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Abl and Canoe/Afadin mediate mechanotransduction at tricellular junctions

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Abstract

Epithelial structure is generated by the dynamic reorganization of cells in response to mechanical forces. Adherens junctions transmit forces between cells, but how cells sense and respond to these forces *in vivo* is not well understood. We identify a mechanotransduction pathway involving the Abl tyrosine kinase and Canoe/Afadin that stabilizes cell adhesion under tension at tricellular junctions in the *Drosophila* embryo. Canoe is recruited to tricellular junctions in response to actomyosin contractility, and this mechanosensitivity requires Abl-dependent phosphorylation of a conserved tyrosine in the Canoe actin-binding domain. Preventing Canoe tyrosine phosphorylation destabilizes tricellular adhesion, and anchoring Canoe at tricellular junctions independently of mechanical inputs aberrantly stabilizes adhesion, arresting cell rearrangement. These results identify a force-responsive mechanism that stabilizes tricellular adhesion under tension during epithelial remodeling.

A universal property of cells is the ability to detect and respond to mechanical stimuli, such as the stiffness of the extracellular environment, shear and osmotic stress from fluid flow, and forces generated by neighboring cells (1,2). Mechanical forces influence cell fate, division, adhesion, and behavior (3,4), and defects in cellular mechanotransduction pathways are implicated in many diseases, including deafness, atherosclerosis, and cancer (5,6). In epithelial tissues, adherens junctions are critical sites of force transmission between cells (7–9) and undergo dynamic assembly and disassembly under tension during cell rearrangement (3,4,7). Interactions between several components of adherens junction complexes are stabilized by mechanical forces *in vitro* and in cultured cells, and have been proposed to reinforce cell adhesion under tension (10–15). However, how adherens junctions sense and respond to mechanical forces *in vivo,* and how these processes influence dynamic cell behaviors in tissues, are not well understood. Mechanical forces are converted into biochemical changes in cells through a variety of mechanisms, including changes in protein

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conformation, localization, activity, interactions, and post-translational modifications (1,2,9). In particular, tyrosine phosphorylation is enhanced at cell-cell and cell-matrix adhesions, two essential force-bearing structures within cells (16,17). Tyrosine phosphorylation can be modulated by mechanical force *in vitro,* with evidence for force sensing at the level of tyrosine kinases (18–22) and substrates (22–25). However, how tyrosine kinase signaling is regulated by mechanical forces *in vivo*, and the effects of this regulation on cell adhesion and epithelial remodeling, are not well understood.

Tyrosine phosphorylation at tricellular junctions is tension-sensitive

To investigate whether tyrosine kinase signaling is regulated by mechanical forces *in vivo*, we analyzed the distribution of tyrosine-phosphorylated proteins in the *Drosophila* embryo. During convergent extension, epithelial cells undergo directional cell rearrangements in response to planar polarized actomyosin contractility (26–30). We observed a striking tissue-wide pattern of phosphotyrosine during this process, with the highest levels of tyrosine phosphorylation at tricellular adherens junctions where three cells meet (Fig. 1A, S1A). Tricellular adherens junctions are specialized structures that contain dedicated adhesion molecules (31–34) and are predicted to be sites of increased tension in epithelia (35–37). Phosphotyrosine was more strongly enriched at tricellular junctions than the core adherens junction proteins E-cadherin and α-catenin or the membrane marker Spider–GFP (Fig. 1B,G, S1H,I, Table S1). The enrichment of phosphotyrosine was further increased at 4-way junctions that form as intermediates during cell rearrangement (Fig. S1B,C). These results indicate that tyrosine phosphorylation is enhanced at sites where three or more cells meet.

To investigate the dynamics of tyrosine kinase signaling at tricellular junctions, we developed a method to visualize tyrosine phosphorylation in living embryos. Fluorophore-conjugated monoclonal antibodies to phosphotyrosine were injected into syncytial embryos and retained in cells after cellularization, allowing direct visualization of tyrosine-phosphorylated proteins (Fig. 1C–E, Movie S1). Phosphotyrosine levels at tricellular junctions were positively correlated with myosin II regulatory light chain (MRLC-GFP), an indicator of cortical contractility (Fig. 1D,I, S1E). Inhibiting cortical myosin activity by injecting the Rho-kinase inhibitor Y-27632 (27,30) rapidly depleted phosphotyrosine levels at tricellular junctions within 5 min of injection, accompanied by an increase in phosphotyrosine levels at bicellular junctions (Fig. 1F,H, S1F,G). The acute release of tension by Y-27632 did not affect the overall level of tyrosine phosphorylation in the embryo (Fig. S1D), adherens junction integrity (Fig. S1H–K), or the integrity of tricellular junctions marked by the tricellular adherens junction protein Sidekick (31–34) (Fig. S1L–O), which is sensitive to actomyosin perturbations over longer timescales (32). These results demonstrate that increased tyrosine phosphorylation at tricellular junctions requires cytoskeletal tension.

Canoe/Afadin is a tyrosine kinase substrate at tricellular junctions

To investigate the molecular basis of tension-sensitive tyrosine phosphorylation at tricellular junctions, we screened >100 Venus-tagged proteins (31) to identify factors that colocalize with phosphotyrosine at tricellular junctions. This screen identified Canoe/Afadin, a conserved actin-binding protein that links cell-surface receptors to the actomyosin

cytoskeleton in *Drosophila* and mammals (38–43). A functional Canoe-Venus fusion expressed from the endogenous locus (31) was enriched at tricellular junctions in wild-type embryos (Fig. 1G), consistent with previous findings (40,42). Canoe-Venus localization at tricellular junctions was highly dynamic, accumulating and dissipating on a timescale of seconds (Movie S2), and these changes were strongly correlated with changes in phosphotyrosine (Fig. 1J–L, S2A–C). Canoe-Venus was tyrosine phosphorylated in embryonic lysates, although Canoe protein accounted for only a minority of tyrosine-phosphorylated proteins in the embryo (Fig. S2D,I,J). Moreover, phosphotyrosine levels at tricellular junctions were significantly reduced in *canoe*^{R2} maternal mutants (referred to as *canoe* mutants) and these levels were restored by reintroducing wild-type N-terminally tagged Canoe protein (Fig. S2E–H). These results indicate that Canoe, or a Canoe-dependent protein, is a substrate for tyrosine kinase signaling at tricellular junctions.

Canoe/Afadin is a critical regulator of epithelial morphogenesis and animal viability in *Drosophila* and mammals (38–41). However, although mammalian Afadin can influence tricellular adhesion under certain conditions in culture (42), an *in vivo* requirement for Canoe/Afadin in regulating tricellular adhesion has not been directly demonstrated. To address this question, we analyzed cell adhesion in Canoe-deficient embryos using E-cadherin and Sidekick-Venus to visualize bicellular and tricellular junctions, respectively. In wild-type embryos, cells were in close contact at tricellular junctions and Sidekick-Venus localization in all three cells appeared as a single spot by confocal microscopy, indicating strong adhesion (Fig. 2A). By contrast, gaps in E-cadherin and Sidekick-Venus localization at tricellular junctions were detected in *canoe* mutants and *canoe* knockdown (KD) embryos that express an shRNA targeting *canoe* (Fig. 2A,I,K, S3A–C). Defects in E-cadherin localization at tricellular junctions were first detected in stage 7 in Canoe-deficient embryos, when E-cadherin localization at bicellular junctions was largely intact. By contrast, defects in E-cadherin localization at bicellular junctions were not prevalent until stage 8 (Fig. S3D–F). These results demonstrate an early, specific requirement for Canoe in regulating cell adhesion at tricellular junctions.

Abl-dependent phosphorylation of Canoe is required for tricellular adhesion

The correlation between Canoe and phosphotyrosine localization at tricellular junctions, and the requirement for Canoe in maintaining tricellular adhesion, raise the possibility that Canoe is part of a tyrosine kinase signaling pathway that stabilizes cell adhesion at tricellular junctions. Consistent with this idea, injection of the tyrosine kinase inhibitor bosutinib rapidly decreased Canoe localization at tricellular junctions (Fig. S4A–G) and disrupted tricellular adhesion (Fig. S4H–J). Abl, a nonreceptor tyrosine kinase that is inhibited by bosutinib in mammals (44), has conserved functions in epithelial remodeling and phosphorylates a large number of substrates involved in actin organization, cell adhesion dynamics, and cell matrix attachment (45–49). We therefore tested whether Abl is required for Canoe localization and function at tricellular junctions using a maternally expressed shRNA that effectively inhibits *Abl* expression and activity (48,49). We found that *Abl* KD embryos displayed a significantly reduced enrichment of Canoe and phosphotyrosine at

tricellular junctions (Fig. 2B,C, S5A–E), as well as defects in tricellular adhesion that were similar to, but not quite as severe as, the defects in Canoe-deficient embryos (Fig. 2I–K). These results demonstrate that Abl is required for Canoe localization and cell adhesion at tricellular junctions.

As Abl is a tyrosine kinase, we hypothesized that Abl could regulate Canoe localization and function by promoting Canoe tyrosine phosphorylation. In support of this model, Abl enhanced Canoe tyrosine phosphorylation when coexpressed with Canoe in *Drosophila* $S2R^+$ cells (Fig. S6A,B). Canoe contains 47 tyrosines, making identifying the Abl target sites in Canoe a daunting task. To expedite this process, we performed a structure/function analysis to identify the minimal sequences required for Canoe function. A truncated protein that contains the N- and C-terminal domains of Canoe (Canoe-NC), but lacks the central region including the PDZ domain that interacts with several cell-surface receptors (39), localized correctly to tricellular junctions (Fig. S7A,D) and fully rescued myosin localization in *canoe* mutants (Fig. S7E,F), a well-established readout for Canoe function (40,41). By contrast, further deletion of the N-terminal domain of Canoe that binds to the Rap1 GTPase (43,50), or the C-terminal domain that binds to F-actin (38,40), eliminated Canoe localization to tricellular junctions and rendered Canoe nonfunctional (Fig. S7A,D–F). Moreover, tyrosine phosphorylation of Canoe-NC, but not the full-length protein, was significantly reduced in *Abl* KD embryos (Fig. S6C). Therefore, even though other tyrosine kinases can phosphorylate the nonessential Canoe central domain, Abl is required for the phosphorylation of the minimal Canoe-NC protein that is fully functional during convergent extension. Further analysis of Canoe-NC variants lacking specific tyrosines demonstrated that Abl-dependent tyrosine phosphorylation of the Canoe-NC protein in $S2R^+$ cells was significantly reduced by mutating the 11 C-terminal tyrosines ($Canoe\text{-}NC^{11YF}$), the last 4 tyrosines ($Canoe\text{-}NC^{4YF}$), or the most C-terminal tyrosine of Canoe ($Canoe\text{-}NC^{Y1987F}$), which is located in a predicted Abl target site (L/I/V-Y-x-x-P) (46) (Fig. 2F,G, S6A–C). These findings indicate that Abl promotes the phosphorylation of the C-terminal tyrosine of Canoe, which is conserved from worms to mammals.

To determine whether tyrosine phosphorylation is required for Canoe function, we tested whether full-length Canoe phosphovariants rescue the defects in *canoe* mutant embryos. Both wild-type Canoe and a full-length Canoe variant lacking the N-terminal tyrosines ($Canoe^{9YF}$) localized correctly to tricellular junctions (Fig. S7A–D) and fully rescued tricellular adhesion in *canoe* mutants (Fig. 2K). By contrast, full-length Canoe proteins lacking the 11 C-terminal tyrosines ($Canoe^{11YF}$) or the most C-terminal tyrosine ($Canoe^{Y1987F}$) were less enriched at tricellular junctions and failed to rescue tricellular adhesion and myosin localization in *canoe* mutants (Fig. 2D,E,H,K, S7A,D–F). $Canoe^{Y1987F}$ embryos had more severe defects in tricellular adhesion than *Abl* KD embryos (Fig. 2J,K), likely due to residual Abl expression or the ability of other kinases to phosphorylate the Canoe C-terminal domain. All Canoe phosphovariants localized correctly to bicellular junctions (Fig. S7A,B), indicating that a general localization to adherens junctions, in the absence of a selective enrichment at tricellular junctions, is not sufficient for Canoe function. These results demonstrate that phosphorylation of the most C-terminal tyrosine of Canoe is necessary for its localization and function *in vivo*.

Canoe localization to tricellular junctions is mechanosensitive

As Canoe is dynamically recruited to and stabilizes adhesion at tricellular junctions, we hypothesized that Canoe could be part of a force-regulated mechanism that strengthens adhesion at tricellular junctions under tension. To test this model, we used three approaches to determine whether Canoe localization is regulated by force. First, we analyzed the relationship between Canoe and myosin localization at tricellular junctions in living embryos. Canoe-Venus localization to tricellular junctions was pulsatile (Fig. 3A,B), a characteristic feature of contractile actomyosin networks (51,52). Moreover, Canoe recruitment to tricellular junctions was strongly correlated with changes in myosin localization, with no delay between Canoe-Venus and MRLC-mCherry recruitment by cross-correlation analysis (Fig. 3C, Movie S3). Consistent with this relationship, the enrichment of Canoe at tricellular junctions was significantly reduced in stage 15 embryos that have less cortical myosin (Fig. S8A–C). By contrast, less Canoe protein was recruited with each myosin pulse in *Abl* KD embryos, even though myosin localization and dynamics at tricellular junctions occurred normally (Fig. S5H–N, Movie S4). Thus, Canoe recruitment correlates with bursts of myosin accumulation at tricellular junctions in wild-type embryos, and Canoe localization appears to be uncoupled from myosin in the absence of Abl activity.

To further investigate this model, we tested whether Canoe localization to tricellular junctions requires actomyosin activity. Reducing actomyosin contractility by injecting a Rho-kinase inhibitor resulted in a rapid loss of Canoe-Venus from tricellular junctions within 5 min of injection and suppressed fluctuations in Canoe-Venus intensity (Fig. 3D,E, S8D–F, Movie S5). By contrast, Rho-kinase inhibitor did not decrease, and in fact slightly increased, Canoe-Venus levels at bicellular junctions (Fig. 3D, S8G,I,J). These results indicate that actomyosin contractility is not necessary for Canoe cortical localization, but is required for the selective accumulation of Canoe at tricellular junctions.

To directly test whether Canoe localization is regulated by mechanical force, we used laser ablation methods (30,35) to acutely release tension at individual tricellular junctions by severing one of the three connected bicellular junctions (Fig. 3F,G, Movie S6). To quantify the effects of releasing tension, we measured Canoe-Venus intensity at the tricellular junctions and bicellular junctions attached to the cut edge before and after ablation. To control for photobleaching, we analyzed Canoe-Venus intensity in cells located two or more cells away from the ablation site for comparison (Fig. S8H). Laser ablation led to a 23±9% (mean±SD) decrease in Canoe-Venus intensity at tricellular junctions within 70 s after ablation, whereas Canoe-Venus localization at bicellular junctions was unaffected (Fig. 3H,I). Taken together, these results demonstrate that Canoe-Venus enrichment at tricellular junctions requires mechanical forces generated by actomyosin contractility.

Dynamic Canoe localization to tricellular junctions is required for cell intercalation

The findings that Canoe is recruited to tricellular junctions by tyrosine phosphorylation and actomyosin contractility raise two questions. First, what is the physiological relevance of coupling Canoe recruitment with changes in myosin activity? Second, does tyrosine

phosphorylation only regulate Canoe localization to tricellular junctions, or is it also required for Canoe function at these sites? To address these questions, we developed a method to target Canoe to tricellular junctions independently of endogenous mechanical inputs. This "vertex trap" method involved expressing VhhGFP4 (VHH), a genetically encoded nanobody with high affinity for GFP (53), fused to the C-terminus of the tricellular junction protein Sidekick (Sdk) (32–34). Sdk-VHH expression is predicted to recruit GFP- and Venus-tagged proteins to tricellular junctions through interactions with the localized nanobody (Fig. 4B). The localization of Canoe-Venus was not significantly altered by Sdk-VHH expression in wild-type embryos (Fig. 4A,E), and cell adhesion and myosin localization in these embryos occurred normally (Fig. S9A–E). By contrast, Sdk-VHH allowed Canoe-Venus to remain associated with tricellular junctions when myosin activity was inhibited by Y-27632 injection, a situation in which this enrichment is normally lost in wild-type embryos (Fig. 4A,F). Thus, the vertex trap system uncouples Canoe localization from cytoskeletal tension, providing an opportunity to investigate the effects of eliminating Canoe mechanosensitivity on cell rearrangement.

To investigate the effects of uncoupling Canoe localization from mechanical inputs, we performed time-lapse imaging of embryos expressing vertex-trapped Canoe. During convergent extension, epithelial cells undergo cell rearrangements that are driven by planar polarized actomyosin contractility (26–30). In a stereotypical cell rearrangement during this process (27), tricellular junctions merge to form 4-way vertices that subsequently resolve to form two new tricellular junctions, a process that requires active remodeling of cell adhesion (Fig. 4C,I, S10A). Protein localization is highly dynamic during this process, with MRLC-mCherry and Canoe-Venus reproducibly dissociating from 4-way vertices just before resolution (Fig. 4C, S10B,C). In embryos that expressed vertex-trapped Canoe, cells were frequently arrested at the 4-way vertex stage, resulting in an increase in the average vertex duration time to 9.1±0.7 min compared with 5.1±0.4 min in wild-type embryos (mean±SEM) (Fig. 4D,G,I, Movie S7). Embryos that expressed vertex-trapped Canoe also often failed to complete resolution and displayed increased cell stretching, consistent with a reduction in cell rearrangement (Fig. S9F,G). Further increasing the levels of Canoe at tricellular junctions by coexpressing Sdk-VHH with overexpressed Venus-Canoe extended the vertex duration time to 17.8±0.9 min, more than three times longer than in wild-type embryos (Fig. 4H, Movie S8). By contrast, *Abl* KD embryos that recruited less Canoe to tricellular junctions accelerated vertex resolution, reducing the average vertex duration time to 2.6±0.2 min (Fig. S10D,E) (48). It was not possible to analyze vertex duration in Canoe-deficient embryos, due to widespread defects in tricellular adhesion. These results indicate that varying Canoe levels at tricellular junctions modulates the timing of cell rearrangement.

We next tested whether tyrosine phosphorylation is solely required for Canoe localization to tricellular junctions, or if it is also necessary for Canoe function at these sites. If tyrosine phosphorylation is only required to recruit Canoe to tricellular junctions, then trapping Canoe phosphovariants at these sites would be predicted to bypass the requirement for phosphorylation. Using the vertex trap assay, we found that Canoe^{11YF}-Venus, which lacks the 11 C-terminal tyrosines of Canoe including Y1987, stabilized cell adhesion at 4-cell vertices and delayed cell rearrangement to the same extent as wild-type Canoe (Fig. 4H, Movie S9). By contrast, deleting the entire C-terminal region of Canoe (Canoe-ΔC)

including the F-actin-binding domain eliminated the effects of vertex-trapped Canoe on cell adhesion (Fig. 4H, S9H,I). Thus, tyrosine phosphorylation is essential for Canoe localization to tricellular junctions, but is not required for Canoe to stabilize adhesion at these sites when Canoe is targeted to tricellular junctions through a heterologous mechanism. Together, these results demonstrate that tyrosine phosphorylation modulates Canoe levels at tricellular junctions, which influences the rate of cell rearrangement during epithelial remodeling.

Discussion

Here we show that Canoe and Abl function in a mechanotransduction pathway that regulates dynamic changes in cell adhesion at tricellular junctions during epithelial remodeling. Canoe localization to tricellular junctions is acutely disrupted by laser ablation, demonstrating that Canoe localization is rapidly modulated by mechanical perturbation. Canoe mechanosensitivity requires Abl-dependent phosphorylation of a conserved tyrosine (Y1987) in the Canoe actin-binding domain, and Canoe localization to tricellular junctions is required to stabilize tricellular adhesion. Conversely, constitutively anchoring Canoe at tricellular junctions arrests cell rearrangement, indicating that Canoe levels tune the rate of junctional remodeling (Fig. 4J). These results demonstrate that the mechanosensitivity of this critical junctional regulator is modulated by phosphorylation of a single tyrosine residue, and reveal an essential role of Canoe in coupling tricellular adhesion with mechanical forces during epithelial remodeling.

Mechanical forces trigger a cascade of molecular events in cells that translate biophysical signals into altered cellular behaviors. However, the mechanosensors that directly change conformation under tension *in vivo* are not well defined. Cell-surface receptors are well-positioned to detect forces generated by neighboring cells, but Canoe localization and function at tricellular junctions do not require the PDZ domain that mediates its interaction with known receptors (39). One possibility is that the Canoe protein itself could act as a mechanosensor. Canoe is predicted to be anchored to the membrane through interactions with Rap1 and the actin cytoskeleton (38–40,43,50). Thus, cytoskeletal tension could stretch the Canoe protein and expose tyrosine 1987 to phosphorylation by Abl. Alternatively, Abl or its upstream activators could be regulated by tension during this process, as the activity of Abl and other tyrosine kinases such as Src and FAK has been shown to be regulated by tension *in vitro* (18–22). In a third possibility, the regulation of Canoe by Abl could allow Canoe to detect force-induced changes in other molecules at tricellular junctions. As tyrosine 1987 is in the Canoe actin-binding domain, phosphorylation at this site could allow Canoe to recognize distinct actin structures at tricellular junctions, force-induced conformational changes in the cadherin-catenin complex (54,55), or other specialized features of tricellular junction composition or geometry (37). Once recruited to tricellular junctions, Canoe could stabilize adhesion at these sites by reinforcing the connection between adherens junctions and the actomyosin cytoskeleton (40–42).

Abl tyrosine kinases influence many structural changes that are driven by mechanical forces in tissues, including epithelial remodeling, tissue invagination, axon guidance, and cell migration (45,46). Abl has been shown to regulate a number of proteins that act at tension-bearing structures in cells in addition to Canoe/Afadin recruitment to tricellular junctions,

including vinculin localization (20,22,25) and β-catenin recycling (48) at bicellular junctions and regulation of membrane curvature by BAR-domain proteins (21). Together, these results raise the possibility that Abl could transduce mechanical forces into a wide range of structural changes within cells. For example, during cell rearrangement, Abl could simultaneously stabilize tricellular adhesion by recruiting Canoe and destabilize bicellular adhesion by enhancing β-catenin turnover (48), which could allow bicellular junctions to complete contraction before tricellular junctions are remodeled. Tricellular junctions serve many important roles in epithelial development and homeostasis, including modulating cell rearrangement (32–34,52), orienting mitotic spindles (56), balancing stem cell proliferation and differentiation (57), and maintaining epithelial barrier function (58). An understanding of how mechanical inputs affect the conformation, localization, activity, and interactions of proteins at tricellular junctions will provide insight into how these structures sense and integrate mechanical forces in epithelial tissues.

Methods summary

The methods used in this study are described in the Supplementary Materials, including *Drosophila* stocks and genetics, cloning and transgenic lines, immunofluorescence, time-lapse imaging, laser ablation, drug and antibody injection, immunoprecipitation, immunoblotting, and mass spectrometry, and quantification and statistical analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data and materials availability:

All data are available in the manuscript or supplementary material.

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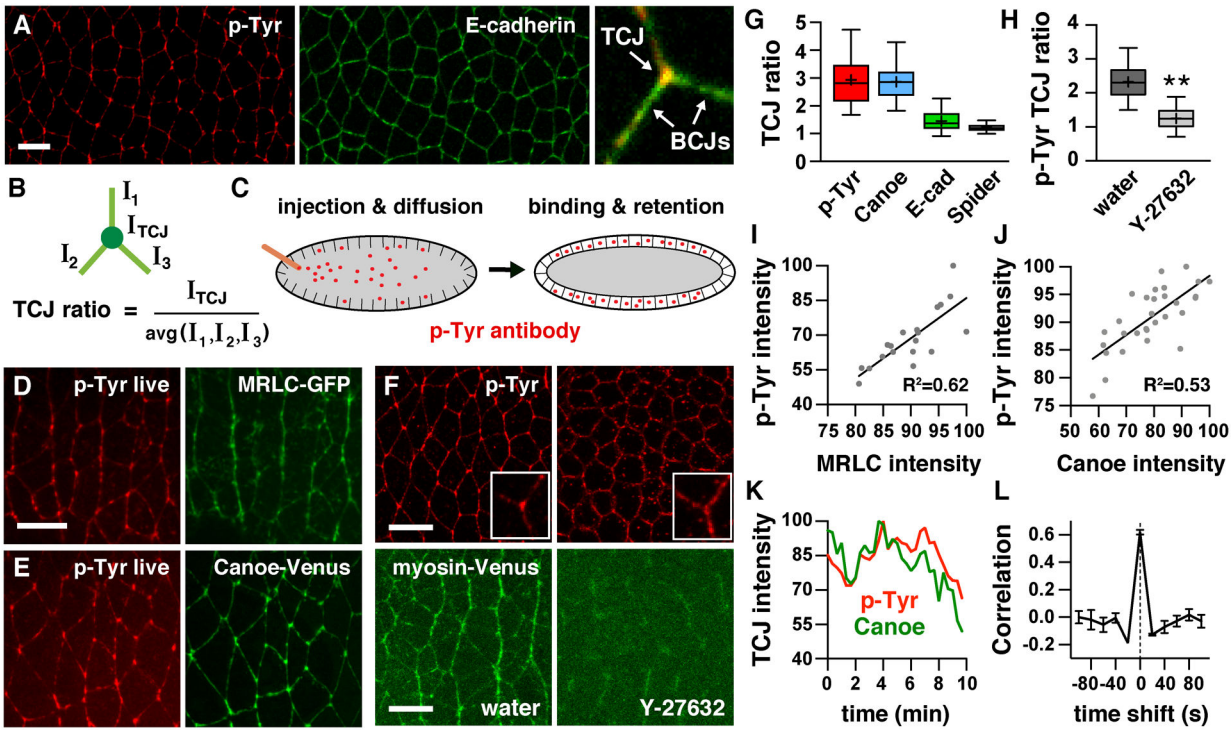

Figure 1. Tension-sensitive localization of phosphotyrosine at tricellular junctions.

(**A**) Phosphotyrosine (p-Tyr) and E-cadherin in the *Drosophila* embryo. Close-up, tricellular junction (TCJ) and bicellular junctions (BCJs). (**B**) TCJ ratio (ratio of the mean TCJ intensity to the mean intensity of the three connected BCJs). (**C**) Alexa-Fluor 594-conjugated p-Tyr antibody was injected into syncytial embryos and retained in cells after cellularization. (**D,E**) p-Tyr in living embryos expressing MRLC-GFP (myosin II regulatory light chain) (D) or endogenous Canoe-Venus (E). (**F**) p-Tyr and myosin-Venus (myosin II heavy chain) in embryos injected with water or Y-27632. Close-ups, single TCJs. (**G**) TCJ ratios of p-Tyr, Canoe-Venus, E-cadherin, and Spider–GFP. (**H**) p-Tyr TCJ ratio in embryos injected with water or Y-27632. (**I,J**) p-Tyr intensity correlates with MRLC-GFP (I) and Canoe-Venus (J) at TCJs. Dots, individual TCJs in a single embryo. (**K**) Canoe-Venus and p-Tyr intensity at a single TCJ over time (imaged every 20 s). (**L**) Correlation coefficient for the rates of change in Canoe-Venus and p-Tyr intensity at TCJs (mean±SEM between embryos). Canoe-Venus data were shifted by the times on the x-axis. Boxes, second and third quartiles; whiskers, 5th–95th percentile; horizontal line, median; +, mean. Intensity is a percentage of the maximum value. ** p<0.003, Welch's t-test. Embryos are stage 7. Living embryos are shown in D, E, F (bottom panels), and I-L. Anterior left, ventral down. Bars, 10 μm. See Table S1 for details.

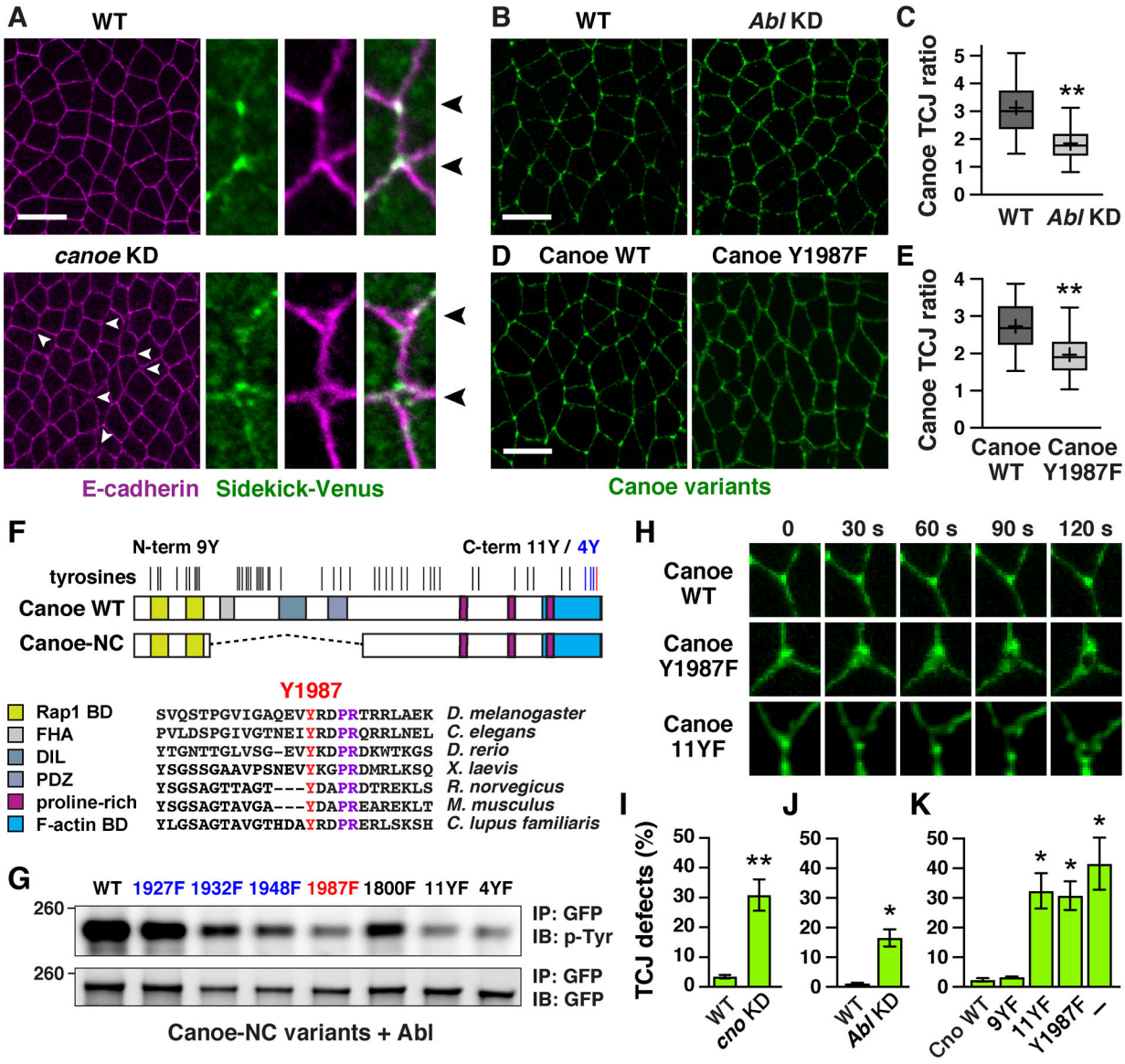

Figure 2. Tyrosine phosphorylation is required for Canoe localization and function at tricellular junctions.

(**A**) E-cadherin and Sidekick-Venus in wild-type (WT) and *canoe* KD embryos. Close-ups, single edges (rotated 30°). Arrowheads, TCJs and 4-way junctions. (**B**) Endogenous Canoe-Venus in WT and *Abl* KD embryos. (**C**) Canoe-Venus TCJ ratios. (**D**) N-terminally tagged Venus-Canoe variants expressed using the Gal4/UAS system in a *canoe* mutant background. (**E**) Venus-Canoe TCJ ratios. (**F**) Canoe domain structure. Tyrosines, vertical lines. (**G**) Venus-Canoe-NC variants in which the indicated tyrosines were mutated to unphosphorylatable phenylalanine (F) residues were coexpressed with HA-Abl in S2R^{+} cells, immunoprecipitated (IP) with anti-GFP, and immunoblotted (IB) with anti-p-Tyr or anti-GFP. Protein size in kDa. The tyrosines mutated in 11YF and 4YF are indicated in (F). (**H**) Stills from movies of *canoe* mutant embryos expressing full-length Venus-Canoe variants using the Gal4/UAS system. (**I-K**) Percentage of TCJs with gaps in E-cadherin

signal in *canoe* KD (I), *Abl* KD (J), or *canoe* mutants expressing full-length Venus-Canoe variants (K) (mean±SEM between embryos). Cno WT, *canoe* mutant expressing Venus-Canoe-WT; –, *canoe* mutant alone. * $p<0.04$, ** $p\leq0.001$, Welch's t-test. Embryos are stages 7–8. Living embryos are shown in D, E, and H. Anterior left, ventral down. Bars, 10 μm.

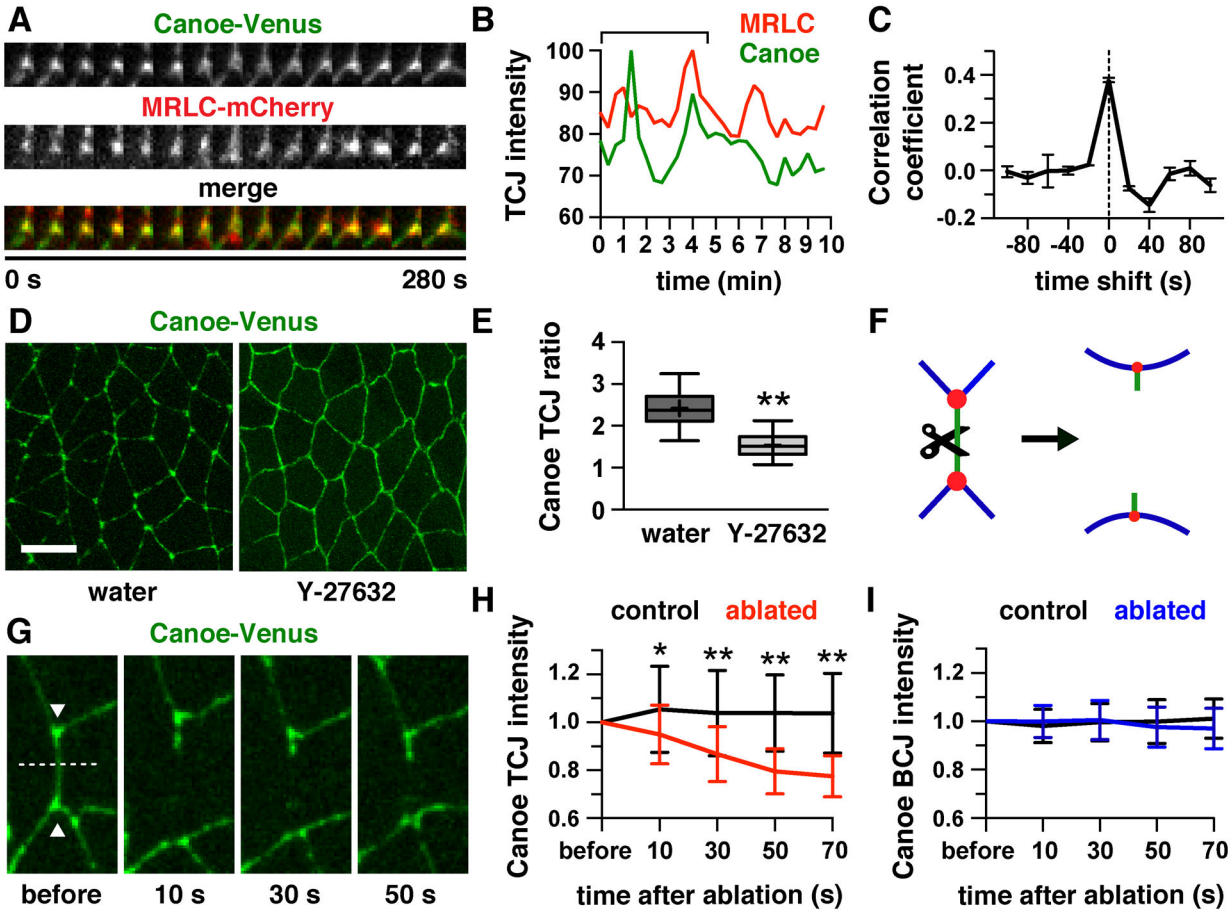

Figure 3. Canoe is recruited to tricellular junctions by cytoskeletal tension.

(**A,B**) Kymograph (A) and plot (B) of Canoe-Venus and MRLC-mCherry intensity at a single TCJ over time (imaged every 20 s). Intensity is a percentage of the maximum value. Bracket, time window in A. (**C**) Correlation coefficient for the rates of change in Canoe-Venus and MRLC-mCherry intensity at TCJs. Canoe-Venus data were shifted by the times on the x-axis (mean±SEM between embryos). (**D**) Canoe-Venus in embryos injected with water or Y-27632. (**E**) Canoe-Venus TCJ ratios. (**F**) Laser ablation schematic. (**G**) Stills from movies of Canoe-Venus before and after ablation of an edge connecting two TCJs (arrowheads) (rotated 30°). (**H,I**) Canoe-Venus intensity at TCJs (H) and BCJs (I) before and after ablation, normalized to the value at t=0 (mean±SD between ablations). * p=0.01, ** p≤0.0001, Welch's t-test (E), one-way ANOVA (H,I). Embryos are stage 7. Living embryos expressing endogenous Canoe-Venus are shown in all panels. Anterior left, ventral down. Bars, 10 μm.

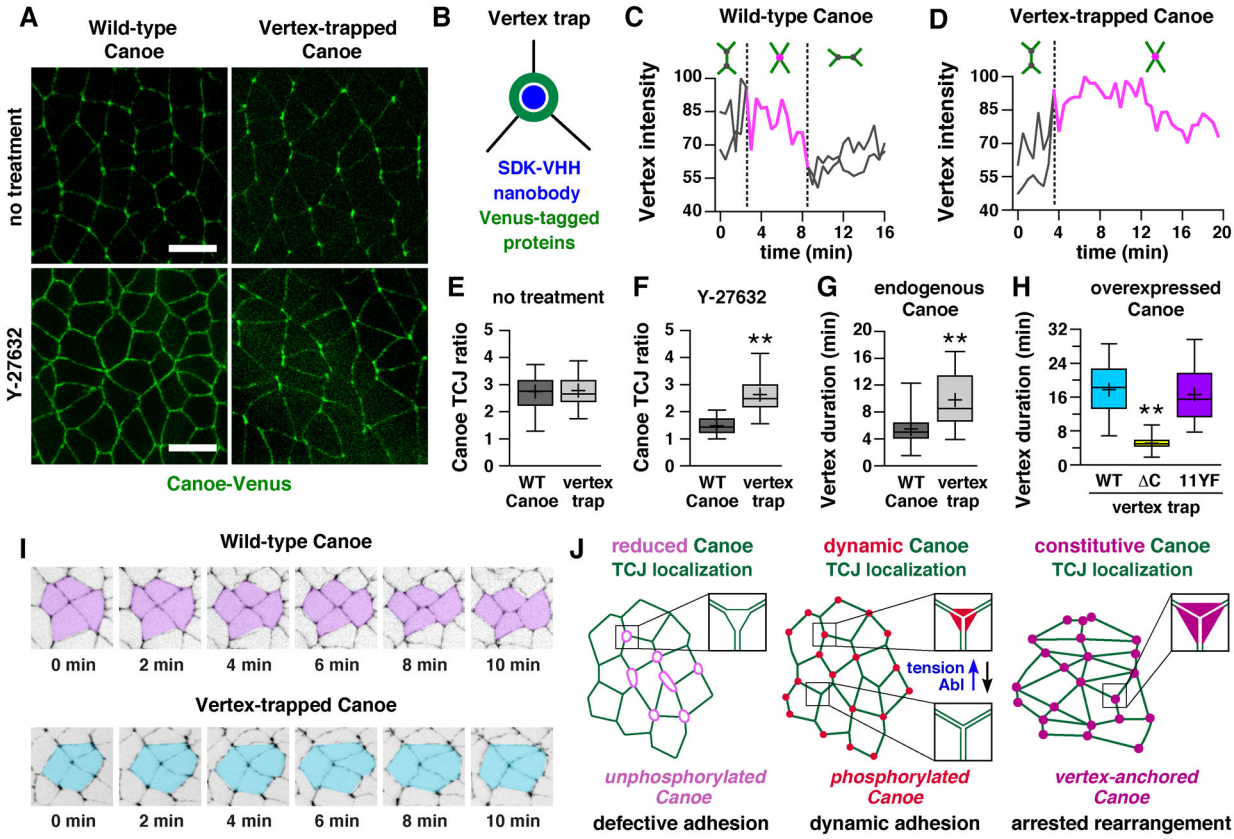

Figure 4. Anchoring Canoe at tricellular junctions impedes cell rearrangement.

(**A**) Embryos expressing endogenous Canoe-Venus (wild-type Canoe, left) or Canoe-Venus trapped at tricellular junctions by Sdk-VHH (vertex-trapped Canoe, right). Top panels, uninjected embryos. Bottom panels, embryos injected with Y-27632. (**B**) Schematic of the vertex trap technique. The GFP nanobody (VHH) fused to the TCJ protein Sidekick (Sdk) recruits Venus-tagged proteins to tricellular junctions. (**C,D**) Endogenous Canoe-Venus intensity in the absence (C) or presence (D) of Sdk-VHH. Two TCJs (gray) converge to form a 4-way vertex (magenta), which resolves to form two new TCJs (imaged every 30 s). (**E-G**) Canoe-Venus TCJ ratio (E,F) and 4-way vertex duration (G) in the absence (WT Canoe) or presence (vertex trap) of Sdk-VHH. (**H**) 4-way vertex duration in embryos that overexpress vertex-trapped Venus-Canoe variants in a wild-type background. (**I**) Stills from movies of embryos expressing endogenous Canoe-Venus in the absence (wild-type Canoe) or presence (vertex-trapped Canoe) of Sdk-VHH. (**J**) Model. Actomyosin contractility and Abl-mediated tyrosine phosphorylation coordinate Canoe levels with mechanical tension at tricellular junctions, promoting the remodeling of cell adhesion during cell rearrangement. ** $p<0.0001$, Welch's t-test. Embryos are stages 7–8. Living embryos are shown in all panels. Anterior left, ventral down. Bars, 10 μm.