The reorganization of cells in response to mechanical forces converts simple epithelial sheets into complex tissues of various shapes and dimensions. Epithelial integrity is maintained throughout tissue remodeling, but the mechanisms that regulate dynamic changes in cell adhesion under tension are not well understood. In *Drosophila melanogaster*, planar polarized actomyosin forces direct spatially organized cell rearrangements that elongate the body axis. We show that the LIM-domain protein Ajuba is recruited to adherens junctions in a tension-dependent fashion during axis elongation. Ajuba localizes to sites of myosin accumulation at adherens junctions within seconds, and the force-sensitive localization of Ajuba requires its N-terminal domain and two of its three LIM domains. We demonstrate that Ajuba stabilizes adherens junctions in regions of high tension during axis elongation, and that Ajuba activity is required to maintain cell adhesion during cell rearrangement and epithelial closure. These results demonstrate that Ajuba plays an essential role in regulating cell adhesion in response to mechanical forces generated by epithelial morphogenesis.

**Introduction**

During development, epithelial cells undergo dynamic changes in cell interactions that are necessary for tissue organization, remodeling, and repair. Adherens junction complexes are essential regulators of cell adhesion that are necessary for cell interactions and epithelial integrity. Functional complexes play critical roles in sustaining and transmitting mechanical forces between cells, but they can also be dynamically assembled and disassembled in response to force (Harris and Tepass, 2010; Lye and Sanson, 2011; Takeichi, 2014). In particular, epithelial cells are exposed to strong mechanical forces during development, when epithelial tissues undergo rapid reorganization in response to developmental signals. Mechanical forces are critical for epithelial morphogenesis in vivo and can influence adherens junction composition, organization, and dynamics in vitro (Gumbiner, 2005; Baum and Georgiou, 2011; Leckband and de Rooij, 2014; Hoffman and Yap, 2015). However, the mechanisms that maintain epithelial integrity under tension, and how these processes are regulated by the physiological forces experienced by cells in vivo, are not well understood.

Mechanical forces can have profound effects on the organization and stability of adherens junction complexes and the nature of the proteins associated with them. In cultured cells, high levels of tension disrupt cell adhesion (Sahai and Marshall, 2002), whereas intermediate levels of tension promote adherens junction clustering (Shewan et al., 2005) and growth (Yamada and Nelson, 2007). Mechanical load produces conformational changes in the core adherens junction protein α-catelin (Yonemura et al., 2010; Yao et al., 2014) and can directly promote the association between α-catelin and F-actin in vitro (Buckley et al., 2014). Many proteins are recruited to sites of increased tension in cells, indicating that a wide range of mechanotransduction mechanisms are activated at adherens junctions (Leerberg and Yap, 2013; Leckband and de Rooij, 2014). One class of proteins that responds to mechanical forces is the zyxin family of LIM domain proteins (Kadrmas and Beckerle, 2004; Schimizzi and Longmore, 2015). Zyxin localizes to sites of increased tension in cells, including adherens junctions, focal adhesions, and actin stress fibers (Yoshigi et al., 2005; Hirata et al., 2008; Sperry et al., 2010; Schiller et al., 2011; Smith et al., 2013; Oldenburg et al., 2015). Other members of the zyxin family, including the Ajuba proteins, localize to adherens junctions and focal adhesions (Marie et al., 2003; Rauskolb et al., 2014; Dutta et al., 2018; Ibar et al., 2018). Ajuba has been implicated in several biological processes, including Hippo signaling (Das Thakur et al., 2010; Reddy and Irvine, 2013; Rauskolb et al., 2014), cell differentiation (Kanungo et al., 2000; Feng et al., 2007), cell migration (Kisseleva et al., 2005; Pratt et al., 2005), and cell proliferation (Kanungo et al., 2000; Hirota et al., 2003). Ajuba binds to the core adherens
junction protein α-catenin (Marie et al., 2003) and localizes to adherens junctions in the Drosophila melanogaster wing disc and in cultured mammalian epithelial cells in a tension-dependent fashion (Rauskolb et al., 2014; Ibar et al., 2018). Loss of Ajuba reduces keratinocyte cell aggregation in culture, in part as a result of altered Rac GTPase signaling (Marie et al., 2003; Nola et al., 2011; McCormack et al., 2017). However, despite intriguing links between Ajuba and adherens junctions, it is not known if Ajuba regulates cell adhesion or the cellular response to mechanical forces in vivo.

In contrast to the three Ajuba proteins in mammals—Ajuba, LIMDI, and WTIP—Drosophila has a single Ajuba protein. Drosophila Ajuba regulates Hippo signaling in the larval wing disc (Das Thakur et al., 2010; Reddy and Irvine, 2013; Rauskolb et al., 2014) but has not been shown to affect cell adhesion in this tissue, in which cells are relatively static (Gibson et al., 2006). During convergent extension in the Drosophila embryo, adherens junctions are dynamically remodeled in the plane of the tissue, inducing spatially regulated cell rearrangements that elongate the head-to-tail body axis (Blankenship et al., 2006; Simões et al., 2010; Levayer et al., 2011; Tamada et al., 2012). Planar polarized actomyosin networks associated with adherens junctions generate contractile forces that drive cell rearrangement (Bertet et al., 2004; Zallen and Wieschaus, 2004; Blankenship et al., 2006; Rauzi et al., 2008; Fernandez-Gonzalez et al., 2009). Here we show that Ajuba localizes to adherens junctions in a spatiotemporally regulated fashion during axis elongation. Ajuba localization is modulated by dynamic changes in actomyosin activity, and the tension-sensitive localization of Ajuba requires its N-terminal domain and two of the three LIM domains. We demonstrate that Ajuba stabilizes adherens junction proteins in regions of high tension during axis elongation, and Ajuba activity is required to maintain cell adhesion during cell rearrangement and epithelial closure in the developing embryo. These results demonstrate that Ajuba is a dedicated junctional regulator that is required to maintain cell adhesion in the presence of mechanical forces during epithelial remodeling.

Results

Ajuba localizes to a subset of adherens junctions during convergent extension

To investigate the role of Ajuba in epithelial remodeling, we first examined Ajuba localization during convergent extension in the Drosophila germband epithelium using a functional Ajuba-GFP fusion (Sabino et al., 2011). In stage 6 embryos before axis elongation, Ajuba-GFP localized primarily to cell vertices (Fig. 1A, −5 min). During axis elongation in stages 7 and 8, Ajuba-GFP localized to cell–cell contacts in a planar polarized fashion (Fig. 1A, 0–15 min). Ajuba-GFP was enriched 1.9 ± 0.1-fold (mean ± SEM) at vertical cell edges, which represent interfaces between neighboring cells along the anterior-posterior (AP) axis, compared with horizontal edges, which correspond to interfaces between dorsal and ventral cells (Fig. 1B). Consistent with findings in other tissues (Marie et al., 2003; Rauskolb et al., 2014), Ajuba junctional localization in the Drosophila embryo requires α-catenin. Reduction of α-catenin levels by RNA interference resulted in a complete loss of Ajuba from cell interfaces, despite the continued presence of β-catenin (Fig. S1, A and B). These results demonstrate that Ajuba localizes to adherens junctions in a dynamic and spatially regulated fashion during convergent extension.

Ajuba junctional localization is regulated by actomyosin contractility

We next investigated whether the spatiotemporal pattern of Ajuba localization is controlled by myosin contractility, as Ajuba localization is regulated by myosin activity in other contexts (Rauskolb et al., 2014; Ibar et al., 2018). During convergent extension, myosin II levels and mechanical tension are selectively increased at vertical cell interfaces (Bertet et al., 2004; Zallen and Wieschaus, 2004; Blankenship et al., 2006; Rauzi et al., 2008; Fernandez-Gonzalez et al., 2009; Kasza et al., 2014). In particular, mechanical tension is highest at linked vertical cell edges that form mechanically integrated, supracellular myosin cables within the tissue (Fernandez-Gonzalez et al., 2009). To determine whether Ajuba localization is sensitive to myosin levels, we analyzed the correlation between Ajuba-GFP and Myo-mCherry (a fusion of the myosin II regulatory light chain to mCherry; Martin et al., 2009). Ajuba and myosin levels at cell interfaces were positively correlated; cell interfaces with high myosin levels also had high levels of Ajuba (Fig. 1, C and D). Notably, Ajuba junctional localization was highest at linked vertical interfaces in supracellular cables (Fig. S2, A and B), which have the highest levels of myosin (Fig. S2 C) and are under the highest tension (Fernandez-Gonzalez et al., 2009). Ajuba did not colocalize with other myosin structures, such as myosin pulses at the medial cell cortex (Fig. S1 C), and Ajuba-GFP displayed a punctate localization at vertical cell interfaces that correlated more strongly with E-cadherin than with myosin (Fig. S1, D–F). These results indicate that Ajuba localizes to a subset of adherens junctions that are predicted to be under high tension during convergent extension.

Myosin localization at the cell cortex during convergent extension is highly dynamic, and pulses of myosin accumulation and dissociation occur on a timescale of seconds (Fig. 1, E and F; Rauzi et al., 2010). Ajuba localization was similarly dynamic: Ajuba levels at adherens junctions rapidly increased after pulses of myosin localization and rapidly diminished as myosin pulses dissipated (Fig. 1, E and F). To investigate the temporal relationship between Ajuba and myosin, we analyzed the relative timing of changes in Ajuba and myosin intensity in time-lapse videos of embryos imaged at 10-s intervals. The strongest correlations between the changes in intensity of Ajuba and myosin were observed when the Ajuba values were shifted back in time by 10 s, suggesting that transient increases in myosin at adherens junctions precede Ajuba localization (Fig. 1 G). Much weaker correlations were detected between myosin and a general membrane marker (Fig. 1 G). These results indicate that Ajuba is rapidly recruited to adherens junctions at the same time as or closely following dynamic increases in myosin localization.

To examine whether the planar polarized localization of Ajuba requires myosin contractility, we used genetic and pharmacological methods to investigate the effects of altering myosin activity. Myosin activity was reduced by peritelline injection of Y-27632, an inhibitor of Rho-kinase, which is required for
myosin cortical localization throughout axis elongation (Bertet et al., 2004; Simões et al., 2010). In addition, we reduced myosin activity partway through elongation by genetically removing the myosin activator Shroom, which is required to maintain myosin cortical localization in stage 8 (Simões et al., 2014). Ajuba-GFP localization to vertical edges was strongly reduced in Y-27632–injected and Shroom mutant embryos, abolishing Ajuba planar polarity (Fig. 2, A, B, D, and E). Conversely, we increased myosin activity by overexpressing the ShroomA isoform of Shroom, which promotes Rho-kinase and myosin localization to adherens junctions (Nishimura and Takeichi, 2008; Bolinger et al., 2010; Simões et al., 2014). ShroomA overexpression enhanced myosin localization at tricellular vertices before axis elongation (Fig. 2, C and F) and at adherens junctions during elongation (Figs. 2 G and S1, D, and E). Shroom overexpression recruited Ajuba to both myosin-positive domains (Fig. 2, C, F, and G; and Fig. S1, D, and E). Ajuba levels at cell interfaces correlated with myosin levels on an embryo-by-embryo basis in Shroom-overexpressing embryos, suggesting that Ajuba junctional localization is proportional to myosin activity (Fig. 2 G). However, the finer-scale, punctate pattern of Ajuba localization correlated better with E-cadherin than with myosin when only vertical edges were considered (Fig. S1, D–F), consistent with the idea that Ajuba associates with a subset of adherens junctions that are under increased tension. Together, these data demonstrate that myosin promotes Ajuba junctional localization and planar polarity during convergent extension.

The preLIM domain and LIM domains 1 and 2 are necessary and sufficient for Ajuba tension-sensitive localization

To understand how Ajuba localization is regulated by myosin activity, we first analyzed the domains required for Ajuba junctional localization. Ajuba is a 718-aa protein that contains a 505-aa N-terminal (preLIM) domain and three C-terminal LIM domains, each consisting of two tandem zinc finger motifs (Fig. 3 A; Michelsen et al., 1993; Goyal et al., 1999). The preLIM domain of mammalian Ajuba interacts with Rac GTPase and F-actin, and the LIM domains (and, to a lesser extent, the preLIM domain) interact with α-catenin (Marie et al., 2003; Nola et al., 2011). We
tagged Ajuba variants with a C-terminal monomeric superfolder Venus (msVenus) tag and expressed them under the control of a maternal Gal4 driver (Fig. 3 A). Variants that contain the Ajuba preLIM domain were expressed at similar levels, but variants lacking this domain were more weakly expressed (Fig. S3 A). Ajuba-msVenus was enriched at vertical edges in a planar polarized fashion (Fig. 3, B–D), similar to the localization of Ajuba-GFP (Fig. 1 A). In contrast, the Ajuba preLIM domain alone displayed weak cortical localization and was primarily cytoplasmic, and the LIM domains alone localized to the nucleus, failing to associate with the cortex even when fused to a nuclear export sequence that restricted the protein fusion to the cytosol (Fig. 3 C). Adding the preLIM domain to LIM domains 1 or 2, but not LIM domain 3, slightly increased Ajuba junctional localization, and adding both LIM domains 1 and 2 (the preLIM+12 variant) strongly enhanced Ajuba localization to both vertical and horizontal edges compared with the preLIM domain alone (Fig. 3, B and C). Despite the strong junctional localization of the preLIM+12 variant, this variant was not planar polarized (Fig. 3 D). This loss of planar polarity was not caused by a loss of myosin planar polarity, which was unaltered in preLIM+12-expressing embryos (Fig. S3, B and C). Other combinations of two LIM domains were not sufficient to promote strong Ajuba junctional localization (Fig. 3, B and C), and none of the Ajuba deletion variants were planar polarized (Fig. 3 D). These results demonstrate that the LIM domains of Ajuba are functionally distinct, and that the preLIM and all three LIM domains are necessary for Ajuba planar polarity.

To determine which domains are required for the modulation of Ajuba localization by actomyosin contractility, we analyzed the response of Ajuba variants to increased tension caused by Shroom overexpression. Shroom overexpression in stage 6 embryos strongly enhanced the localization of Ajuba-msVenus to cell vertices (Fig. 4, A and B). In contrast, most of the Ajuba deletion variants responded weakly or not at all to Shroom overexpression (Fig. 4, A and B). The one exception was the Ajuba preLIM+12 variant, which was strongly recruited to vertices in Shroom-overexpressing embryos, to a similar extent as the full-length protein (Fig. 4, A and B). These results indicate that the preLIM domain and LIM domains 1 and 2 are necessary and sufficient for Ajuba to respond to increased tension caused by Shroom overexpression.
Ajuba regulates dynamic cell rearrangements during convergent extension

Thus far, we have shown that Ajuba localizes to adherens junctions in a tension-dependent fashion during convergent extension, during which planar polarized contractile forces drive oriented cell rearrangements within the tissue (Bertet et al., 2004; Zallen and Wieschaus, 2004; Blankenship et al., 2006; Rauzi et al., 2008; Fernandez-Gonzalez et al., 2009). These results raise the possibility that Ajuba could play a role in regulating cell adhesion in response to actomyosin contractility. To test this idea, we performed time-lapse imaging of convergent extension in embryos expressing β-catenin-GFP (McCartney et al., 2001; Videos 1 and 2). Embryos that lack maternal Ajuba expression were generated using two null alleles: AjubaII, which removes Ajuba and the neighboring gene (AMP-deam; Das Thakur et al., 2010), and Ajuba54, a targeted deletion of the Ajuba ORF that we generated using CRISPR mutagenesis (Fig. S4, A and B; Port et al., 2014). The complete loss of maternal and zygotic Ajuba function caused 100% penetrant lethality by the pupal stage, with some lethality at earlier stages (Fig. S4, C and D). The effects on convergent extension were similar in the presence or absence of zygotic Ajuba activity (Fig. S5, A, B, and D–G), and these embryos were combined for analysis (Figs. 5, 6, and 7).

Cell intercalation during convergent extension in Drosophila is driven by two types of cell rearrangement: rearrangements among four cells, in which a single vertical edge contracts to form a four-cell vertex, also known as a T1 process (Weaire and Rivier, 1984; Bertet et al., 2004), and rosette rearrangements in which multiple linked edges contract to bring five or more cells together at a single point (Blankenship et al., 2006; Fig. 5 A). In both cases, the formation of new contacts between cells that were previously separated promotes vertex resolution, which completes the cell rearrangement. As Ajuba preferentially localizes to linked shrinking edges in forming rosettes (Fig. S2, A and B), we analyzed rosette behaviors in Ajuba mutants. In WT embryos, T1 processes and rosettes occur in an ∼2:1 ratio (Fig. 5 B; Farrell et al., 2017). In contrast, more shrinking edges joined rosettes in Ajuba mutants, resulting in an increase in the number of rosette structures and nearly equal frequencies of rosettes and T1 processes (Fig. 5, B–D). This phenotype was rescued by expressing full-length Ajuba, demonstrating that the cell rearrangement defects in Ajuba mutants are caused by the loss of Ajuba activity (Fig. S3, D and E). In addition, the increased rosette formation in Ajuba mutants was rescued by the Ajuba preLIM+12 variant, but not the preLIM+3 variant (Fig. S3, D and E). These results indicate that the localization of Ajuba to adherens junctions, but not
necessarily its planar polarized distribution, is important for its functions in cell rearrangement.

To identify the mechanisms that lead to increased rosette formation in Ajuba mutants, we analyzed cell rearrangements in more detail. Rosettes could form in one of two ways. In one mechanism, two or more connected edges could contract simultaneously (Fig. 6, A and B, top). Alternatively, edges could contract sequentially, first producing a four-cell vertex that remains stable for an extended period of time (defined here as ≥3 min) before joining a rosette (Fig. 6, A and B, bottom). Simultaneous contraction is the predominant mechanism of rosette formation in WT (Fig. 6 C). In addition, the percentage of shrinking edges that formed rosettes through simultaneous contraction was unchanged in Ajuba mutants. In contrast, Ajuba mutants displayed a more than threefold increase in the percentage of shrinking edges that formed rosettes through sequential contraction (Figs. 6 C and S5 E), accompanied by a decrease in the frequency of Ti processes (Figs. 5 C and S5 B). Therefore, the loss of Ajuba results in an increase in rosette formation through the conversion of Ti processes into rosettes through sequential contraction, a mechanism that rarely leads to rosette formation in WT.

The higher number of rosettes in Ajuba mutants could be caused by an increase in actomyosin contractility, which is required for rosette formation, or a defect in cell adhesion, which is dynamically regulated during rosette formation and resolution. To distinguish between these possibilities, we used laser ablation, a measure of the relative forces acting at cell edges, to investigate whether myosin contractility is altered in Ajuba mutants. The initial retraction velocity in response to ablation is predicted to be proportional to the force acting on that edge before ablation, assuming that the viscoelastic properties of the tissue are constant (Hutson et al., 2003). Retraction velocities after ablation are higher at vertical edges than at horizontal edges in WT embryos, demonstrating that mechanical forces in this tissue are planar polarized (Rauzi et al., 2008; Fernandez-Gonzalez et al., 2009). We observed a similar spatial pattern of retraction velocities in Ajuba mutants, indicating that mechanical forces are correctly generated and organized in the absence of Ajuba activity (Fig. S5, H and I). Consistent with these results, the total number of shrinking edges was not altered in Ajuba mutants. In WT embryos, 87 ± 1% of edges that were vertical at the onset of elongation contracted to a vertex, compared with 82 ± 2% in Ajuba<sup>fl</sup> and 87 ± 2% in Ajuba<sup>54</sup> embryos (mean ± SEM, n = 12 embryos, 50 edges tracked per embryo). These results indicate that Ajuba does not affect the level or distribution of myosin contractility. To test the alternative possibility that Ajuba mutants have defects in cell

Figure 4.  Ajuba variants are differentially regulated by tension. (A) Images of Ajuba-msVenus variants in stage 6 WT and ShroomA-overexpressing (Shroom OE) embryos. LIM only and LIM-NES images were acquired at a higher gain and in a more basal plane to show nuclear localization (LIM only) or exclusion (LIM-NES). Images are anterior left, ventral down. Bar, 10 µm. (B) Vertex enrichment of Ajuba variants in WT (gray) and Shroom OE (white) embryos. Vertex intensities were divided by the cytoplasmic intensity to calculate the vertex enrichment. The mean ± SEM between embryos is shown (n = 8 embryos/variant, 30 vertices analyzed/embryo); *, P < 0.05; **, P < 0.001 compared with full-length Ajuba (Ajuba-FL); one-way ANOVA with Fisher’s least significant difference test. See also Fig. S3.
adhesion, we analyzed the formation of new contacts between cells. We found that high-order vertices were slower to resolve for both T1 processes and rosettes in Ajuba mutants (Figs. 6 F and S5 F). In addition, there was a significant increase in the percentage of rosettes that did not resolve by the end of elongation (Figs. 6 G and S5 G). Despite these defects, a majority of rosette structures completed resolution, and the extent of tissue elongation was not affected in Ajuba mutants (Fig. S5 C). These results indicate that Ajuba regulates the nature and dynamics of cell rearrangement during convergent extension.

Ajuba is required to maintain cell adhesion in regions of high tension

The defective rosette behaviors that occur despite normal levels of myosin contractility in Ajuba mutants are suggestive of a role for Ajuba in regulating cell adhesion. However, it is not immediately clear how defects in cell adhesion could lead to increased rosette formation and reduced rosette resolution. In one model, cell adhesion at multicellular vertices could be increased in Ajuba mutants, allowing cells to remain in higher-order configurations for a longer time. Alternatively, cell adhesion could be reduced in Ajuba mutants, producing gaps between cells that impede the formation of new cell contacts required for vertex resolution. To distinguish between these possibilities, we analyzed cell behaviors in time-lapse videos of Ajuba mutant embryos. In WT embryos, adherens junction proteins are tightly apposed at interfaces between adjacent cells (Fig. 6 D and Video 3). This distribution indicates that cell adhesion is normally maintained throughout junctional remodeling. In contrast, Ajuba mutants often displayed aberrant gaps between cells that appeared to represent breaks in adhesion (Fig. 6 D and Video 4). These defects were most pronounced in rosettes: 40 ± 4% of rosettes in AjubaII mutants and 37 ± 3% of rosettes in Ajuba54 mutants displayed a visible gap between cell interfaces at late stages of rosette formation, compared with 9 ± 2% of rosettes in WT embryos (Figs. 6 E and S5 D). Transient gaps between cells in Ajuba mutants could account for the delay in the resolution of high-order vertices (Fig. 6 F and G). These results indicate that Ajuba is required to maintain cell adhesion at late stages of rosette formation, when cells are predicted to be under the highest level of tension within the tissue.

We next asked if the cell adhesion defects in Ajuba mutants are caused by a mislocalization of adherens junction proteins. The apical distributions of the core adherens junction proteins α-catenin and β-catenin were not obviously affected in Ajuba mutants (Fig. 7, A, B, and E). However, unlike WT embryos, α-catenin
and β-catenin were often absent from rosette vertices in Ajuba mutants (Fig. 7, A and B). In addition, E-cadherin localization at shrinking edges was often faint and diffuse, and cortical myosin structures in neighboring cells often appeared as two parallel lines in Ajuba mutants, in contrast to a single line in WT (Fig. 7 C). Defects in myosin localization at shrinking edges were not associated with a loss of myosin planar polarity or with obvious gaps between cells until late stages of rosette formation. These results suggest that subtle defects in protein localization at shrinking edges in Ajuba mutants precede a more pronounced separation of cells at or near rosette vertices. Although α-catenin localization was visibly disrupted at the apical cell surface, cell contacts appeared intact in more basal planes (Fig. 7 D). These results indicate that Ajuba is required to stabilize cell adhesion and apical adherens junction localization during convergent extension.

Dorsal closure defects in Ajuba mutants are enhanced by reducing E-cadherin

We next sought to determine whether the roles of Ajuba during convergent extension reflect a broader requirement for Ajuba activity in stabilizing cell adhesion under tension. In particular, Ajuba function is expected to be particularly important for tissues in which the level of tension is high or the level of adhesion is low. To investigate this possibility, we analyzed epithelial remodeling in another context in which cells are exposed to strong mechanical forces during development. Dorsal closure is a morphogenetic process driven by actomyosin contractility in the late Drosophila embryo (Kiehart et al., 2000; Hutson et al., 2003). During this process, lateral epidermal sheets on both sides of the embryo come together over the amnioserosa and fuse at the dorsal midline (Fig. 8 B and Video 5; Harris, 2017). High levels of tension are generated at the interface between the lateral epidermis and the amnioserosa, but, despite this tension, these tissues remain tightly apposed throughout closure. Mutants defective for adherens junction proteins disrupt this attachment, resulting in the separation of the amnioserosa from the lateral epidermis (Gorfinkiel and Martinez-Arias, 2007). We found that Ajuba-GFP localizes to the leading edge of the lateral epidermis in a punctate fashion (Fig. 8 A), coinciding with adherens junctions that are associated with a
tensile actomyosin cable (Kiehart et al., 2000; Gorfinkiel and Martinez-Arias, 2007). Time-lapse imaging of dorsal closure in stage 14 embryos revealed the formation of small gaps between the lateral epithelium and the amnioserosa during the final zipper phase of dorsal closure in Ajuba mutants (mild defect, Fig. 8 D and Video 7). In addition, a subset of embryos displayed a larger separation of the epidermis from the amniosera at earlier stages of closure (moderate defect, Fig. 8 E and Video 8). These results indicate that Ajuba is required to maintain cell adhesion under tension during dorsal closure. If this is the case, then reducing cell adhesion is predicted to enhance the defects in Ajuba mutants. To test this, we introduced one mutant allele of E-cadherin (Drosophila shotgun (shg)) into Ajuba maternal and zygotic mutants to reduce zygotic E-cadherin levels. Reducing zygotic E-cadherin expression by half did not disrupt dorsal closure on its own (Fig. 8 C and Video 6). However, this significantly enhanced the dorsal closure defects in Ajuba mutants, resulting in a catastrophic separation of the epidermis from the amniosera early in dorsal closure (severe defect, Fig. 8, F–H; and Videos 9 and 10). These results demonstrate that Ajuba is required to stabilize cell adhesion during multiple epithelial remodeling events in the Drosophila embryo.

**Discussion**

The conserved LIM domain protein Ajuba has been shown to localize to adherens junctions in a tension-sensitive fashion, but has not previously been demonstrated to regulate cell adhesion in vivo. Here we show that Ajuba is required to stabilize adherens junctions under tension during multiple epithelial remodeling events in the Drosophila embryo. Ajuba localizes to adherens junctions in a dynamic and planar polarized fashion that is spatially and temporally regulated by actomyosin contractility. The Ajuba preLIM domain and LIM domains 1 and 2 are necessary for Ajuba to localize strongly to adherens junctions and respond to changes in myosin activity. Ajuba mutants display defects in the localization of adherens junction proteins in multicellular rosette structures, altering the nature and dynamics of cell rearrangement during convergent extension. In addition, the loss of Ajuba activity results in the formation of large gaps between tissues during dorsal closure in embryos with reduced levels of E-cadherin. These results demonstrate that Ajuba is required to maintain cell adhesion at sites of dynamic changes in cell interactions induced by mechanical forces during epithelial remodeling.

These studies demonstrate a novel role for Ajuba in regulating dynamic cell behaviors in actively remodeling epithelial
Ajuba regulates adhesion under tension. Cell adhesion is not generally disrupted in Ajuba mutants. Instead, we demonstrate that the ability to rapidly resolve higher-order interactions among cells requires Ajuba activity. In particular, Ajuba is specifically required for dynamic changes in adhesion in multicellular rosette structures, which are under high levels of tension during convergent extension. Although increased actomyosin contractility can enhance rosette formation (Kasza et al., 2014), myosin activity was not affected in Ajuba mutants. Instead, we show that Ajuba mutants have defects in cell adhesion and adherens junction localization at high-order vertices, which could impede the formation of new contacts between cells and delay the resolution of T1 processes, resulting in the conversion of these structures into rosettes. Rosette behaviors, originally discovered in Drosophila (Blankenship et al., 2006), are commonly observed during convergent extension in vertebrates, including the mouse and Xenopus laevis kidney (Lienkamp et al., 2012), the mouse cochlea and limb bud (Chacon-Heszele et al., 2012; Lau et al., 2015), the chick primitive streak (Rozbicki et al., 2015), and the chick and mouse neural plate (Nishimura and Takeichi, 2008; Nishimura et al., 2012; Williams et al., 2014). Rosettes also occur in other processes of epithelial remodeling, such as in the zebrafish lateral line (Lecaudey et al., 2008), during wound healing (Razzell et al., 2014), and during ommatidial rotation (Mirkovic et al., 2011). These structures likely represent specialized sites of increased tension within tissues, which may place unique demands on adherens junctions and necessitate distinct mechanisms of junctional regulation. Notably, the loss of Ajuba causes striking defects in embryos with reduced cell adhesion, resulting in the large-scale separation of tissues during dorsal closure. This process is also characterized by strong mechanical forces (Kiehart et al., 2000; Hutson et al., 2003). We propose that Ajuba activity may be generally required to reinforce cell adhesion in situations when cell adhesion is low or myosin contractility is high, which are common characteristics of actively remodeling epithelia.

Ajuba localization is highly dynamic and correlates with changes in junctional myosin levels, following peaks of myosin localization within seconds. How Ajuba responds to tension on such a rapid timescale is unclear. In one model, Ajuba could act as a mechanosensor, undergoing a conformational change in response to mechanical force that stabilizes its association with adherens junctions. Alternatively, Ajuba could be recruited to adherens junctions by force-dependent changes in other proteins. A likely candidate for recruiting Ajuba to adherens junctions is α-catenin, which binds to Ajuba and is required for Ajuba junctional localization in vitro (Marie et al., 2003) and in vivo (Rauskolb et al., 2014; this study). Mechanical stretching of α-catenin exposes a central domain in α-catenin that allows it to bind to vinculin (Ishiyama et al., 2013; Yao et al., 2014). In addition, tension stabilizes the interaction between α-catenin and F-actin through a catch bond mechanism (Buckley et al., 2014). Other known binding partners of Ajuba family proteins include Shroom (Chu et al., 2018) and the actin cytoskeleton (Marie et al., 2003; Nola et al., 2011). As Ajuba localization in the Drosophila embryo requires both α-catenin and actomyosin contractility, these results raise the possibility that both actomyosin structures and junctional components contribute to Ajuba recruitment to adherens junctions under tension. Consistent with this possibility, we show that Ajuba localization, function, and response to mechanical forces requires the preLIM domain, which binds to F-actin, as well as the first two LIM domains, which are part of a region that interacts with α-catenin. These two structural requirements for force-dependent Ajuba localization indicate that multiple binding sites are required to recruit Ajuba to adherens junctions under tension.

Figure 8. Loss of Ajuba enhances the dorsal closure defects in cell adhesion mutants. (A) Ajuba-GFP localization in a living embryo during dorsal closure. (B–G) Stills from time-lapse videos of stage 14 embryos expressing β-catenin-GFP. WT (B), E-cadherin heterozygote (shg2/+) (C), Ajuba54MZ maternal/zygotic mutant (D), Ajuba54MZ maternal/zygotic mutant (E), AjubaIIMZ maternal/zygotic mutant that is also heterozygous for E-cadherin (F; Ajuba54MZ, shg2/+), and AjubaIIMZ maternal/zygotic mutant that is also heterozygous for E-cadherin (G; AjubaIIMZ, shg2/+). The lateral epidermis (annotated in blue) is connected to the amnioserosa (annotated in yellow) in WT but becomes separated in Ajuba mutants. (H) Percentage of embryos showing no defect or mild, moderate, or severe dorsal closure phenotypes. Images are dorsal views, anterior left. Bars, 30 µm. See also Fig. S4 and Videos 5–10.
Interactions between adherens junctions and the actin cytoskeleton are essential for cell adhesion and tissue integrity (Yonemura et al., 2010; Desai et al., 2013). In particular, the interaction between α-catenin and F-actin has been proposed to function as a catch bond that is stabilized in the presence of force (Buckley et al., 2014). This raises the question of why additional proteins such as Ajuba are required to stabilize adhesion complexes, if conformational changes in α-catenin alone are sufficient to strengthen the association between adherens junctions and F-actin. One possibility is that the interaction between α-catenin and F-actin is not stable enough to withstand the strong mechanical forces required for complex junctional remodeling events involving multiple cells. Ajuba could act as an additional, stabilizing link between adherens junctions and the cortical actomyosin network in high-stress regions, reminiscent of the role of vinculin, which stabilizes the interaction between α-catenin and F-actin (le Duc et al., 2010; Huveneers et al., 2012; Twiss et al., 2012). Alternatively, Ajuba could recruit other proteins to adherens junctions that modulate junctional organization or dynamics. Further studies are needed to define the mechanisms that promote the dynamic remodeling of adhesion under tension. Ajuba has been shown to function in a wide range of processes, including cell differentiation, cell proliferation, cell migration, and Hippo signaling. It will be interesting to determine how the effects of Ajuba on dynamic transitions in cell adhesion during tissue remodeling are related to its diverse roles in epithelial development.

Materials and methods

Fly stocks and genetics

Stocks used for live or fixed imaging were the Ajuba-GFP BAC (Sabino et al., 2011), Myo-mCherry (sqh-mCherry, the myosin regulatory light chain fused to mCherry expressed from the sqh promoter; Martin et al., 2009), Resilie-GFP (gift of A. Debec, Institut Jacques Monod, Paris, France), Myo-GFP (sqh-GFP expressed from the sqh promoter; Royou et al., 2004), E-cadherin-mTomato (shg-mTomato expressed from the endogenous promoter; Huang et al., 2009), β-catenin-GFP (arm-GFP expressed from the endogenous promoter; McCartney et al., 2001), gap43-mCherry (expressed from the sqh promoter; Martin et al., 2010), Shroom<sup>III</sup> (Simões et al., 2014), Df(2R)Exel7131 (Parks et al., 2004), UAST-ShroomA (Bolinger et al., 2010), UASp-Ajuba-msVenus variants (this study), Ajuba<sup>II</sup> (Das Thakur et al., 2010), Ajuba<sup>54</sup> (this study), and shg<sup>II</sup> (E-cadherin; Nüsslein-Volhard et al., 1984). Shroom mutants were the progeny of Shroom<sup>III+Df(2R)Exel7131</sup> females and males that were heterozygous for Ajuba-GFP BAC or sqh-mCherry (III). Shroom-overexpressing embryos were the F2 progeny of UAST-ShroomA; sqh-mCherry; Ajuba-GFP BAC or UAST-ShroomA; E-cadherin-mTomato; Ajuba-GFP BAC males × matatub67;15 Gal4 females (gift of D. St Johnston, University of Cambridge, Cambridge, UK). The localization of Ajuba-msVenus variants was analyzed in the F2 progeny of UASp-Ajuba-msVenus variant males × matatub67;15 Gal4 females. The localization of Ajuba-msVenus variants in Shroom-overexpressing embryos was analyzed in the F2 progeny of UAST-ShroomA; UASp-Ajuba-msVenus variant males × matatub67;15 Gal4 females. The localization of sqh-mCherry in Ajuba-msVenus variant–expressing embryos was analyzed in the F2 progeny of sqh-mCherry; UASp-Ajuba-msVenus variant males × matatub15 Gal4 females. The α-catenin knockdown (KD) embryos were the progeny of ShroomΔ11/Df(2R)Exel7131 attP2 males (generated by the Transgenic RNAi Project at Harvard Medical School; Perkins et al., 2015). Crosses and embryo collections for imaging Ajuba-GFP, Ajuba-msVenus variants, and Shroom mutants were performed at 25°C. Shroom-overexpressing embryos and controls were collected at 18°C, and Ajuba mutant embryos and controls were collected at 20°C. All embryos were imaged live at room temperature except for the fixed embryos in Figs. 7, 8, 9, 11, and 13 (D and E).

Ajuba<sup>III</sup> and Ajuba<sup>54</sup> (simplified to Ajuba<sup>II</sup> here and in Fig. S4) germline clone embryos were made by heat-shocking flies of the following genotypes as larvae:

Ajuba<sup>II</sup>, FRT19A/ovo<sup>D2</sup>, FRT19A; arm-GFP, hs-flp/+; Ajuba<sup>III</sup>, FRT19A/ovo<sup>D2</sup>, FRT19A; sqh-GFP, hs-flp/+; Ajuba<sup>54</sup>, FRT19A/ovo<sup>D2</sup>, FRT19A; hs-flp/+; matatub15/Ajuba-msVenus variants

These females were crossed to FM7a, Dfd-YFP/Y males, and progeny were imaged live or fixed for immunostaining. For live imaging, embryos that were maternally mutant for Ajuba (genotyped by the presence of Dfd-YFP signal) and embryos that were maternally and zygotically mutant for Ajuba (genotyped by the absence of Dfd-YFP signal) were analyzed separately (Fig. S8 and Fig. S5, A, B, and D–G) or combined for analysis (Figs. 5, 6, 7, and 8 and S5, C and D). For embryo collections for imaging Ajuba-GFP, Ajuba-msVenus variants, and Shroom mutants were performed at 25°C. Shroom-overexpressing embryos and controls were collected at 18°C, and Ajuba mutant embryos and controls were collected at 20°C. All embryos were imaged live at room temperature except for the fixed embryos in Figs. 7, 8, 9, 11, and 13 (D and E).

Ajuba deletion allele

The Ajuba<sup>54</sup> allele was generated using CRISPR mutagenesis (Fig. S4, A and B; Port et al., 2014). Two gRNAs were ubiquitously expressed in transgenic embryos using the pCFD4 plasmid backbone. One gRNA targeted the 5′ end of the Ajuba ORF, just after the start codon, and the other gRNA targeted the Ajuba 3′ UTR. The pCFD4-Ajuba-gRNA plasmid was cloned by two-part Gibson assembly of BbsI-digested pCFD4 with a gel-purified PCR fragment amplified from pCFD4 using Ajuba gRNA primers 5′-TATATAGGAAGATATCCGGTGAACCTCTGGAATTGCCCGGGTACGGCCTTTATTAGCGTAGAAATAGCAAG-3′ and 5′-ATTTTAACCTGCTATTTCTAGCTCTAAACGCTACTGCAAAGGTGGAATTACGGCCTTAAATATATAGCTGAC-3′ with NEBuilder HiFi 2× Master Mix (New England Biolabs) according to the protocol on http://www.crisprflydesign.org. The pCFD4-Ajuba-gRNA plasmid was inserted into the attP2 landing site on chromosome III. Transgenic males bearing pCFD4-Ajuba-gRNA were crossed to
Transgenic lines
To generate the UASp-Ajuba-msVenUS constructs, the full-length Ajuba ORF (2,154 nucleotides) was PCR-amplified and cloned into pEntr/D-TOPO (Invitrogen) using the following primers: 5′-CAC CATGACACCCCCGAGCGCACAAGA-3′ and 5′-TCCGATATCTGGGTAGAAGGCT-3′. The full-length Ajuba coding sequence was PCR amplified from pEntr-Ajuba and cloned into a UASp C-terminal msVenUS backbone digested with BamHI by Gibson assembly (NEBuilder HiFi 2x Master Mix; New England Biolabs). The monomeric superfolder Venus (msVenUS) tag was a gift of B. Glick (University of Chicago, Chicago, IL). Ajuba deletion variants were cloned as two- or three-part Gibson assembly reactions using the same backbone with a combination of PCR fragments amplified from UASp-Ajuba-msVenUS and gBlock (IDT) sequences for the deletion variants. Transgenes were inserted into the attP2 site on chromosome III. The following C-terminally msVenUS-tagged constructs were generated in this study: Ajuba-FL (aa 1–718), Ajuba preLIM (aa 1–505), Ajuba LIM only (aa 506–718), Ajuba LIM-NES (aa 506–718 plus the NES sequence from PKI, LALKLA GLDI [Wen et al., 1995], fused at the C terminus after msVenUS), Ajuba preLIM+1 (aa 1–559 and 693–718), Ajuba preLIM+2 (aa 1–505, 571–623, and 693–718), Ajuba preLIM+3 (aa 1–505 and 631–718), Ajuba preLIM+12 (aa 1–623 and 693–718), Ajuba preLIM+23 (aa 1–505, 571–718), and Ajuba preLIM+13 (aa 1–570 and 631–718).

Live imaging
Embryos were dechorionated for 2 min in 50% bleach and mounted in a 1:1 mixture of halocarbon oils 27 and 700 (Sigma) or halocarbon oil 27 alone on a gas-permeable membrane (YSI). For imaging the Ajuba-GFP BAC in WT, Shroom mutant, and or halocarbon oil 27 alone on a gas-permeable membrane (YSI). Embryos were dechorionated for 2 min in 50% bleach and mounted in a 1:1 mixture of halocarbon oils 27 and 700 (Sigma) or halocarbon oil 27 alone on a gas-permeable membrane (YSI). For imaging the Ajuba-GFP BAC in WT, Shroom mutant, and or halocarbon oil 27 alone on a gas-permeable membrane (YSI). Embryos were dechorionated for 2 min in 50% bleach and mounted in a 1:1 mixture of halocarbon oils 27 and 700 (Sigma) or halocarbon oil 27 alone on a gas-permeable membrane (YSI).

Drug injections
For injections of Rho-kinase inhibitor (Y-27632), stage 6 embryos were dechorionated for 2 min in 100% bleach, attached to coverslips with heptane/glue, and desiccated for 7–9 min in Drierite. Embryos were covered in a 1:1 mixture of halocarbon oils 27 and 700 (Sigma) and microinjected laterally into the perivitelline space with 1 mM Y-27632 (Millipore) diluted in water, or water alone as a control. The Y-27632 concentration is predicted to be diluted 50-fold in the embryo. Embryos were imaged live 2–8 min after injection.

Immunoblotting
For Western blot analysis, 40 stage 6–8 embryos were hand-selected, crushed with a glass needle, and boiled in 20 µl of 1.5× SDS buffer with 2% β-mercaptoethanol. Samples were run on Bis-Tris 4–12% protein gels (NuPage; Invitrogen) and transferred
onto polyvinylidene fluoride membrane (Immobilon-P; Millipore). Antibodies used were mouse anti-GFP (1:2,000, Roche) and mouse anti-armadillo/β-catenin (1:250; Developmental Studies Hybridoma Bank). Protein bands were detected using chemiluminescence with goat anti-mouse IgG HRP-conjugated secondary antibodies (Jackson Laboratory) and Amersham ECL prime reagent (GE Healthcare). Immunoblots were imaged using a Fujifilm LAS-3000 imager.

**Edge and vertex intensity measurements**

Protein localization was analyzed in maximum-intensity projections of 1.5–4.5 µm in the region of the apical adherens junctions using SIE STA software (Fernandez-Gonzalez and Zallen, 2011) or ImageJ. Lines on cell edges were drawn by the user in the anterior and central regions of the germband, and the mean pixel intensity and orientation were measured for each edge. Intensities were averaged for all edges in a 0°–15° angular range (horizontal edges) and 75°–90° angular range (vertical edges) relative to the AP axis. Vertex and edge intensities were divided by the cytoplasmic intensity to calculate the relative enrichment. Ajuba-GFP BAC vertex enrichment (Fig. 2F) was lower than for UAS-Ajuba FL-msVenus (Fig. 4B) because different transgenes were used. The cytoplasmic pixel intensity was calculated in SIE STA as the mean intensity of all pixels not included in the edge measurements (Fig. 1B and Fig. 2, D and E) or in ImageJ by averaging the mean intensity of a circular cytoplasmic region for ≥10 cells (Fig. 2, F and G; Fig. 3, B and D; Fig. 4B; and Fig. S3C). Planar polarity was calculated as (mean pixel intensity at vertical edges – mean cytoplasmic pixel intensity)/ (mean pixel intensity at horizontal edges – mean cytoplasmic pixel intensity) (Fig. 3D). In Fig. S2, lines on cell edges were manually drawn on single frames from a time-lapse video (Fig. S2B) or on images of immunostained embryos (Fig. S2, C and D). Vertex intensities were measured in ImageJ using circular regions of interest of 2.4 µm (Fig. 2F) or 1.0 µm (Fig. 4B) in diameter. Only tricellular vertices were analyzed.

**Correlation analysis**

To measure the spatial correlation between Ajuba-GFP and Myo-mCherry (Fig. 1D), edge intensities were measured using SIE STA (Fernandez-Gonzalez and Zallen, 2011), and the R value for the linear best fit line was calculated in Prism (GraphPad). To measure the temporal correlation between Ajuba-GFP and Myo-mCherry, or Resille-GFP and Myo-mCherry (Fig. 1G), we used previously described methods (Martin et al., 2009). Time-lapse videos were made of five embryos each. Z-stacks were acquired at 0.5-µm z-steps and 10-s intervals on an Ultraview Vox spinning disk confocal with a Zeiss Plan Neofluor 63×/1.4-NA oil immersion objective. A maximum-intensity projection of a z-stack encompassing 4–7 µm in the region of the adherens junctions, which includes the majority of Ajuba-GFP and Myo-mCherry signal, was used for analysis. The mean intensity at each edge was measured in ImageJ. Edge intensity was averaged over a window of three consecutive time points and assigned to the central frame. The changes in Ajuba-GFP, Resille-GFP, and Myo-mCherry smoothed intensities were plotted as a scatter plot, and the R value for the linear best fit line was calculated in Prism.

The Ajuba-GFP or Resille-GFP datasets were shifted backward or forward in time, and new R values from these plots were calculated in the same way. To measure the spatial correlation between Ajuba and myosin or E-cadherin (Fig. S1F), lines were drawn on 50 near-vertical edges per image in ImageJ, the intensity of all pixels under these lines was plotted as an individual scatter plot for each embryo, and the R value was calculated using linear regression analysis.

**Edge tracking and analysis**

To analyze cell behaviors in WT and Ajuba mutant embryos (Figs. 5, 6, and S5), 50 vertical cell interfaces that were present at the beginning of germband extension in stage 7 (t = 0) were tracked for up to 30 min in each embryo (n = 12 embryos/genotype). Edges were assigned to T1 processes if they contracted into a four–cell vertex that went on to resolve. Edges were assigned to simultaneous rosettes if they contracted into a four–cell vertex that joined a vertex of five or more cells within 3 min, or if they contracted into a vertex of five or more cells that had not previously been a four–cell vertex for more than 3 min. Edges were assigned to sequential rosettes if they contracted into a four–cell vertex that joined a vertex of five or more cells after more than 3 min, or if they contracted into a vertex of five or more cells that had previously been a four-cell vertex for more than 3 min. The rosette:T1 ratio in Figs. 5B and S5A was the number of shrinking edges that formed rosettes divided by the number of shrinking edges that formed T1 processes. Rosettes were scored as having an adhesion defect if there was a visible gap in the β-catenin-GFP signal in time-lapse videos. These defects were observed only at late stages of rosette formation. Vertex lifetime and the percentage of unresolved vertices were scored for each pair of cells that shared a shrinking edge. Vertex lifetime was the time from when that edge contracted to a vertex to the time when the two cells no longer shared a vertex. A vertex was scored as unresolved if the two cells still shared a vertex at t = 30 min. The rosette:T1 ratio in Fig. S3E was calculated at a single time point in fixed embryos immunostained for E-cadherin and was the total number of rosette intermediates (groups of five or more cells that were connected by a common vertex or by short edges that were <1.2 µm long) divided by the total number of T1 intermediates (groups of four cells that were connected by a common vertex or by a short edge that was <1.2 µm). To analyze tissue elongation, two cells that were located at least 30–40 cells apart along the AP axis and 1–2 cell diameters away from the ventral furrow at end of the fast phase of germband extension in late stage 8 were manually tracked back to the beginning of germband extension in stage 7. Tissue elongation was calculated as the fold-change in distance between these cells along the AP axis.

**Online supplemental material**

Fig. S1 shows the localization of Ajuba in WT, α-catenin KD, and Shroom-overexpressing embryos. Fig. S2 provides additional analysis of Ajuba-GFP localization to different subsets of junctions. Fig. S3 shows the expression levels of Ajuba-msVenus variants and their effects on myosin localization and rosette formation. Fig. S4 describes the genetic crosses used to generate the Ajuba54 allele and the lethality of Ajuba mutants. Fig. S5 shows
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