Spatiotemporal control of epithelial remodeling by regulated myosin phosphorylation

Karen E. Kasza, Dene L. Farrell, and Jennifer A. Zallen

Spatiotemporally regulated actomyosin contractility generates the forces that drive epithelial cell rearrangements and tissue remodeling. Phosphorylation of the myosin II regulatory light chain (RLC) promotes the assembly of myosin monomers into active contractile filaments and is an essential mechanism regulating the level of myosin activity. However, the effects of phosphorylation on myosin localization, dynamics, and function during epithelial remodeling are not well understood. In Drosophila, planar polarized myosin contractility is required for oriented cell rearrangements during elongation of the body axis. We show that regulated myosin phosphorylation influences spatial and temporal properties of contractile behavior at molecular, cellular, and tissue length scales. Expression of myosin RLC variants that prevent or mimic phosphorylation both disrupt axis elongation, but have distinct effects at the molecular and cellular levels. Unphosphorylatable RLC produces fewer, slower cell rearrangements, whereas phosphomimetic RLC accelerates rearrangement and promotes higher-order cell interactions. Quantitative live imaging and biophysical approaches reveal that both phosphovariants reduce myosin planar polarity and mechanical anisotropy, altering the orientation of cell rearrangements during axis elongation. Moreover, the localized myosin activator Rho-kinase is required for spatially regulated myosin activity, even when the requirement for phosphorylation is bypassed by the expression of phosphomimetic myosin RLC. These results indicate that myosin phosphorylation influences both the level and the spatiotemporal regulation of myosin activity, linking molecular properties of myosin activity to tissue morphogenesis.

Contractile assemblies of actin filaments and the nonmuscle myosin II motor protein produce mechanical forces that generate changes in cell shape during cytokinesis, cell movements during cell migration, and the dynamic remodeling of multicellular tissues (1–3). During development, spatiotemporal patterns of actomyosin contractility remodel simple epithelia into functional tissues with complex form and structure. Contractile force generation requires the assembly of inactive myosin monomers into active bipolar filaments (4). Phosphorylation of the myosin II regulatory light chain promotes myosin filament assembly and the movement of the myosin motor along actin filaments in vitro (5, 6) and is necessary for cytokinesis (7–9), oogenesis (7, 10, 11), and tissue morphogenesis (12–15). However, the in vivo effects of regulatory light chain phosphorylation on myosin localization, dynamics, and force generation, and how these properties are integrated to achieve complex changes in the shapes of tissues, are not well understood.

Epithelial elongation in the Drosophila embryo is driven by cell rearrangements that are coordinated by spatiotemporal patterns of actomyosin contractility. Myosin contractility is spatially regulated in the plane of the epithelium (termed planar polarity), driving the planar polarized contraction of cell interfaces oriented close to perpendicular to the anterior–posterior axis (AP edges) (16–20). New interfaces preferentially form between dorsal and ventral cells (DV edges), resulting in oriented cell rearrangements that rapidly elongate the body axis of the animal from head to tail (16–18, 21). The myosin II activator Rho-associated protein kinase (Rho-kinase) localizes to regions of cells that display strong actomyosin activity during development (14, 15, 22) and promotes myosin contractility by phosphorylating the myosin regulatory light chain (RLC) and inactivating myosin phosphatase (23). Rho-kinase is required for localized myosin contractility during Drosophila axis elongation (14), neural tube closure in chick (22), and in other epithelia that undergo planar polarized cell behaviors (24, 25). Localized phosphorylation by Rho-kinase could therefore serve as an instructive cue that determines where and when myosin is activated within the cell. However, constitutively active myosin variants that evade this upstream regulation are sufficient to support normal myosin function during cytokinesis (26, 27), oogenesis (7), and wing hair formation (12), indicating that in some contexts regulated myosin phosphorylation is dispensable for morphogenesis. This raises the alternative possibility that the primary requirement for phosphorylation is to activate myosin, and additional mechanisms regulate the spatiotemporal patterns of myosin localization within epithelia.

Here we use time-lapse imaging and biophysical approaches to analyze the role of myosin phosphorylation in polarized cell rearrangements during Drosophila axis elongation. Myosin II regulatory light chain variants that prevent or mimic phosphorylation both disrupt axis elongation, but have distinct effects on myosin localization, dynamics, and cell behavior. These results indicate that regulated myosin phosphorylation provides an instructive cue that directs both the magnitude and spatiotemporal pattern of myosin activity during epithelial remodeling, linking molecular-scale myosin activity to cell behavior and tissue morphogenesis.

Results

Myosin II Regulatory Light Chain Phosphovariants Disrupt Drosophila Axis Elongation. To systematically examine the effects of myosin II RLC phosphorylation on cell behavior in a multicellular tissue, we

Significance

Cells in the fruit fly Drosophila require forces generated by the myosin II motor to reorganize the embryo and elongate the body axis from head to tail. It has been proposed that regulated myosin phosphorylation generates global patterns of myosin activity that shape tissues. However, the mechanisms that determine where and when forces are generated are not well understood. We show that myosin variants that evade upstream regulation by preventing or mimicking phosphorylation disrupt the spatiotemporal pattern of forces during axis elongation. Mimicking myosin phosphorylation accelerates cell rearrangements, but these rearrangements are poorly oriented and produce less efficient elongation. These results indicate that regulated myosin phosphorylation provides an instructive cue that determines where and when myosin generates force to shape multicellular tissues.

Author contributions: K.E.K. and J.A.Z. designed research; K.E.K. performed research; D.L.F., and J.A.Z. analyzed data; and K.E.K. and J.A.Z. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

1To whom correspondence should be addressed. Email: zallenj@mskcc.org.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1400520111/-/DCSupplemental.
generated a collection of transgenic *Drosophila* lines that express RLC variants with altered molecular properties. Phosphorylation of the *Drosophila* RLC (Sqh, encoded by spaghetti squash) at S21 and T20 is required for myosin filament assembly and contractile activity, with S21 being the primary site (7, 12). We generated GFP-tagged RLC variants with negatively charged glutamate substitutions at one or both sites to mimic phosphorylation (RLC-EE, RLC-E20, RLC-E21) or alanine substitutions to mimic the unphosphorylated form (RLC-AA, RLC-A21) (Fig. SL4) (28). Transgenes were expressed at comparable levels in the progeny of females bearing sqh<sup>1</sup> mutant germ lines that have a 90% reduction in endogenous RLC levels (referred to as sqh<sup>1</sup> mutants) (29) (Fig. SL1 B and C), making it possible to modulate myosin activity without eliminating it. Phosphomimetic RLC is expected to be constitutively active, whereas only a fraction of wild-type RLC is predicted to be in the phosphorylated form (28, 30). Therefore, phosphomimetic variants are predicted to shift the population toward the noncontractile, monomeric form (Fig. L4), with potential consequences for cell behavior and tissue structure.

We analyzed the effects of myosin RLC phosphoryvants on axis elongation in sqh<sup>1</sup> mutants. Embryos mutant for sqh<sup>1</sup> display severely reduced elongation (Fig. 1 C and D), whereas sqh<sup>1</sup> mutants expressing wild-type RLC (RLC-WT) elongated at the same rate and to the same extent as wild-type embryos, indicating that RLC-WT functions normally in elongation (Fig. 1 B–D) (21, 31). These embryos were therefore used as wild-type controls. In contrast, sqh<sup>1</sup> embryos expressing unphosphorylatable RLC-AA or RLC-A21 failed to elongate, whereas phosphomimetic RLC-EE or RLC-E21 produced an intermediate amount of elongation (Fig. 1 B–D). Particle image velocimetry analysis of tissue deformation in confocal movies showed that embryos expressing RLC-WT elongated 1.70 ± 0.10-fold, RLC-EE elongated 1.55 ± 0.17-fold, and RLC-AA elongated 1.15 ± 0.05-fold (Fig. 1D). Thus, RLC variant expression effectively varies the extent of tissue elongation over a broad range, providing an opportunity to investigate the mechanisms by which regulated myosin activity contributes to efficient elongation.

**Myosin II RLC Variants Display Altered Localization and Dynamics.** Actomyosin contractility is required for polarized cortical contraction and junctional remodeling during axis elongation. Dynamic myosin structures are present at cell–cell boundaries near adherens junctions and at the apical cell cortex (19, 20, 32–34). To assess the localization of myosin RLC phosphoryvants to these force-generating structures, we first examined myosin II localization in fixed embryos. We focused on RLC-EE and RLC-AA, which produced epithelia that were morphologically intact but displayed significantly reduced tissue elongation. In confocal sections, RLC-WT was present in the cytoplasm, at the medial cell cortex, and near adherens junctions at the apicolateral boundaries of cells, where it was preferentially enriched (2.2 ± 0.1-fold) (Fig. 2 A and D and Fig. S2A). RLC-AA was less enriched at adherens junctions compared with RLC-WT (1.4 ± 0.1-fold, P = 0.003) (Fig. 2 C and F and Fig. S2A), with residual junctional accumulation likely due to coassembly into filaments with low levels of endogenous RLC (26). In contrast, RLC-EE was more strongly enriched at junctions relative to the medial/cyttoplasmic population (2.8 ± 0.4-fold, P = 0.002) (Fig. 2 B and E and Fig. S2A), consistent with results in cultured cells (35). In addition, RLC-EE displayed a larger reduction in apical levels and accumulated at the basal side of the epithelium, where it appeared to localize to the central yolk cell (Fig. S2 B–E). Myosin II heavy chain displayed a similar localization to the RLC variants, suggesting that the RLC variants reflect the distribution of myosin motor complexes (Fig. S3).

Myosin phosphorylation decreases myosin turnover in cultured cells (27, 30, 35), but the effect of phosphorylation on myosin dynamics in multicellular tissues in vivo has not been examined. To investigate how phosphorylation influences myosin dynamics, we performed fluorescence recovery after photobleaching (FRAP) experiments on the junctional myosin population at cell–cell boundaries. RLC-WT is highly dynamic (20), with a recovery half-time of ~6 s and a mobile fraction of 68 ± 3% (Fig. 2 G–I). RLC-EE displayed a similar recovery half-time, but the mobile fraction was reduced to 47 ± 3% (Fig. 2 G–I), suggesting that a larger fraction of RLC-EE protein is assembled into filaments that are stably associated with the cortex. In contrast, RLC-AA turned over faster, with a recovery half-time of ~4 s, but a similar mobile fraction to RLC-WT (Fig. 2 G–I). These results support the idea that phosphorylation stabilizes myosin association with the cortex.

**Myosin II RLC Variants Alter the Number and Speed of Cell Rearrangements.** We next investigated the effects of changes in myosin regulation and dynamics on the cell rearrangements that drive axis elongation. First, we examined whether the RLC phosphoryvants influence the number of cell rearrangements initiated in these embryos. In contrast, fewer edges contracted in RLC-AA and sqh<sup>1</sup> (68 ± 2% and 85 ± 2%, P ≤ 0.002), as expected for reduced myosin activity. The fraction of contracting edges was positively correlated with the extent of elongation on an embryo-by-embryo basis (Fig. 3F), consistent with an important contribution of cell rearrangement to elongation.

We next tested whether RLC variants alter rearrangement speed by measuring the rates of the two steps of cell rearrangement, interface contraction and vertex resolution (Fig. 3 A and B). A similar fraction of AP edges contracted in RLC-WT and RLC-EE embryos (96 ± 1% and 95 ± 1%) (Fig. 3F), indicating that similar numbers of rearrangements are initiated in these embryos. In contrast, fewer edges contracted in RLC-AA and sqh<sup>1</sup> (88 ± 2% and 85 ± 2%, P ≤ 0.002), as expected for reduced myosin activity. The fraction of contracting edges was positively correlated with the extent of elongation on an embryo-by-embryo basis (Fig. 3F), consistent with an important contribution of cell rearrangement to elongation.
Myosin II RLC phosphovariants disrupt planar polarized myosin localization and activity. The myosin activator Rho-kinase is present in a planar polarized distribution during axis elongation (14) (Fig. 1B), raising the possibility that myosin contractility is spatially controlled by localized myosin phosphorylation. If this is the case, then RLC phosphovariants would be predicted to disrupt this spatial regulation. To test this, we developed computational methods to analyze the planar polarized localization of myosin RLC phosphovariants in living embryos (Fig. 4A–F and Movies S1–S3). RLC-WT became enriched at AP edges ~10 min before the onset of elongation and reached a maximum relative AP enrichment of 2.34 ± 0.15 (Fig. 4A, B, and D and Fig. S4). RLC-EE planar polarity initiated around the same time prior to elongation, but RLC-EE displayed a 25% reduction in peak planar polarity (2.01 ± 0.13) (Fig. 4A, B, and E and Fig. S4). In contrast, RLC-CA displayed a 83% reduction in peak planar polarity (1.23 ± 0.03) (Fig. 4 A, B, and F and Fig. S4). These results indicate that additional changes in cell behavior contribute to the tissue elongation defects in RLC-EE embryos.

Although tissue elongation is positively correlated with increased rosette formation in embryos expressing wild-type myosin, RLC-EE embryos did not follow this trend—embryos with more rosettes elongated less (Fig. 3H). Moreover, while an increase in the speed of cell rearrangements would be expected to enhance tissue elongation, faster rearrangements are not translated into efficient elongation in RLC-EE (Figs. 1D and 3C–E). These results indicate that additional changes in cell behavior contribute to the tissue elongation defects in RLC-EE embryos.

Phosphomimetic Myosin II RLC Promotes Higher-Order Rearrangements. Tissue elongation occurs through both local (T1 process) and higher-order (rosette) cell interactions, which are mediated by myosin-driven contraction of single or multiple cell interfaces, respectively (Fig. 3 A and B) (17, 18, 36). To test whether RLC phosphovariants influence the frequency or relative contributions of these two behaviors, we quantified the frequency of T1 processes and rosettes in these embryos. On average in RLC-WT, contracting AP edges participated relatively equally in T1 processes (48 ± 1% of AP edges) and rosettes (47 ± 2%). We exploited natural variations in the embryo population (Fig. 1D) to analyze the relationship between cell behaviors and tissue elongation on an embryo-by-embryo basis. In RLC-WT, rosette behaviors were positively correlated with tissue elongation—embryos with more rosettes elongated to a greater extent (Fig. 3H). In contrast, the number of T1 processes showed a negative correlation with the extent of elongation (Fig. 3G). This indicates that a shift in the cell rearrangement distribution toward higher-order rearrangements is associated with increased elongation.

In RLC-EE, a larger fraction of contracting AP edges formed rosettes (54 ± 1%, P = 0.003), accompanied by a decrease in T1 processes (41 ± 1%, P = 0.001). In contrast, fewer T1 processes occurred in RLC-AA (38 ± 1%, P < 0.002) and a greater fraction of edges completely failed to contract (Fig. 3F). Variations in the number of contracting AP edges and the nature of cell rearrangements in RLC-AA and sqh1 embryos did not correlate with differences in tissue elongation (Fig. 3 F–H), suggesting that both types of rearrangement are ineffective in these embryos. Therefore, the cell rearrangement distribution is shifted toward fewer rearrangements in RLC-AA and more rosettes in RLC-EE, suggesting that increased myosin activity in RLC-EE embryos promotes higher-order cell interactions.
results demonstrate that regulated phosphorylation at these sites is essential for myosin planar polarity.

We hypothesized that Rho-kinase might be responsible for the residual planar polarized myosin localization in sqh1 mutants expressing RLC-EE, either directly through phosphorylation of endogenous RLC protein or indirectly by regulating the underlying actin cytoskeleton. To test this, we expressed RLC-EE and RLC-WT proteins in embryos maternally mutant for Rho-kinase, in which endogenous RLC phosphorylation is strongly reduced and myosin does not localize to the cortex (12, 14). In Rok− maternal mutants, RLC-WT was cytoplasmic (Fig. 4H), similar to endogenous myosin in these embryos. In contrast, RLC-EE was cortically localized but failed to become planar polarized (Fig. 4F), consistent with an essential role for Rho-kinase in controlling the spatial pattern of myosin localization.

The functional outcome of myosin planar polarity is to promote the contraction of AP interfaces to drive oriented cell rearrangement (16–20). To test if the RLC variants disrupt spatially regulated force generation during axis elongation, we used a UV laser to sever the cortical cytoskeleton at single cell interfaces (Fig. 5A and B) (20). The peak retraction velocity of the two connected vertices away from the ablation site is proportional to interface tension before ablation and inversely proportional to frictional drag (37). Because myosin can influence the mechanical properties as well as the forces generated in tissues (38, 39), we compared the ratio of retraction velocities at AP and DV interfaces between genotypes as a measure of mechanical anisotropy. In RLC-WT, the mean peak retraction velocities were 0.61 ± 0.04 μm/s at AP interfaces and 0.15 ± 0.03 μm/s at DV interfaces, producing a mechanical anisotropy (AP/DV velocity ratio) of 4.1 ± 0.9 (P < 10−3) (Fig. 5 C and D). In contrast, anisotropy was decreased in RLC-EE (2.8 ± 0.6, P < 10−3) and abolished in RLC-AA (1.5 ± 0.6, P = 0.3) (Fig. 5 C and D), correlating with peak RLC planar polarity in time-lapse movies (Fig. 5E). These results demonstrate that RLC phosphovariants disrupt the establishment of strongly planar polarized myosin localization and activity.

**Myosin II RLC Variants Disrupt Spatially Regulated Cell Behavior.** The ultimate outcome of regulated myosin localization and activity is to induce oriented cell rearrangements that drive axis elongation. We reasoned that the myosin RLC phosphovariants may influence the spatial organization of cell behaviors. In particular, decreased myosin planar polarity and mechanical anisotropy could result in rearrangements that fail to promote, or that even oppose, tissue elongation. To investigate this possibility, we analyzed the orientation of cell rearrangements. In RLC-WT embryos, cell rearrangements display a striking planar asymmetry: the majority of contracting edges are concentrated in the orientation range within 15° of perpendicular to the anterior–posterior axis (the 75°–90° sector in Fig. 4C). Classifying edges in the 75°–90° sector as well oriented and all others as poorly oriented, 35% of contracting edges were poorly oriented in RLC-WT whereas 42% and 48% of contracting edges were poorly oriented in RLC-EE and RLC-AA, respectively (P < 0.005) (Fig. 6A). Thus, myosin RLC variants increase cell interface contraction at orientations that are not optimal for elongation.

The mechanisms governing the formation of new interfaces between cells during vertex resolution are unknown. We next tested if the reduced planar polarity of RLC phosphovariants influences interface formation. Vertex resolution in RLC-WT occurs with high spatial fidelity through the formation of DV edges that contribute to tissue elongation (Fig. 6 B and E). The fraction of vertices that failed to resolve was the same in RLC-WT, RLC-EE, and RLC-AA (7 ± 2%). In contrast, a significantly increased fraction of vertices resolved aberrantly in RLC-EE and RLC-AA (P < 10−3) (Fig. 6F). In RLC-AA, most errors involved edges that reformed with an AP orientation, suggestive of ineffective contraction. In contrast, errors in RLC-EE included new edges that were correctly oriented but unstable, suggestive of a defect in the stabilization of new interfaces (Fig. 6 B and F). Unstable DV edges in RLC-EE embryos were often associated with an aberrant accumulation of RLC-EE (Fig. 6F).
The spatial organization of cell behaviors was further disrupted when RLC-EE was expressed in 

expression was in maternal mutants, in which RLC-EE protein was cortically localized but not planar polarized (Fig. 4). These embryos displayed increased errors in both contraction and resolution (Fig. 6C), consistent with an essential role for Rho-kinase in controlling spatially localized actomyosin contractility.

The total fraction of AP edges that productively contribute to tissue elongation through correctly oriented interface contraction and vertex resolution was well correlated with elongation on an embryo-by-embryo basis (Fig. 6D). This correlation across genotypes suggests that the number and orientation of cell rearrangements can account for the major effects of RLC variants on tissue elongation (Fig. 6G).

Discussion

The levels and spatiotemporal patterns of myosin contractility are actively regulated to generate tissue shape and structure. Here we show that both properties of myosin contractility are controlled at the level of myosin II regulatory light chain phosphorylation. We investigated the role of myosin phosphorylation in a multicellular system by expressing myosin RLC phosphovariants in Drosophila embryos with reduced levels of endogenous RLC. Variants that prevent or mimic phosphorylation both disrupt axis elongation, but differ in their effects on myosin dynamics and cell behavior. Phosphomimetic RLC-EE accelerates and sqh1 and RLC-AA slow cell rearrangement, indicating that the levels of active myosin are rate limiting in this context. Strikingly, RLC-EE reduces myosin planar polarity and mechanical anisotropy, and RLC-AA abolishes both.

Thus, whereas cells rearrange faster in RLC-EE, interface contraction and vertex resolution are poorly oriented, resulting in reduced tissue elongation. These results highlight that even partial perturbations to planar polarized myosin activity can significantly disrupt the spatial organization of cell behaviors.

The misoriented cell rearrangements in embryos expressing myosin RLC phosphovariants provide direct evidence that spatiotemporally regulated phosphorylation is essential for localized actomyosin contractility. In the Drosophila embryo, Rho-kinase is planar polarized and required for axis elongation (14). The reduced RLC-EE planar polarity in sqh1 mutants, and the lack of RLC-EE planar polarity in Rok2 mutants, argue that Rho-kinase is an essential spatial input regulating localized actomyosin contractility during epithelial remodeling. Supporting this idea, phosphomimetic myosin RLC also disrupts myosin dynamics and radial cell polarity in apically constricting cells (40). It is also necessary to consider that mutating phosphorylation sites could fail to fully mimic the structure of phosphorylated myosin or eliminate dynamic cycling between phosphorylated and dephosphorylated states that may be important for spatial control of contractility. Notably, eliminating myosin phosphorylation by expression of RLC-AA abolishes the ability of cortical myosin to respond to planar spatial cues. Thus, although several inputs influence myosin planar polarity during axis elongation, including the actin cytoskeleton (18), the polarity protein Par-3 (14), cell adhesion (41), and mechanical tension (20), the spatial systems that generate planar polarity require regulated RLC phosphorylation to control myosin localization. These results demonstrate that regulated myosin phosphorylation is essential for spatiotemporally controlled cell behaviors during epithelial elongation, linking molecular properties of myosin contractility to tissue morphogenesis.

Higher-order rosette behaviors are a conserved feature of tissue elongation (18, 20, 22, 42–45). In Drosophila, several mutants with reduced elongation have fewer rosettes (18, 44), but phosphomimetic RLC-EE is the only known perturbation that enhances rosette formation. Because the myosin cables that lead to rosette formation sustain increased tension (20), increased rosette formation is likely to be an outcome of enhanced myosin activity in RLC-EE embryos. Notably, we find that the extent of axis elongation in wild-type embryos is positively correlated with rosette formation, revealing that rosettes are closely associated with the essential mechanisms that drive elongation. Embryos expressing RLC-EE do not follow this trend. This may arise from two independent consequences of increased myosin activation: more rosettes form due to increased myosin activity, but more errors in rearrangement orientation occur because myosin is not correctly localized. Alternatively, too many overlapping rosettes could disrupt the coherence of cell rearrangements, impeding efficient remodeling. These results highlight the role of regulated myosin phosphorylation in controlling both the overall levels and patterns of myosin activity to direct cell behaviors.

Myosin contractility is a conserved mechanism for cortical contraction and junctional remodeling in epithelia (3). The increase in vertex resolution errors in embryos expressing RLC phosphovariants reveals a role for regulated myosin activity in the formation of new interfaces between cells, in addition to the well-appreciated role for myosin in cell interface contraction. These errors may result from ectopic myosin activity, which could promote contraction and oppose adhesion at newly formed interfaces. In the Drosophila wing, aberrant myosin accumulation at new contacts that form during cell rearrangement in PTEN mutants is associated with disrupted cell packing (46). Alternatively, errors in vertex resolution could be a consequence of the increased
rate of rearrangements in RLC-EE, if faster rearrangements do not allow enough time for cells to respond to biochemical signal, yielding specific timescales of cell rearrangement that are optimal for elongation. Finally, RLC phosphovariants could produce changes in the mechanical properties of the epithelium that constrain cell rearrangements, altering both their speed and orientation. Increased mechanical tension is associated with increased stiffness in cells and tissues (47), and RLC phosphorylation is therefore likely to alter tissue mechanics (39, 48). Future studies of how myosin activity influences the mechanical properties of epithelia in vivo will be required to understand the extent to which intrinsic properties of myosin contractility account for emergent patterns of tissue structure and mechanics.

Materials and Methods

Unless otherwise noted, embryos were maternally mutant for sqh (29) or Rok
2 (12) and had one maternal copy each of a GFP-tagged myosin RLC variant and gap43:mCherry (49) expressed from the sqh promoter. Fixed embryo imaging and FRAP were performed on a laser scanning confocal microscope. Live imaging and laser ablation were performed on a spinning disk confocal microscope outfitted with a Micropoint laser. Time-lapse movies were analyzed computationally using custom MATLAB routines (Figs. 4 and 6A–D). Tissue elongation was quantified by particle image velocimetry. Error bars are the SEM. Details can be found in SI Materials and Methods.

ACKNOWLEDGMENTS. We are grateful to F. Wirtz-Peitz for pSqh-attB-mEGFP; A. Mainieri and L. Fairchild for movie segmentation; O. Weitz for computational methods; R. Fernandez-Gonzalez, S. Simoès, M. Tamada, and A. Vichas for helpful discussions; and R. Fernandez-Gonzalez, A. Martin, C. Vasquez, D. Robinson, D. Warshaw, and J. Zallen for critical manuscript readings. This work was supported by National Institutes of Health/National Institute of General Medical Sciences R01 Grant GM102803 (to J.A.Z.) and a Howard Hughes Medical Institute–Helen Hay Whitney Fellowship and Burroughs Wellcome Career Award at the Scientific Interface (to K.E.K.). J.A.Z. is an Early Career Scientist of the Howard Hughes Medical Institute.

Supporting Information

Kasza et al. 10.1073/pnas.1400520111

SI Materials and Methods

Fly Stocks and Methods. Embryos were generated and studied at 25 °C. Alleles of spaghetti squash and Rho-kinase were sqh \(^1\) (1) and Rok \(^2\) (2), respectively. Germ-line clones were generated with the FLP-DFS system, a technique combining site-specific yeast flipase recombination and dominant female sterile mutations, and ovo \(^1\) FRT101 (3) or ovo \(^2\) FRT19A (gift of N. Tolwinski, National University of Singapore, Singapore). To visualize cell membranes, gap43:mCherry (4) was recombined with hs-FLP38 on the second chromosome. Female larvae of the following genotypes were heat shocked and crossed to sibling males. All progeny were maternally mutant for sqh or Rok and had one maternal copy of a C-terminal GFP-tagged myosin II regulatory light chain (RLC) variant expressed from the endogenous sqh promoter.

\[
\text{sqh}\(^1\) \text{ FRT101/ovo} \text{ D1} \text{ FRT101; hs-FLP38, gap43:mCherry/}, \\
\text{sqh}\(^1\) \text{ FRT101/ovo} \text{ D1} \text{ FRT101; hs-FLP38, gap43:mCherry/+}, \\
\text{sqh-RLC-WT:GFP/+}, \\
\text{sqh}\(^1\) \text{ FRT101/ovo} \text{ D1} \text{ FRT101; hs-FLP38, gap43:mCherry/}, \\
\text{sqh-RLC-EE:GFP/+}, \\
\text{sqh}\(^1\) \text{ FRT101/ovo} \text{ D1} \text{ FRT101; hs-FLP38, gap43:mCherry/}, \\
\text{sqh-RLC-AA:GFP/+}, \\
\text{sqh}\(^1\) \text{ FRT101/ovo} \text{ D1} \text{ FRT101; hs-FLP38, gap43:mCherry/}, \\
\text{sqh-RLC-E21:GFP/+}, \\
\text{sqh}\(^1\) \text{ FRT101/ovo} \text{ D1} \text{ FRT101; hs-FLP38, gap43:mCherry/}, \\
\text{sqh-RLC-E20:GFP/+}, \\
\text{sqh}\(^1\) \text{ FRT101/ovo} \text{ D1} \text{ FRT101; hs-FLP38, gap43:mCherry/}, \\
\text{sqh-RLC-A21:GFP/+}, \\
\text{Rok}\(^2\) \text{ FRT19A/ovo} \text{ D2} \text{ FRT19A; hs-FLP38, sqh-RLC-WT:GFP/+}, \\
\text{Rok}\(^2\) \text{ FRT19A/ovo} \text{ D2} \text{ FRT19A; hs-FLP38, sqh-RLC-EE:GFP/+}, \\
\text{Rok}\(^2\) \text{ FRT19A/ovo} \text{ D2} \text{ FRT19A; hs-FLP38, gap43:mCherry/+}, \\
\text{sqh-RLC-WT:GFP/+}, \\
\text{sqh-RLC-EE:GFP/+}, \\
\text{sqh-RLC-AA:GFP/+}, \\
\text{sqh-RLC-E21:GFP/+}, \\
\text{sqh-RLC-E20:GFP/+}, \\
\text{sqh-RLC-A21:GFP/+}. \\
\]

Transgenic Lines. The following myosin II RLC variants were generated by cloning the full-length sqh cDNA into the pENTR/D TOPO cloning vector followed by site-directed mutagenesis (Stratagene Quik-Change II XL system): RLC-WT (wild type), RLC-EE (T20E, S21E), RLC-AA (T20A, S21A), RLC-E21 (S21E), RLC-E20 (T20E), and RLC-A21 (S21A). Clones were recombined into pSqh-attB-mEGFP (gift of F. Wirtz-Peitz, Harvard Medical School, Boston) with the Gateway system (Invitrogen) to produce C-terminal GFP-tagged RLC variants expressed from the endogenous sqh promoter. To ensure comparable expression levels, transgenes were inserted into the genome at the attP2 site on chromosome III (in sqh \(^1\) and Rok \(^2\) mutants) or the attP4 site on chromosome II (in Rok \(^2\) mutants). RLC-EE produced slightly smaller embryos, presumably due to effects on oogenesis (5), but absolute egg size does not affect relative elongation.

Immunohistochemistry. Primary antibodies were: rabbit anti-GFP, 1:200 (Torrey Pines); mouse anti-Neurotactin, 1:200 (DSHB); and rabbit anti-Zipper, 1:500 (gift of D. Kiehart, Duke University, Durham, NC) (6). Secondary antibodies conjugated to Alexa-488 or Alexa-568 and Alexa-568–conjugated phalloidin (Molecular Probes) were used at 1:500. Embryos were fixed 20 min in 4% (vol/vol) formaldehyde (EMS) in PBS/heptane and devitellinized in heptane/methanol. Embryos were mounted in Prolong Gold (Molecular Probes) and imaged on a Zeiss LSM700 laser scanning confocal microscope with a 40x/1.3 NA objective. The 1.0-μm Z slices were acquired at 0.5-μm steps. Maximum intensity projections of 2–3 μm were taken in the apical junctional plane. RLC phosphovariant enrichment at junctions relative to the medial/cyttoplasmic population was quantified using SIESTA, a tool for scientific image segmentation and analysis (7).

Western Blotting. Embryos aged 2–4 h were lysed in sample buffer and boiled for 5 min. Lysate from ~15 embryos per lane were run on a 4–12% (vol/vol) Bis-Tris SDS/PAGE gel (NuPAGE, Invitrogen). Protein was transferred to a 0.45-μm nitrocellulose membrane by standard protocols. Primary antibodies were used at the following concentrations: mouse anti-GFP, 1:1,000 (Roche); mouse anti-β-tubulin, 1:1,000 (DSHB); and guinea pig anti-Sqh1P, 1:1,000 (gift of R. Ward, University of Kansas, Lawrence, KS) (8). Secondary antibodies were mouse and guinea pig HRP-conjugated antibodies, 1:10,000 (Jackson Immuno-Research Laboratories) and were detected by chemiluminescence imaging (ECL Plus Western Blotting Detection Reagents; GE Healthcare).

Time-Lapse Imaging. Embryos were dechorionated 2 min in 50% (vol/vol) bleach, washed in water, and mounted in halocarbon oil 27 (Sigma) between a coverslip and an oxygen-permeable membrane (YSI). The anterior ventrolateral region of the germ band was imaged with an Ultraview RS5 spinning disk confocal microscope (Perkin-Elmer) controlled by Metamorph software (Universal Imaging) using a 40x/1.3 NA oil-immersion objective (Zeiss). Two-color Z stacks were acquired at 1-μm steps and 15-s time intervals. Maximum intensity projections of 2–6 μm in the apical junctional plane were analyzed. Particle image velocimetry (PIV) was performed using PIVlab (version 1.2) in MATLAB. Tissue flow was analyzed in a 175-μm × 228-μm region at the anterior end of the ventrolateral germ band. Each image was divided into small template windows (128 × 128 pixels, covering ~20 cells) that overlapped by 50%. Using smaller windows did not significantly affect the results. A vector field was obtained by performing a cross-correlation between each window in the current time point and the next time point to determine the displacements for each window and time frame. Tissue elongation was measured by quantifying the cumulative sum of the anterior-directed tissue displacements at the anterior end of the region and the posterior-directed tissue displacements at the posterior end of the region.

Fluorescence Recovery After Photobleaching. The ventrolateral germ band of stage 7/8 embryos was imaged with a Zeiss LSM700 laser scanning confocal microscope using a 63×/1.4 NA oil-immersion objective. Embryos expressing GFP-tagged RLC variants were imaged every 0.5 s at an optical slice thickness of 1.7 μm to include a majority of apical junctional myosin signal. A 1.0-μm diameter region at the cell cortex was photobleached, and fluorescence intensity in the bleached region was measured at each time point using custom MATLAB routines. Intensities were background corrected by subtracting the postbleach fluorescence. Kymographs were compressed along the time axis for visualization.

Laser Ablation. The ventrolateral germ band of stage 7/8 embryos was imaged with an Ultraview RS5 spinning disk confocal microscope on a Zeiss LSM700 laser scanning confocal microscope using a 63×/1.4 NA oil-immersion objective. The Rhodamine laser (633 nm) was transmitted through the microscope objective and focused to a spot of ~500 nm. The anterior ventrolateral region of the germ band was imaged with a Ultraview RS5 spinning disk confocal microscope with a 40x/1.3 NA objective. Time LAPSE imaging (ECL Plus Western Blotting Detection Reagents; GE Healthcare).


Kasza et al. www.pnas.org/cgi/content/short/1400520111

1 of 5

PNAS
micron (Perkin-Elmer). An N2 Micropoint laser (Photonics Instruments) tuned to 365 nm was used to abllate cell interfaces labeled with the GFP-tagged RLC phosphovariants or gap43: mCherry. Imaging was performed before and after ablation using a 63×/1.4 NA oil immersion lens (Zeiss) that was also used to focus the Micropoint laser. Three Z slices were acquired at 0.5-µm steps and 2-s time intervals and maximum intensity projections were taken. Vertices connected to the ablated interface were identified and manually tracked in ImageJ.

The peak retraction velocity following ablation is proportional to the tension at the interface just before ablation and inversely proportional to the frictional drag. Thus, changes in peak retraction velocity can reflect changes in both of these mechanical properties of the tissue. As a measure of the mechanical anisotropy, we compare the mean peak retraction velocities at anterior–posterior (AP) and dorsal and ventral (DV) cell interfaces.

**Automated Image Segmentation.** Two-color time-lapse movies were segmented and analyzed computationally using custom MATLAB routines. Errors were corrected manually with an interactive user interface. Embryos were temporally registered by setting \( t = 0 \) as the time in early stage 7 when the derivative of the elongation curve intersects zero, then adjusting to maximize the overlap for several geometric and topological measurements. Cells tracked for \( \geq 12.5 \) min after \( t = 0 \) were analyzed. To calculate RLC intensity, vertex positions were locally optimized to maximize overlap with the RLC signal. RLC intensity for each pixel was the maximum pixel intensity along a 5-pixel line normal to the long axis of the interface; interface intensity was the average of the maximum pixel intensities along the interface. Background was subtracted on a per-interface basis and was based on intensity in the central (cytoplasmic) regions of the 20 nearest cells. For each embryo, the mean RLC intensity at all interfaces binned by angular orientation was calculated at each time point.


For planar polarity measurements, the ratio of the average intensity of interfaces oriented at 60°–90° relative to the anterior–posterior axis and interfaces oriented at 0°–30° were calculated on a per-cell basis. Per-cell planar polarity measurements were averaged to yield a single value for each embryo at each time point. \( P \) values were calculated using the \( t \) test followed by the appropriate \( t \) test using the area under the curve from \( -5 \) to \( 20 \) min as the test statistic.

**Cell Rearrangement Analysis.** To track cell rearrangement dynamics, automated image segmentation and analysis was complemented with manual analysis of cell behaviors. Embryos were temporally registered by setting \( t = 0 \) as the time in early stage 7 when the derivative of the elongation curve intersects zero. All AP cell interfaces oriented between 75° and 90° relative to the AP axis just before the onset of elongation were identified and tracked manually over time. AP interface contraction rate was quantified as the length of the interface at the first time point when it began to contract, divided by the time from the onset of contraction to vertex formation. The time required for vertex resolution was calculated as the time from the formation of a vertex at which four or more cells meet until the time that a newly formed edge was \( > 1 \) µm long for \( \geq 5 \) min. Manual tracking was used in Figs. 3 and 6 (except 6A), automated image segmentation and analysis was used in Figs. 4 and 6 (the orientation of all interfaces \( \geq 3 \) µm long, tracked \( \geq 5 \) min, and contracted to vertex that existed \( \geq 1 \) min), and PIV was used to quantify tissue elongation.

**Statistical Analysis.** Unless otherwise noted, error bars are the SEM. Mean values of normal distributions were compared using a Student's t test, and nonnormal distributions were analyzed using a Mann–Whitney test.

---

**Fig. S1.** Myosin II regulatory light chain phosphosignatures. (A) Myosin II regulatory light chain (RLC) phosphovariants used in this study. (B and C) Western blot of lysates from 2- to 4-h embryos probed with anti-GFP, anti-Sqh1P (monophosphorylated Drosophila RLC), or anti-\( \beta \)-tubulin (loading control). (B) GFP-tagged RLC variant transgenes are expressed at similar levels. (C) Levels of endogenous monophosphorylated RLC are strongly reduced in sqh-1 embryos compared with wild type (OreR), consistent with previous reports of a 90% reduction in RLC mRNA and protein (1, 2).

Fig. S2. Subcellular RLC phosphovariant localization. (A–D) GFP-tagged myosin II regulatory light chain (RLC) phosphovariants visualized in fixed embryos with antibodies to GFP. (A) Quantification of RLC phosphovariant intensity at apical cell junctions relative to nonjunctional regions, comprising the medial cortex and cytoplasm ($n = 10$ RLC-WT, 9 RLC-EE, 3 RLC-AA embryos). (B–D) Cross-sections of the germ-band epithelium during elongation (early stage 7). An increased fraction of RLC-EE localizes to the basal side of cells and appears to be retained in the yolk cell following cellularization. (E) Still images of apical (projection of 0–5 μm from the apical surface) and basal (projection of 30–35 μm from the apical surface) sections from confocal movies of RLC-WT and RLC-EE embryos. Consistent with fixed embryos (B–D), more RLC-EE is visible in the basal 30- to 35-μm section compared with RLC-WT. During elongation, the basal RLC-EE contracts from large regions spanning 10- to 20-cell diameters at $t=0$ min to small, bright clusters at $t=28$ min (dashed lines). Similar clusters are also visible in RLC-WT embryos (dashed lines). These contractions are uncorrelated with the movements of apical junctional myosin and suggest that the basal RLC is localized in the central yolk cell, which directly underlies the germ-band epithelium. (Scale bars, 10 μm.)

Fig. S3. Localization of myosin II heavy chain. (A–D’) Myosin II heavy chain (MHC) visualized in fixed embryos with antibodies to Drosophila MHC (Zipper), and cell outlines visualized with antibodies to neurotactin (Nrt). MHC shows similar localization to the RLC variants in sqh$^1$ embryos expressing RLC-WT (A), RLC-EE (B), and RLC-AA (C). $n = 3–9$ embryos per condition. Images, 50 μm × 50 μm. Compare with Fig. 2.
Fig. S4. RLC phosphorylation affects the dynamics of planar polarity establishment. Mean intensities of GFP-tagged RLC phosphorylation at interfaces binned by orientation in examples of single embryos. (A) Single embryo analysis shows that RLC-WT (Left column) is enriched at AP interfaces (oriented at 75°–90° relative to the anterior–posterior axis) and is present at lower levels at DV interfaces (0°–15°). (B) RLC planar polarity is consistently decreased in RLC-EE (Center column). (C) Little to no planar polarity is observed in RLC-AA.

Movie S1. Axis elongation in a RLC-WT-expressing embryo. Embryo expressing RLC-WT:GFP (green) and gap43:mCherry (red) in a sqh^{+} background. Anterior left, ventral down.

Movie S1
Movie S2. Axis elongation in a RLC-EE-expressing embryo. Embryo expressing RLC-EE-GFP (green) and gap43:mCherry (red) in a sqh¹ background. Anterior left, ventral down.

Movie S2

Movie S3. Axis elongation in a RLC-AA-expressing embryo. Embryo expressing RLC-AA-GFP (green) and gap43:mCherry (red) in a sqh¹ background. Anterior left, ventral down.

Movie S3