Thus, tissue tension-dependent subcellular localization is specific to a subset of ABPs, including AIP1 and myo-II.

In addition to mechanical anisotropy, the wing develops a planar cell polarity (PCP) along the in-plane axis of the epithelium, which may bias AIP1 localization. However, AIP1-dependent subcellular localization is specific to a subset of ABPs, including AIP1 and myo-II. In addition to mechanical anisotropy, the wing develops a planar cell polarity (PCP) along the in-plane axis of the epithelium, which may bias AIP1 localization. However, AIP1-dependent subcellular localization is specific to a subset of ABPs, including AIP1 and myo-II.
Fig. 1 AIP1 localizes to remodeling AP junctions in the Drosophila pupal wing. a AIP1-GFP (left) and an AJ marker, Dcx-tag-TagRFP (right) in the WT wing at 24 h APF. Blue arrowheads indicate AP junctions. b Directional bias of AIP1-GFP signal intensity (left) and Dcx-tag-TagRFP signal intensity (right) along the junctions. Each junction is divided into twelve bins according to its angle relative to the PD axis, and the average signal intensity in each bin is plotted. Blue arrows indicate the length $R_i$ and orientation $\theta$ of an angular mean vector (Methods and Supplementary Figure 1e)\(^3\), c, d Top-view (c) and side-view (d) of cells expressing AIP1-GFP (left) and Dcx-tag-TagRFP (right) at 24 h APF. The vertical section along the dashed arrow in panel c is shown in d. Alphabets indicate corresponding coordinates in different views. e, g Selected snapshots from movies of AIP1-GFP (gray in top panels, green in bottom panels) and Dcx-tag-TagRFP (red in bottom panels). Blue arrowheads indicate remodeling (e) and stable (g) junctions. f, h Plot of AIP1-GFP signal intensity (green, left y-axis) and length (blue, right y-axis) of AP junctions undergoing cell rearrangement (f) or not (h). In f, $t = 0$ indicates the time point when the junction was reconnected. Data shown in h indicate that bleaching was negligible. i Snapshots of utrABD-GFP before and after photobleaching (square indicates ROI). $t = 0$ indicates the time point when photobleaching was conducted. j, k utrABD-GFP in ROIs examined before and after photobleaching along the AP (j) and PD (k) junctions. Color maps are shown in the upper right. i The stable fraction of utrABD-GFP. Welch’s t-test: AP vs. PD, $P < 0.01$. The number of junctions examined is indicated (b, f, h, i). Data are presented as the mean ± s.e.m. (b) and as the mean ± s.d. (f, h, i). Scale bars: 5 µm (a, e, g), 2 µm (c, d, i), and 0.5 µm (j).

Fig. 2 AIP1 and coflin are required for the efficient hexagonal cell packing and directional cell rearrangement. a-c Images of DE-cad-GFP with the indicated genotypes (a WT at 32 h APF; b flr RNAi at 32 h APF; and c tsr RNAi at 28 h APF at 29 °C, which corresponds to ~32 h APF at 25 °C). Cells are colored according to the number of junctions (red, square; green, pentagon; gray, hexagon; blue, heptagon; and magenta, octagon). d Table of the fraction of hexagonal cells for each indicated genotype. Welch’s t-test: WT (25 °C) vs. flr RNAi, $P < 0.001$, WT (29 °C) vs. tsr RNAi, $P < 0.001$. e Schematic of cell rearrangement analysis. We tracked individual junctions that appeared in a movie and measured their angle relative to the PD axes ($\theta$) of newly generated junctions based on cell rearrangement. $\theta_{final}$ and $\theta_{all}$ are the sets of angles from the final round and all rounds of cell rearrangement, respectively, from which the magnitude of directional bias $R_{final}$ and $R_{all}$ were calculated (METHODS). $N_{seq}$ and $N_{all}$ indicate the numbers of remodeled junctions and cell rearrangements, respectively. f-h Quantification of cell rearrangement for each genotype based on time-lapse data captured at 24–27 h APF at 25 °C (WT, flr RNAi) and time-lapse data acquired at 20.5–23 h 20 m APF at 29 °C (tsr RNAi). i PD (red) and other (gray) fractions of $\theta_{final}$. Classification of $\theta_{final}$ is illustrated with a semicircle. j tsr RNAi. Quantification of cell rearrangement for each genotype. k Dunnett’s test: WT vs. flr RNAi, $P < 0.001$, WT vs. tsr RNAi, $P < 0.001$. h Dunnett’s test: WT vs. flr RNAi, $P < 0.01$, WT vs. tsr RNAi, $P < 0.01$. The number of pupae (d), and the numbers of events and pupae examined (f-h) are indicated. Data are presented as the mean ± s.d. (d, f-h). Scale bar: 20 µm (a).

AIP1-coflin are required for efficient cell rearrangement. Next, we examined whether and how the depletion of AIP1 affects directional cell rearrangement. We expressed dsRNA against flr using the Gal4-UAS system combined with a temperature shift (Supplementary Note 2). Under experimental conditions, GFP-tagged endogenous AIP1 was not detected in flr RNAi cells (Supplementary Figure 2d-f). We also confirmed that flr RNAi did not alter tissue stress anisotropy per se (Supplementary Figure 4; Dunnett’s test, $P > 0.9$)\(^4\). The angles of new junctions generated by cell rearrangement were measured in time-lapse movies obtained at 24–27 h APF. When junctions underwent multiple rounds of remodeling, final and preceding rounds of junctional remodeling were considered separately for analysis ($\theta_{final}$ and $\theta_{all}$ and $N_{seq}$ and $N_{all}$ in Fig. 2e; METHODS). This is because the former affects the steady alignment of hexagonal cells along the PD axis that accelerates the formation of a hexagonal pattern (Supplementary Figure 1d), whereas the latter is an intermediate process of searching for an efficient relaxation pathway\(^5\). flr RNAi did not lower the frequency of cell rearrangement (Supplementary Figure 5; Dunnett’s test, $P > 0.05$ for $N_{seq}/N_{cell}$ and $P > 0.05$ for $N_{all}/N_{cell}$). Both $\theta_{final}$ and $\theta_{all}$ became less biased toward the PD axis following flr RNAi, and the difference between WT and flr RNAi was larger for $\theta_{final}$ than for $\theta_{all}$ (Fig. 2f–h; Dunnett’s test, $P > 0.001$ for $R_{final}$ and $P < 0.01$ for $R_{all}$; semicircle in Fig. 2f shows angle classification). Contraction-elongation was affected by flr RNAi as expected from disoriented cell rearrangement (Supplementary Figure 6a, b).

As AIP1 is a cofactor of coflin, we examined whether coflin is involved in regulating AIP1 localization and PD cell rearrangement. In vitro studies, the binding affinity of AIP1 for F-actin...
is much weaker in the absence of coflin, and coflin binds to actin filaments for a longer period of time when F-actin severing is suppressed through the loss of cofactor proteins, including AIP1. In S. cerevisiae, coflin hypomorph mutation leads to the diffusive distribution of AIP1 in the cytosol, whereas aip1 null mutation moderately alters the subcellular localization of coflin. We determined whether these phenomena were also observed in the Drosophila pupal wing. In tsr dsRNA-expressing cells, in which GFP-tagged endogenous coflin fluorescence was not detected (Supplementary Figure 2j), AIP1 was diffusely distributed in the AJ plane, with little accumulation at the AJ plane along the apico-basal axis (Supplementary Figure 3b-e). Fmi dsRNA-expressing cells exhibited abnormal aggregates of coflin in the cytosol (Supplementary Figure 3f-k). The enrichment of coflin at the junction is more evident along the plane of AJ, presumably because coflin remained in actin cables along the AJ
plane for a longer period of time. Collectively, AIP1 and cofilin are mutually dependent for their localization, and phenotype quality and strength were conserved between fly and yeast. Next, we addressed the requirement for cofilin in directional cell rearrangement. *tsr* RNAi, without changing the inferred tissue stress anisotropy (Supplementary Figure 4; Dunnett’s test, \( P > 0.9 \)), caused defects in hexagonal cell packing (Fig. 2c, d; Welch’s \( t \)-test, \( P < 0.001 \)), directional cell rearrangement (Fig. 2f–h; Dunnett’s test, \( P < 0.001 \) for \( R_{\text{final}} \) and \( P < 0.01 \) for \( R_{\text{initial}} \)), and contraction-elongation (Supplementary Figure 6c), similar to that caused by *flr* RNAi.

### The acute delocalization of AIP1 impairs cell rearrangement

We also pharmacologically inhibited the functions of AIP1/cofilin using the F-actin stabilization drug Jasplakinolide (Jasp). Jasplakinolide facilitates F-actin by competitively inhibiting cofilin binding to F-actin and decreasing the monomer off rate at filament ends.\(^{38,49}\)

As expected from the uniform distribution of AIP1 in *tsr* RNAi cells (Supplementary Figure 3c), the biased distribution of AIP1 was lost in wings incubated with Jasplakinolide for 4 h (Fig. 4a–c). In contrast, the average signal intensity of AIP1-GFP along junctions was not significantly affected (Fig. 4d; Welch’s \( t \)-test, \( P > 0.2 \)). Under these conditions, the directionality of cell rearrangement was weakened (Fig. 4e–g; Welch’s \( t \)-test, \( P < 0.01 \) for \( R_{\text{final}} \) and \( P < 0.05 \) for \( R_{\text{initial}} \)), and the overall extent of contraction-elongation was decreased (Supplementary Figure 6d, e). In summary, the acute delocalization of AIP1 resulted in disoriented cell rearrangement, highlighting the importance of the subcellular localization of AIP1 in regulating directional cell rearrangement and suggesting that *flr* 1-o-f phenotypes are unlikely to reflect early developmental defects.

Thus far, we showed that tissue tension specifies AIP1 localization in a cell, and AIP1 and its cofactor cofilin are required for efficient directional cell rearrangement. Thus, next undertook experiments to answer three questions. First, how do AIP1 and cofilin promote directional cell rearrangement? Second, whether and how do AIP1 and cofilin facilitate a resistance to tissue tension during directional cell rearrangement? Third, how are AIP1 localization and function related to tissue tension?

### Fmi and Dsh polarities are largely normal in *flr* RNAi cells

Previous studies reported that hexagonal cell packing is regulated through the PCP pathway via cadherin trafficking in the *Drosophila* pupal wing, and AIP1 is required for PCP establishment.\(^{34,37,50}\) Thus, AIP1 potentially controls cell...
rearrangement via the PCP pathway in the wing. However, we observed the enrichment of Fmi and Disheveled (Dsh) at AP (vertical) junctions in flr RNAi cells, similar to WT cells (Supplementary Figure 7b, d, f, h). To quantitatively evaluate these observations, we measured the orientation of Fmi and Dsh polarities in each cell using PCP nematics (Supplementary Figure 7a). According to this analysis, an off-axis fraction was detected for the Fmi polarity of flr RNAi cells (Supplementary Figure 7c, e; Watson’s test, P < 0.001), and Dsh polarity distribution was indistinguishable between WT and flr RNAi (Supplementary Figure 7g, i; Watson’s test, P > 0.4). The discrepancy with previous studies may result from different I-o-f conditions employed (see Supplementary Note 2 for details).

To address how the Fmi polarity map affects cell rearrangement, we transiently overexpressed fmi-GFP, which has been shown to be a functional allele, and measured the orientation of cell rearrangement in such tissues. fmi-GFP overexpression abolished Fmi polarity (Supplementary Figure 8a, b; Watson’s test, P < 0.001), whereas the directionality of cell rearrangement was only mildly affected compared with that of flr RNAi cells (Supplementary Figure 8c-e; Welch’s t-test, P > 0.2 for $R_{\text{fmi}}$ and P < 0.01 for $R_{\text{flr}}$). From these results, we concluded that cell rearrangement defects observed in the flr RNAi wing is unlikely to reflect PCP signaling dysfunction.

Fig. 5 The global myo-II polarization pattern is not affected by flr RNAi. a, c myo-II-GFP (left) and the AJ marker Dv-cat-TagRFP (right) in WT (a) and flr RNAi (c) wings at 24 h APF. Yellow and blue arrowheads indicate PD and AP junctions, respectively. b, d Directional bias of myo-II-GFP signal intensity (left) and Dv-cat-TagRFP signal intensity (right) along junctions in WT (b) and flr RNAi (d) wings was quantified as have been done in Fig. 1b. The number of junctions examined (b, d) is indicated. Data are presented as the mean ± s.e.m. (b, d). Scale bars: 5 μm (a, c).

The detachment of myosin cables along remodeling junctions. We next focused on myo-II, a well-established regulator of cytoskeletal dynamics during junctional remodeling. Previously, we showed that myo-II is localized on PD junctions in response to tissue stretching. This global myo-II polarization pattern was not affected by flr RNAi (Fig. 5). However, a local structural change in junctional actomyosin was observed during cell rearrangement, myo-II formed a small ring-like structure, accompanied by D-α-catenin (D-α-cat) signal loss, in the short junctions of WT cells (Fig. 6a; hereafter called the myo-II ring), and much larger myo-II rings were detected in flr or tsr RNAi cells (Fig. 6b, c, j; Steel test, WT vs. flr RNAi, P < 0.001, WT vs. tsr RNAi, $P < 0.001$). The septate junction (SJ) protein Discs large (Dlg) was present along short junctions (Fig. 6d–f), indicating that the myo-II ring structure did not represent cell extrusion; instead, it is generated by the detachment of myosin cables around the cell vertex (a similar subcellular structure was reported in ref.53), and the larger myo-II ring may represent its precocious formation. Time-lapse analysis showed that the myo-II ring was only transiently formed immediately prior to the reconnection of cell contact surfaces in WT cells. In contrast, the myo-II ring was present for a longer duration in flr RNAi cells and occasionally failed to convert into stable junctions, potentially resulting in disoriented cell rearrangement (Fig. 6l–n).

Mechanical rescue of myo-II ring enlargement. Interestingly, myo-II ring enlargement in flr RNAi cells was partially rescued by relaxing tissue stretch beginning at 15 h APF, when the extrinsic force begins to stretch the wing (Fig. 7a, b; Steel–Dwass test, WT vs. flr RNAi, P < 0.001, WT vs. flr RNAi + hinge cut, P > 0.4, flr RNAi vs. flr RNAi + hinge cut, P < 0.001). This result suggests that AIP1 prevents the detachment of junctional actomyosin, which runs perpendicular to the direction in which the wing is stretched, by anisotropic tissue tension (Fig. 7c).

Actin turnover ensures mechaeresistance of junctions. To characterize the molecular mechanism by which AIP1 and coflin regulate the reorganization of junctional actomyosin, we searched for molecules that connect actomyosin to the AJ in an AIP1/cofilin-dependent manner. Canoe (Cno) (Drosophila Afadin), which maintains the linkage between the actin cytoskeleton and the AJ in epithelial cells, is one such candidate. We observed the specific loss of Cno in short junctions following flr or tsr RNAi (Fig. 6g–i). Moreover, cno RNAi induced the detachment of junctional actomyosin in wing cells (Fig. 6k). Together, these data indicate that disruption of the Cno-mediated linkage between actomyosin and the AJ leads to precocious formation of the myo-II ring.

This observation is similar to that of a previous study in which the inhibition of actin turnover destabilized the attachment of the actomyosin network to AJs during apical cell constriction. FRAP analysis consistently showed that AIP1 and coflin were required for efficient actin turnover along AP junctions (Fig. 8a–e; Steel test, WT vs. flr RNAi, P < 0.01, WT vs. tsr RNAi, P < 0.001). In addition, ectopic F-actin accumulation, which may potentially reflect sites of impaired actin turnover, was detected in RNAi cells (Supplementary Figure 9). Application of the F-actin

\[ \text{mechanical force} \]
stabilization drug Jasp from 15 to 24 h APF induced myo-II ring enlargement and the loss of Cno from the short AP junctions (Fig. 8f, g, i–k). Furthermore, myo-II ring size returned to normal values following tissue stretch relaxation (Fig. 8h, i; Steel–Dwass test, DMSO vs. Jasp, \( P < 0.001 \), DMSO vs. Jasp + hinge cut, \( P > 0.8 \), Jasp vs. Jasp + hinge cut, \( P < 0.001 \)). Collectively, these data show that AIP1 and coflin promote actin turnover to regulate the Cno-dependent reorganization of junctional actomyosin in a specific region and at the appropriate time during cell rearrangement. This mechanism is essential for cells to resist the mechanical load imposed on the remodeling AP junction in a process of complete directional cell rearrangement.

Cooperative coflin binding links tissue tension and AIP1. Finally, we addressed how tissue tension is related to AIP1 localization and function (Fig. 9). Actin filaments alter their helical pitch in response to applied forces, and these structural changes are amplified by positive feedback between coflin binding and F-actin twisting (Fig. 10a)\(^{15,17–19} \). This suggests that in the pupal wing, F-actin along the PD axis, which is under tension, adopts a less twisted configuration, leading to the preferential binding of coflin and AIP1 to more twisted F-actin. This is under tension, adopts a less twisted configuration, leading to the preferential binding of coflin and AIP1 to more twisted F-actin. To examine this hypothesis, we took advantage of an actin mutant that inhibits cooperative coflin binding to F-actin (yeast act5C\(^{14,16} \))\(^{37} \). Following misexpression of the corresponding mutant Drosophila actin5C (act5C) gene, AIP1-GFP did not accumulate at AP junctions (Fig. 9a, b). In contrast, the global polarity of myo-II distribution was not significantly affected, although myo-II-GFP signals became diffuse along the junction (Fig. 9c, d). Moreover, the misexpression of mutant act5C caused the detachment of junctional actomyosin and the AJ (Fig. 9e, f; Wilcoxon rank sum test, \( P < 0.001 \)), resulting in impaired hexagonal cell packing (Fig. 9g–i; Welch’s \( t \)-test, \( P < 0.001 \)). These developmental defects were specifically
induced by the mutant Actin but not its wild-type counterpart (Supplementary Figure 10). Thus, our data support the hypothesis that the cooperative binding of cofilin to twisted, mechanically relaxed F-actin links anisotropic tissue tension and AIP1 localization and function (Fig. 10b).

**Discussion**

In addition to global patterning determined by signaling molecules, global mechanical patterning represents another strategy for facilitating the long-range coordination of tissue development. This concept raises the questions as to how tissue-scale forces regulate biochemical signaling within or between cells and how cells subjected to tissue-scale forces maintain structural integrity when undergoing morphogenetic processes. The results of the present study highlight that actin regulation mediated through AIP1 and cofilin achieves an optimal balance between resistance to anisotropic tissue tension and morphogenesis.

The current working hypothesis (Fig. 10b) is that cells respond to anisotropy in tissue tension via the cooperative binding of cofilin to twisted F-actin, leading to AIP1 accumulation along remodeling AP junctions. AIP1 and cofilin facilitate a resistance to anisotropic tissue tension and maintain junction stability, thereby supporting PD cell rearrangement. Our observation that the misexpression of mutant act5C induced the delocalization of AIP1 and phenocopied flr RNAi is consistent with the current working hypothesis that postulates the positive feedback between AIP1 binding and F-actin twisting. Future development of a biophysical method to visualize the helical pitch of actin filaments inside a cell will enable a more direct test.

We speculate that the myo-II ring is required to reconnect cell contact surfaces at four-way junctions. AIP1 and cofilin likely protect this temporally loosened junctional structure from anisotropic tissue tension by promoting actin turnover and strengthening the linkage between actomyosin and AJ via Cno, the Drosophila Afadin protein. Detailed molecular mechanisms that link actin turnover, junctional actomyosin, and E-cad/α-cat/Afadin remain elusive. Rapid actin turnover can prevent the aggregation of a contractile actin network and thereby maintain its uniform network structure, which may be required for stable binding of F-actin to E-cad/α-cat/Afadin. AIP1-mediated actin turnover can also activate biochemical signaling that

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**Fig. 7** myo-II ring enlargement in flr RNAi cells is rescued by relaxing tissue stretch. **a** Images of myo-II-GFP (gray in a left panel, green in a right panel) and Dα-cat-TagRFP (gray in a middle panel, red in a right panel) in a mechanically relaxed flr RNAi wing. The wing was detached from the hinge at 15 h APF and observed at 24 h APF. The blue arrowhead indicates a myo-II ring on the short remodeling junction. **b** The myo-II ring area is plotted for each genotype (WT and flr RNAi data were same as that shown in Fig. 6j). Steel-Dwass test: WT vs. flr RNAi, P < 0.001, WT vs. flr RNAi + hinge cut, P > 0.4, flr RNAi vs. flr RNAi + hinge cut, P < 0.001. The difference between flr RNAi and flr RNAi + hinge cut was statistically significant after excluding outliers above 1.2 μm². **c** Schematic of the defects resulting from genetic or pharmacological manipulations to stabilize F-actin. AJ (E-cad) (yellow), α-catenin (green), Cno (light blue), actomyosin (red), cofilin (magenta), AIP1 (orange), Dlg (gray), and tissue tension generated by the extrinsic stretching force (blue arrows). Top (top) and side (bottom) views are shown. Left: In WT tissue, cells maintain AJ and actomyosin integrity along the AP junction in the presence of anisotropic tissue tension. Right: F-actin stabilization weakens Cno-mediated linkage between actomyosin and AJ. Subsequently, actomyosin detaches from the AP junction. The number of ROIs examined is indicated (b). Data are presented as the mean ± s.d. (b). Scale bar: 2 μm (a).
controls linker proteins, including α-cat and Afadin. In addition, it may potentially speed up lateral mobility of E-cad clusters to AIP1; however, this observation also implies the existence of distribution during development according to tissue tension patterns. First, tissue tension acts as a mechanical cue to bias protein localization and morphogenetic cell processes, thereby strengthening adjustability and flexibility to regulate development. The conversion of tissue tension into structural changes in the actin filament/network via ABP interactions may be a conserved strategy for mechanotransduction, although conclusive, direct evidence in vivo is required. Second, global mechanical patterning must be reconciled to maintain the structural integrity of the cell/tissue. Future studies should explore whether the molecular mechanisms identified in the present study potentially function in other developmental contexts, such as cell proliferation and apoptosis, and/or in other epithelial tissues.

**Methods**

**Generation of transgenic flies.** To construct pUAST-attB-act5CWT, pUAST-attB-act5CG147V, and pUAST-attB-flr, act5C was PCR-amplified from a cDNA clone (Drosophila Genomics Resource Center #LD18090). The G147V mutation was introduced using the GeneArt® Site-Directed Mutagenesis System (Thermo Fisher Scientific) and introduced into YC480 (AttB1) using the GATEWAY® Site-Directed Mutagenesis System (Thermo Fisher Scientific) and introduced into YC480 (AttB1) using the GATEWAY® System (Invitrogen). The resulting act5CG147V and act5C WT constructs were digested using SacI and NotI, ligated into pUAST-attB with the same restriction enzymes, and transformed into the ERW2 strain. Act5C RNAi was generated using the GeneArt® RNAi Expression Kit (Thermo Fisher Scientific) and introduced into the ERW2 strain.

**Image analysis**

The area of myo-II rings was plotted for each condition. Steel–Dwass test: DMSO vs. Jasp, P < 0.001. DMSO at 24 h APF; and Jasp-treated (k) wings at 24 h APF. Blue arrowheads indicate the short AP junctions. The number of junctions examined (d, e) and the number of ROI examined (i) are indicated. Data are presented as the mean ± s.d. (d, e, i). Scale bars: 0.5 μm (a) and 2 μm (f, j).
Cooperative binding of cofilin to F-actin is required for the biased distribution of AIP1, the integrity of junctional actomyosin, and hexagonal cell packing. a AIP1-GFP (left) and the AJ marker Dα-cat-TagRFP (right) in an actSC147V misexpressed wing at 20.5 h APF at 29 °C, which corresponds to ~24 h APF at 25 °C. b Directional bias of AIP1-GFP signal intensity (left) and Dα-cat-TagRFP signal intensity (right) along junctions in actSC147V misexpressed cells was quantified as described for Fig. 1b. c myo-II-GFP (left) and the AJ marker Dα-cat-TagRFP (right) in an actSC147V misexpressed wing at 20.5 h APF at 29 °C. Yellow and blue arrowheads indicate PD and AP junctions, respectively. d Directional bias of myo-II-GFP signal intensity (left) and Dα-cat-TagRFP signal intensity (right) along junctions in an actSC147V misexpressed wing. e Images of myo-II-GFP (gray in a left panel, green in a right panel) and Dα-cat-TagRFP (gray in a middle panel, red in a right panel) in an actSC147V misexpressed wing. Blue arrowhead indicates myo-II ring. f Images of DE-cad-GFP with the indicated genotypes at 28 h APF at 29 °C, which corresponds to ~32 h APF at 25 °C (g, WT; h, actSC147V misexpression). Cells are colored according to the number of junctions. i Table listing the fractions of hexagonal cells for each genotype. Welch’s t-test: WT vs. actSC147V, p < 0.001. j, k Images of AIP1-GFP (left) and the AJ marker Dα-cat-TagRFP (right) in an actSC147V misexpressed wing at 20.5 h APF at 29 °C, which corresponds to ~24 h APF at 25 °C. Yellow and blue arrowheads indicate PD and AP junctions, respectively. Images of AIP1-GFP (gray in a left panel, green in a right panel) and Dα-cat-TagRFP (gray in a middle panel, red in a right panel) in an actSC147V misexpressed wing. Blue arrowhead indicates myo-II ring. l Images of DE-cad-GFP with the indicated genotypes at 28 h APF at 29 °C, which corresponds to ~32 h APF at 25 °C (g, WT; h, actSC147V misexpression). Cells are colored according to the number of junctions. i Table listing the fractions of hexagonal cells for each genotype. Welch’s t-test: WT vs. actSC147V, p < 0.001. j, k Images of AIP1-GFP (left) and the AJ marker Dα-cat-TagRFP (right) in an actSC147V misexpressed wing at 20.5 h APF at 29 °C, which corresponds to ~24 h APF at 25 °C. Yellow and blue arrowheads indicate PD and AP junctions, respectively. Images of AIP1-GFP (gray in a left panel, green in a right panel) and Dα-cat-TagRFP (gray in a middle panel, red in a right panel) in an actSC147V misexpressed wing. Blue arrowhead indicates myo-II ring. l Images of DE-cad-GFP with the indicated genotypes at 28 h APF at 29 °C, which corresponds to ~32 h APF at 25 °C (g, WT; h, actSC147V misexpression). Mean ± s.d. (%). j, k, n = 380 for each genotype.

Western blotting. Pupae of yw, flareGFP, or tsrGFP at 24–28 h APF were lysed in Laemmli’s sample buffer (62.5 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 0.001% bromophenol blue, and 713 mM β-mercaptoethanol). The lysates were boiled for 20 min and these samples were loaded into a 10% polyacrylamide gel. After SDS-PAGE, samples were transferred to a PDMS membrane (Bio-Rad), blocked with 3% BSA-TBS (20 mM Tris (pH 7.2), 150 mM NaCl) and incubated with anti-GFP (1/1000, SantaCruz sc-9996) or anti-α-tubulin (1/2000, MBL PM054) diluted in 3% BSA-TBS overnight at 4 °C. The membrane was washed with TBS-0.05% tween20 and incubated with HRP-conjugated secondary antibodies (ThermoFisher) diluted in 3% BSA-TBS for at least 1 h at room temperature. Blots were developed using ECL Western Blotting Detection Reagents (GE healthcare) and imaged using LAS-4000 IR multi color (FUJIFILM). An original scan of the western blot is shown in Supplementary Figure 11.

Drosophila genetics. Fly strains and a list of genotypes are summarized in Supplementary Methods.
Fig. 10 Summary and working hypothesis. a Schematic showing tension on actin filaments and their twisting, which have been reported by previous studies using in vitro reconstituted actin filaments5,17-19. Actin filaments (red) alter their helical pitch in response to applied forces (blue arrows). collin (magenta) preferentially binds to twisted, mechanically relaxed actin filaments. b Summary of the working hypothesis. See the main text for details. Upper and bottom dashed arrows indicate an unidentified pathway regulating directional cell rearrangement downstream to AIP1/collin or actin turnover, which may potentially function via E-cad transport36.

Immunohistochemistry and phalloidin staining. Anti-Coo (1/400)63, anti-Dlg (1/500)64, anti-Dah (1/1000)65, and anti-Fmi (1/10)45 antibodies were used. Pupae at appropriate ages were dissected, and wings were fixed at room temperature for 30 min in PBS containing 4% paraformaldehyde. After washing with PBS containing 0.1% Triton X-100, these preparations were incubated overnight at 4 °C with the indicated antibodies.

To visualize F-actin in wing cells, dissected wings were incubated overnight at 4 °C with Alexa Fluor 546 Phallolid (1/1000, invitrogen A22283). The condition of dissecting and fixing pupae was same as above.

Image collection. To prepare the Drosophila pupal wing samples for image collection, pupae at appropriate ages were fixed to double-sided tape and the pupal case above the left wing was removed. The pupae were then placed on a small drop of water or Immersol W 2010 (Zeiss 444969-0000-000) in a glass bottom dish with the left side facing downward45,46,48. The fixed time-point images other than Fig. 3a, b and time-lapse images shown in Fig. 6, m were acquired using an inverted confocal microscope (A1R; Nikon) equipped with a ×60/NA1.2 Plan Apochromat water-immersion objective at 25 °C. Other images were acquired using an inverted confocal spinning disk microscope (Olympus IX83 combined with Yokogawa CSU-W1) equipped with an iXon3 888 EMCCD camera (Andor), an Olympus ×60/N1.2 Splanapo water-immersion objective, and a temperature chamber (TOKAI HIT), using IQ 2.9.1 (Andor)66. After imaging, we manually assessed when necessary. By repeating this automatic detection and subsequent time points immediately prior to photobleaching and subtracted the residual value immediately after photobleaching. A FRAP recovery curve was constructed by fitting the normalized FI to F(t) = (1-exp(-r−t)) using MATLAB, from which the stable fraction of utrABD-GFP was calculated.

Image analysis

Subcellular distribution of proteins. To characterize directional bias in the subcellular distribution of AIP1-GFP, myo-II-GFP, and the AJ marker Dac-tagRFP, fluorescent signals along the AJ plane were extracted from a snapshot of live cells (0.094 µm/pixel). Subsequently, the following procedure was applied. First, the background signal was subtracted using the “subtract background” command (r = 100) in ImageJ. Second, to avoid counting the signals at the vertices for all associated junctions, we omitted the signals for 2 pixels at the ends of the junctions when calculating the mean signal intensity along each junction. Each junction was divided into twelve bins according to its angle relative to the PD axis, and the average signal intensity in each bin was plotted. In addition, we calculated the magnitude Rθ and the orientation θ of the polarity, defined as R(θ) = |e(θ)| = |sin θ - cos θ| < 0.5 /

Subcellular distribution of proteins. To characterize directional bias in the subcellular distribution of AIP1-GFP, myo-II-GFP, and the AJ marker Dac-tagRFP, fluorescent signals along the AJ plane were extracted from a snapshot of live cells (0.094 µm/pixel). Subsequently, the following procedure was applied. First, the background signal was subtracted using the “subtract background” command (r = 100) in ImageJ. Second, to avoid counting the signals at the vertices for all associated junctions, we omitted the signals for 2 pixels at the ends of the junctions when calculating the mean signal intensity along each junction. Each junction was divided into twelve bins according to its angle relative to the PD axis, and the average signal intensity in each bin was plotted. In addition, we calculated the magnitude Rθ and the orientation θ of the polarity, defined as R(θ) = |e(θ)| = |sin θ - cos θ| < 0.5 /

Statistics. Data are presented as the mean ± s.e.m. in plots of the signal intensity of AIP1-GFP, myo-II-GFP, and Dac-tagRFP in each angular bin and as the mean ± s.d. in the other plots. P-values were calculated in R based on Welch’s t-test.
Figure 4, Supplementary Figure 5a, b, the Steel test (Fig. 6j, Fig. 8e), the Steel test was less than six and/or data follow the normal distribution. The Dunnett or Steel test was performed when analyzing multiple comparisons between the WT and other groups (the Steel test is a non-parametric equivalent of Dunnett’s test). The Steel-Dwass test was performed when comparing each pair in all groups.

Data availability. The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary files, and available from the corresponding author upon reasonable request. Plasmids and fly stocks generated in this study are available from the corresponding author.

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Author contributions

K.I. and K.S. designed the research and analyzed the data. K.I. performed the experiments and K.S. assisted with the experiments. K.S. drafted the manuscript, with inputs from K.I. All authors approved the final manuscript.

Additional information

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