Combinatorial patterns of graded RhoA activation and uniform F-actin depletion promote tissue curvature

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Abstract

During development, gene expression regulates cell mechanics and shape to sculpt tissues. One example is epithelial folding, which proceeds through distinct cell shape changes that occur in different regions of the tissue. How transcription factors combinatorially coordinate cell shape across a tissue is poorly understood. Here, using Drosophila melanogaster as a model, we investigate how cell shape changes are coordinated to promote tissue bending during gastrulation. By quantifying the multicellular patterns of RhoA activation, F-actin, and myosin-2 and perturbing RhoA activation, we find that Snail and Twist regulate distinct patterns of graded F-actin accumulation and uniform F-actin depletion, which synergize to create zones of high and low F-actin levels within the Drosophila mesoderm. Graded actomyosin results from a gradient in RhoA activation and the width of this zone regulates tissue curvature. Thus, changes in tissue shape are regulated through the choreographed interplay of distinct gene expression patterns.
Introduction

During development, the three-dimensional shape of a complex organism is generated based on patterning that is encoded by the one-dimensional sequence of nucleotides in the genome. Patterns of gene expression, and resulting signaling processes, overlap and interact in space and time to define each cell’s function. For example, morphogen gradients encode positional information for specific cell fates (Rogers and Schier, 2011; Wolpert, 1969). For tissues to obtain their final shape and functional state, not only do cell fates need to be positionally specified, but also cell shapes and mechanical properties; mechanical properties and force generation are often patterned within a group of cells of the same fate (Mongera et al., 2018; Sui et al., 2018; Sumigray et al., 2018). Each tissue shape change requires coordinated changes in cell shape and position across the tissue, which have to be tailored to the tissue’s morphological and functional requirements while exhibiting robustness and reproducibility between individual embryos/organisms (Chanet et al., 2017; Hong et al., 2016; von Dassow and Davidson, 2009).

Mesoderm invagination in the early Drosophila melanogaster embryo is an established model system for gene expression patterning and morphogenesis (Leptin, 2005). This system is powerful to study tissue folding because of its relative simplicity; in this process, a single epithelial sheet at the surface of the embryo folds, driven by geometric shape change of non-dividing cells (i.e., the number of cells is constant). Mesoderm invagination requires apical constriction, a cell shape change driven by actomyosin contraction that converts columnar epithelial cells to a wedge shape and promotes tissue curvature (Leptin and Grunewald, 1990; Sweeton et al., 1991). Importantly, apical constriction is coordinated across the presumptive mesoderm; there is a spatial, ventral-lateral gradient of apical non-muscle Myosin-2 (myosin) and apical constriction that is centered within the mesoderm (Heer et al., 2017; Lim et al., 2017; Spahn and Reuter, 2013) (Figure 1 A). A zone of maximal apical constriction extends from the ventral midline up to 2 cell diameters on each side, while apical constriction beyond cell row 2 gradually declines up to 5 cells from the midline (Heer et al., 2017). At the border of this gradient, about 2 - 4
lateral cell rows (row ~ 6 – 9) stretch their apical surface and bend toward the forming furrow (Heer et al., 2017; Leptin and Grunewald, 1990; Sweeton et al., 1991). In contrast, more lateral cells, which are part of the neighboring ectoderm, maintain almost constant apical area throughout the folding process (Rauzi et al., 2015). We investigated how this tissue-wide pattern of cell shape is established and its relevance to achieving proper tissue shape. Mesoderm cell shape change and cell fate are initiated by the transcription factors Twist and Snail, which exhibit distinct patterns of expression (Boulay et al., 1987; Furlong et al., 2001; Leptin, 1991; Thisse et al., 1988). In the early syncytial blastoderm, twist mRNA is expressed in a narrow band along the anterior-posterior axis on the ventral side of the embryo, which expands dorsally into a wider band at the cellular blastoderm stage (Leptin, 1991). Measurements of the transcription dynamics of Twist target genes, T48 and fog, demonstrate that downstream Twist targets are expressed first at the ventral midline and also expand laterally (Lim et al., 2017). This temporal progression of gene expression results in the graded accumulation of T48 transcripts and protein along the ventral-lateral axis (Heer et al., 2017; Lim et al., 2017; Rahimi et al., 2019). Snail can both activate and repress gene expression (Rembold et al., 2014). One gene that is activated by Snail is the G-protein coupled receptor (GPCR), Mist (Manning et al., 2013). In contrast to graded Twist activity, Snail activity as assessed by Mist mRNA expression is uniform across the mesoderm (Lim et al., 2017). Furthermore, Snail expression exhibits a sharp boundary between mesoderm and ectoderm, whereas Twist expression is graded, extending up to ~2-3 cells across this boundary into the ectoderm (Leptin, 1991). How these overlapping but distinct expression patterns combinatorially contribute to cell shape change across the tissue is not understood. The Twist target gene Fog activates the Mist GPCR and a uniformly expressed GPCR, Smog (Costa et al., 1994; Kerridge et al., 2016; Manning et al., 2013). This GPCR pathway, in parallel with T48 expression, results in apical myosin activation (Kolsch et al., 2007). The GPCR pathway and T48 act via the guanine nucleotide exchange factor (GEF) RhoGEF2, which activates RhoA (Barrett et al., 1997; Hacker and Perrimon, 1998). Functioning in opposition to RhoGEF2 is the RhoA GTPase-activating protein (GAP) C-GAP
(Cumberland-GAP or RhoGAP71E) (Mason et al., 2016). RhoA coordinately activates myosin via Rho-associated and coiled coil kinase (ROCK), and F-actin assembly via the formin Diaphanous (Dawes-Hoang et al., 2005; Homem and Peifer, 2008). Myosin activation occurs in a gradient that is narrower than the mid-cellularization gradient of Twist mRNA accumulation (Heer et al., 2017). How the Twist activity pattern translates to RhoA activation and how RhoA controls the tissue-wide pattern of actomyosin are not known.

During apical constriction, F-actin is apically enriched in ventral cells and proper F-actin organization depends on RhoA signaling (Fox and Peifer, 2007). Conversely, upregulation of F-actin turnover and decreased F-actin levels have been shown in the mesoderm, which allows dynamic actomyosin network reattachment to junctions during constriction (Jodoin et al., 2015). How F-actin is regulated across the mesoderm tissue and how this is associated with patterned myosin and cell shape change are not understood.

Here we show that the tissue-level pattern of F-actin levels is distinct from myosin activation and Twist activity. Snail activity uniformly depletes F-actin across the mesoderm. Graded Twist activity promotes an actomyosin gradient within the zone of F-actin depletion, creating distinct zones of constriction and stretching. We show that the width of this actomyosin gradient is tuned by the activity of the RhoA pathway, which regulates tissue curvature and lumen size in the mesodermal tube structure that results from this fold. Our results show how the combination of overlapping transcriptional pre-patterns coordinates cell shapes across the mesoderm to promote proper tissue curvature.
Results

Myosin and F-actin exhibit distinct tissue-level patterns during mesoderm invagination

Apical myosin activation occurs in a multicellular gradient around the ventral midline (Figure 1 A) (Heer et al., 2017; Lim et al., 2017; Spahn and Reuter, 2013). However, how F-actin is patterned is unknown. To determine the distribution of F-actin levels during mesoderm invagination, we stained embryos with labeled phalloidin and measured cortical F-actin levels by cell row, similarly to how we had measured multicellular patterns of myosin (Heer et al., 2017). This method involved binning cells at different positions from the ventral midline in order to obtain an average protein signal per cell at a given position along the ventral-lateral axis. In contrast to myosin, for which the activated pool accumulates at the apical surface, F-actin (as detected by phalloidin) is most apparent at intercellular junctions. Junctional F-actin levels were higher in the ectoderm than the neighboring mesoderm cells during ventral furrow formation (Jodoin et al., 2015). The difference in F-actin levels between mesoderm and ectoderm was already present before ventral furrow initiation: there was a clear boundary between low (mesoderm) and high (ectoderm) F-actin concentration zones starting at mid-late cellularization, before apical constriction had started (Figure 1 B and D and Supplement to Figure 1 A). During ventral furrow formation, junctional F-actin at the center of the mesoderm increased to form a gradient that extended from cell 2 to cell 6, similar to apical myosin (Figure 1 C and D).

The result of F-actin accumulation in ventral midline cells and persistent F-actin depletion in marginal mesoderm cells was a zone of junctional F-actin depletion in cell rows ~ 6 - 9 of the mesoderm and a discrete boundary between mesoderm and ectoderm (Figure 1C), forming a pattern distinct from that of myosin (Figure 1A). This pattern was also observed in live embryos expressing the actin-binding domain of utrophin fused to green fluorescent protein (Utrophin::GFP) or mCherry (Utrophin::mCherry), but not with a general
membrane marker, Gap43-mCherry, indicating that this measurement is not a fixation artifact or due to changes in plasma membrane structure (Supplement to Figure 1 B). The lower levels of F-actin were not as striking in Utrophin::GFP as in phalloidin-stained embryos, likely because Utrophin interferes with F-actin remodeling (Spracklen et al., 2014); however, we were able to reproducibly detect differences between mesoderm and ectoderm.

The observed tissue-wide pattern of F-actin was strikingly similar to the pattern of apical constriction and stretching. Cell rows that maintained low F-actin levels were the same cell rows at the margin of the mesoderm that stretched during folding (Figure 1 A, C). This led us to hypothesize that F-actin levels could influence cell mechanics and the ability to stretch under stress, which has been shown in other contexts (Salbreux et al., 2012; Stricker et al., 2010). To test the strength of this relationship, we plotted the average cell junctional F-actin intensity as a function of apical cell area for both mesoderm and adjacent ectoderm cells (Figure 1 E). We found that F-actin levels were anti-correlated with cross-sectional apical area. In summary, unlike apical myosin, junctional F-actin exhibited a clear difference between mesoderm and ectoderm, which could explain why lateral mesoderm cells stretch while neighboring ectoderm cells maintain their apical area during tissue folding.

Snail and Twist regulate distinct components of the F-actin pattern

To determine whether patterned gene expression regulates F-actin across the mesoderm, we tested how the transcription factors snail and twist affect the tissue-level pattern of F-actin levels. Snail activity in the mesoderm, as measured by mist transcription, is uniform (Lim et al., 2017). We found that the Snail expression boundary co-localized precisely with the boundary in F-actin depletion (Figure 2 A). Therefore, Snail expression and cortical F-actin depletion are correlated.
Next, we determined whether cortical F-actin depletion is dependent on snail. Unlike control (heterozygous) embryos, snail homozygous mutant embryos expressing Utrophin::GFP did not exhibit patterned cortical F-actin levels in the ventral region (Figure 2 B). Quantification of subapical F-actin levels across the ventral and ventral-lateral domain in snail mutants revealed no boundary, but uniform intensity throughout the region (Figure 2 C). In addition, snail mutants lacked the ventral gradient in F-actin accumulation, consistent with Snail being required for downstream effects of Twist, such as apical myosin activation and dynamics (Kolsch et al., 2007; Martin et al., 2009). Because snail mutant embryos were imaged live, we were able to confirm that we were examining ventral cells based on germband extension movements in the neighboring ectoderm and premature cell divisions that occurred in the uninternalized mesoderm, which is mitotic domain 10 (Foe, 1989; Grosshans and Wieschaus, 2000). Therefore, Snail promotes uniform F-actin depletion across mesoderm cells prior to gastrulation.

In contrast to Snail, the pattern of Twist activity across the mesoderm temporally evolves, with Twist targets initiating first along the ventral midline and then expanding laterally, which results in a gradient of accumulated transcripts (Lim et al., 2017; Rahimi et al., 2019). To determine the effects of twist, we examined F-actin levels in a twist null mutant that has been shown to disrupt myosin stabilization (Martin et al., 2009). In contrast to snail mutants, twist mutant embryos exhibited F-actin depletion in the mesoderm and a clear boundary to more lateral tissue with higher F-actin levels (Figure 2 D, E). The zone of F-actin depletion was decreased to about half the normal width, consistent with previous studies showing that twist mutants reduce the width of snail expression (Leptin, 1991). However, graded F-actin accumulation around the ventral midline was absent in twist mutants, suggesting that it specifically depends on the Twist pathway, which includes RhoA activation. Therefore, mesodermal control of F-actin by Twist and Snail is comprised of two nested layers: 1) prior to ventral furrow formation, uniform Snail activity depletes F-actin across the mesoderm, and 2) during ventral furrow formation, graded Twist activity increases
F-actin accumulation in a ventral-lateral gradient (similar to apical myosin), but marginal mesoderm cells maintain lower cortical F-actin.

RhoA activation occurs in a gradient

We next investigated how the gradient in Twist activity is transduced into a gradient of actomyosin. Fog and T48 transcripts accumulate in a gradient (Lim et al., 2017; Rahimi et al., 2019), but how downstream RhoA activation is patterned was not known. Fluorescently tagged versions of RhoA's activator RhoGEF2 (under an endogenous promoter, Figure 3 B), the Anillin Rho-binding domain, an active RhoA sensor (Figure 3 D), and the RhoA effector ROCK (Figure 3 F) became apically enriched in ventral cells during ventral furrow formation, consistent with previous studies (Kolsch et al., 2007; Mason et al., 2013; Mason et al., 2016). Quantification of apical fluorescence by cell row revealed that all three markers for RhoA pathway activation were graded along the ventral-lateral axis, exhibiting strong fluorescence at the ventral midline and gradually decreasing to baseline after 5 cells (Figure 3 C, E, and G). In contrast, endogenously tagged C-GAP-GFP was largely cytoplasmic and appeared uniform across the ventral domain during folding (Supplement to Figure 3 A).

The width of the gradient in RhoA activation, about 5 cells from the ventral midline, is narrower than that of Twist mRNA, which extends 10-12 cells from the ventral midline (Heer et al., 2017). Thus, RhoA activation occurs in a gradient, but the gradient does not mirror Twist activity, suggesting that additional factors may modulate the downstream activity of the Twist pathway.

Neither graded myosin activation nor F-actin depletion depend on intercellular mechanical connections.
Our data suggest that Snail and Twist promote uniform mesodermal F-actin depletion prior to apical constriction and subsequent graded actomyosin activation within the mesoderm during furrow formation, respectively. In the ventral furrow and the related process of Drosophila posterior midgut formation, it has been proposed that mechanical feedback between constricting cells is required to induce myosin accumulation, which may contribute to a gradient or wave in contractility (Bailles et al., 2019; Mitrossilis et al., 2017). Therefore, it was possible that mechanical induction of myosin also contributes to the gradient.

To determine the contributions of mechanical induction to multicellular patterns of myosin activation and cortical F-actin levels, we examined embryos in which intercellular cytoskeletal coupling was disrupted. We did this by depleting the adherens junction protein α-catenin by RNA interference (α-catenin-RNAi). α-catenin-RNAi has been shown to dramatically uncouple the cytoskeletal meshworks of cells from the junctions and disrupt the formation of supracellular structures in the mesoderm (Figure 4A, Supplemental Movies S1, 2) (Fernandez-Gonzalez and Zallen, 2011; Martin et al., 2010; Yevick et al., 2019). In α-catenin-RNAi embryos, ventral cell apical area remains at pre-gastrulation levels (about 40 μm²), but myosin contracts into spot-like structures at the apical cell surface (Figure 4A, B, E) (Martin et al., 2010). Quantification showed that, despite the lack of mechanical coupling, active myosin reproducibly accumulated in a gradient around the ventral midline (Figure 4C, E). Therefore, graded myosin activation across the tissue does not depend on force transmission between cells. However, the shape of the gradient appeared less sigmoidal in α-catenin-RNAi, suggesting that mechanical feedback could affect gradient shape.

We then asked whether mesodermal F-actin depletion depends on intact adherens junctions. Adherens junctions are a known target of Snail in the mesoderm (Chanet and Schweiguth, 2012; Dawes-Hoang et al., 2005; Kolsch et al., 2007) and adherens junction components exhibit a similar tissue-level pattern to F-actin (Supplement to Figure 4A). We examined F-actin by staining α-catenin-RNAi embryos with phalloidin. Similar to wild-type embryos, F-actin in α-catenin-RNAi embryos was depleted across the mesoderm with a
sharp boundary to the ectoderm before the start of contractile behavior (Figure 4 D, E). This suggested that F-actin regulation by Snail does not depend on intact junctions. Depletion in lateral mesoderm cells was maintained over time despite a lack of stretching in those cells. Interestingly, junctional F-actin levels around the midline did not appear to be increased the same as in wild-type embryos during constriction. However, medio-apical F-actin foci appeared, which co-localized with myosin, presumably because actomyosin was uncoupled from intercellular junctions (Supplement to Figure 4 B). These results indicated that F-actin depletion in the mesoderm does not require intact adherens junctions and is not caused by cell stretching.

RhoA activation level modulates actomyosin gradient width within F-actin depleted zone

The above results suggested a Twist activity gradient leads to a RhoA activation gradient. However, the gradient in Twist mRNA and accumulated T48 transcripts extends beyond the 5-6 cell rows from the ventral midline where we detect RhoA activation (Heer et al., 2017; Lim et al., 2017; Rahimi et al., 2019). Therefore, we hypothesized that the gradient in Twist activity is not simply transmitted, but is shaped by the signaling network downstream of Twist. Mesodermal RhoA signaling involves a signaling circuit, which includes an activator/inhibitor pair (Figure 3A). The activator RhoGEF2 is required for high levels of apical myosin accumulation (Dawes-Hoang et al., 2005; Nikolaidou and Barrett, 2004). The inhibitor, C-GAP, has been shown to disrupt proper subcellular myosin localization when severely depleted in ventral cells (Mason et al., 2016). To test the importance of this circuit in regulating the coordination of contractility, we quantified actomyosin contractility in embryos where either C-GAP or RhoGEF2 levels were tuned to an extent that did not fully disrupt subcellular myosin localization, but did change tissue-wide patterning.
First, we examined apical, activated myosin after elevating net RhoA activation by either depleting C-GAP by RNAi (C-GAP-RNAi) or overexpressing RhoGEF2 (RhoGEF2 O/E). These perturbations elevated myosin in mesoderm cells, but did not elevate contractility in the ectoderm at this stage (Figure 5A). C-GAP-RNAi does not prevent basal myosin loss in mesoderm cells (Mason et al., 2016), suggesting that this perturbation specifically affects contractility on the apical side. In control embryos, apical myosin is activated within 5 cells from the ventral midline. In contrast, C-GAP-RNAi and RhoGEF2 O/E extended myosin activation to 7-8 cells away from the midline, with 3-4 cells on each side of the ventral midline having uniformly high myosin intensity (Figure 5B). C-GAP-RNAi also extended the width of RhoA activation as detected by the active RhoA sensor (Supplement to Figure 5A). Consistent with the expansion of contractility, measurements of apical area demonstrated that C-GAP-RNAi and RhoGEF2 O/E resulted in a wider zone of uniform apical constriction, which changed from 2 cells on either side of midline up to 4 cells on each side (Figure 5C). Thus, our data suggested that increasing RhoA activation through C-GAP depletion or RhoGEF2 overexpression widens the activated myosin gradient.

Next, we examined how increasing RhoA activation affected the F-actin pattern. We found that, similar to its effect on myosin, increased net RhoA activation expanded the zone of higher F-actin levels around the ventral midline, which is consistent with RhoA regulating actin (Fox and Peifer, 2007). The increased width of F-actin elevation resulted in a narrower region of depleted F-actin (Figure 5D) without changing the position of the mesoderm-ectoderm boundary (Supplement to Figure 5B). In some cases, RhoGEF2 O/E completely eliminated the pattern of F-actin depletion and elevation across the mesoderm during folding (Figure 5D), presumably because ventral F-actin accumulation expanded to the whole mesoderm. In contrast, RhoGEF2 depletion resulted in a wider region of depleted F-actin (Figure 5E). Of note, RhoGEF2 depletion decreased the density of myosin-accumulating cells and overall myosin levels, but did not visibly narrow the myosin gradient (Supplement to Figure 5C), suggesting that F-actin and myosin induction might be differently sensitive to
RhoA levels in our experimental setup. By quantifying the number of cell rows with depleted F-actin, we found that the width of F-actin depletion appears inversely related to net RhoA activation (Figure 5 F). Thus, the level of RhoA activation set by the GEF/GAP balance determines the width of the F-actin gradient within the larger zone of F-actin depletion.

Myosin pulses elicit different area responses in midline versus margin mesoderm cells

So far, we focused on the patterns of average intensity and area in cell rows at a single timepoint; however, myosin contractility in the ventral furrow is highly dynamic and exhibits pulses (Martin et al., 2009). Myosin pulses are discrete events in which there is a burst of myosin accumulation and often constriction of the cell apex. RhoA activity levels determine the contractile outcome of a myosin pulse in individual cells (Mason et al., 2016). Given that RhoA activity is graded across the furrow (Figure 3), we examined the behavior of myosin pulses throughout the mesoderm. There are distinct classes of myosin pulses that are known to occur in constricting cells during furrow formation. ‘Ratcheted pulses’ are events where apical, active myosin persists after a pulse and decreased apical area is sustained (Figure 6 A). In contrast, ‘unratcheted pulses’ exhibit myosin dissipation after the pulse and exhibit cell relaxation. There is a continuum of behaviors from ratcheted to unratcheted, which are associated with high and low RhoA activity, respectively (Mason et al., 2016).

Myosin pulse behavior in stretching cells is not well understood.

To determine whether the ventral-lateral gradient in RhoA patterns myosin pulse type, we examined myosin persistence across different cell rows. Consistent with previous measurements of contractile pulses in the middle of the ventral furrow (Xie and Martin, 2015), pulses close to the ventral midline exhibited persistent myosin; the myosin level after the pulse was higher than the baseline before the pulse (Figure 6 B, cell row 1). Myosin persistence was associated with a sustained decrease in apical area (Figure 6 C, cell row 1).
In contrast, myosin pulses in more lateral mesoderm cells (Figure 6C, cell row 4) did not exhibit strong myosin persistence (Figure 6B, cell row 7). These myosin pulses were associated with area constriction, but cells partially relaxed after each pulse. In marginal mesoderm cells (Figure 6C, cell row 7), myosin pulses still occurred, but exhibited even less persistence. These myosin pulses accompanied cell stretching and did not robustly result in cell apex constriction (Figure 6C, cell row 7). Comparing pulse behavior across different ventral-lateral positions, we found a graded decrease in myosin persistence and area stabilization after pulses with distance from the ventral midline (Figure 6D), with marginal cells increasing their area during myosin pulses.

To confirm the differences in myosin pulses that we observed by determining the average pulse behavior, we examined the cross-correlation between the constriction rate (positive for apical area decrease) and the rate of myosin density change in cells along the ventral-lateral axis, independently of pulse detection. Consistent with cell apical constriction occurring during a myosin pulse, cells closest to the ventral midline exhibited a strong positive correlation (Figure 6E). Peak correlation decreased gradually up to 5 cells from the ventral midline (Figure 6E). Cells ~7-8 rows from the midline, in which apical area increases during a myosin pulse (Figure 6C), exhibited a negative correlation, indicating that these cells with lower F-actin levels (Figure 1) stretch specifically when myosin increases (Figure 6F). This behavior was specific to mesoderm cells at this stage because ectoderm cells did not exhibit either a positive or negative correlation (Figure 6G, cell rows 11, 12). Thus, there are different myosin pulse behaviors depending on ventral-lateral position in the mesoderm.

**The actomyosin gradient width regulates furrow curvature and lumen size**

To examine the role of the wild-type contractile pattern in ventral furrow formation, we tested how disrupting this pattern affected tissue shape. In wild-type embryos with graded constriction, the ventral furrow is a sharp, v-shaped fold with high curvature at its center.
Previous work showed that globally changing cell fate to create a much wider mesoderm and contractile domain lowered the curvature of the furrow (Heer et al., 2017). However, these experiments disrupted cell fate across the entire embryo. Here, we investigated the role of the actomyosin gradient in folding, independently of cell fate. Upon identifying C-GAP, we had found that some C-GAP-RNAi embryos have a more C-shaped, less sharp fold (Mason et al., 2016). Therefore, we directly tested how widening the gradient with C-GAP-RNAi or RhoGEF2 O/E affected the curvature of the ventral furrow. C-GAP-RNAi and RhoGEF2 O/E embryos with a wider myosin gradient often had lower central curvature than in wild-type (Figure 7 B and C, Supplement to Figure 7A and B). However, the RhoGEF2 O/E phenotype was variable and the mean curvature was not significantly different from control embryos (Figure 7 B and C, Supplement to Figure 7A and B). Most embryos with low central curvature still fold successfully, although in some extreme cases the mesoderm was not internalized. Successful folding in C-GAP-RNAi and RhoGEF2 O/E embryos was associated with a significantly enlarged lumen when invaginated mesoderm formed a tube (Fig. 7C).

Conversely, decreased RhoA activation in RhoGEF2-RNAi caused higher central curvature (Supplement to Figure 7A, B). Although these embryos appeared to fold successfully, in some cases, cells at the embryo surface, around the ventral midline, underwent cell division immediately after invagination (Supplemental Movies S3,4). Because the mesoderm is mitotic domain 10 (Fo, 1989), this suggests that a narrower band of mesoderm invaginated and that some marginal mesoderm cells were left outside.

To explore the relationship between the contractile pattern and fold curvature further, we tested whether the contractile pattern of an individual embryo is correlated with tissue curvature in that embryo at a later time during folding. We segmented and binned cells from 12 live-imaged embryos (4 per genotype) to obtain their tissue-wide myosin and constriction patterns. The same embryos were subsequently followed to measure cross-sectional tissue curvature (averaged across three points along the anterior-posterior axis) at about 10 µm.
invagination depth during folding and lumen size after folding. Linear regression analysis revealed that the half-maximal gradient width was inversely correlated with curvature (Figure 7D). Consistent with the actomyosin gradient width determining the number of F-actin depleted cell rows, the number of F-actin depleted cell rows was also correlated with curvature in a separate experiment (Supplement to Figure 7C). Thus, gradient width and the number of F-actin depleted cell rows are defining factors of the curvature of the ventral furrow.
Discussion

Here, we showed that tissue folding is coordinated by cell shape changes across the tissue that emerge from the combination of overlapping transcription factor and signaling patterns (Figure 7 E). First, we found that there is a Snail-mediated uniform decrease in cortical F-actin levels across the mesoderm. This decrease persists in cells at the lateral margin of the mesoderm, with a sharp boundary to higher F-actin-containing ectoderm cells. Second, we found that Twist activity that is refined by RhoA regulation gives rise to a gradient of actomyosin. We showed that RhoA activation occurs in a tissue-level gradient and that modulating the balance of RhoGEF2 to C-GAP shifts the width of this gradient. Importantly, the levels and dynamics of myosin and F-actin in distinct cell groups are correlated with their shape changes, leading us to speculate that differences in F-actin density and myosin persistence between cell groups determine apical constriction vs. stretching behavior across the ventral domain (Figure 7 E).

Snail-mediated uniform mesodermal F-actin depletion

We observed that during folding, cells with high F-actin levels tend to maintain their shape or constrict, whereas low-F-actin cells stretch. The role of F-actin cortex density in a cell’s response to mechanical stress is well documented (Stricker et al., 2010). In the marginal mesoderm, lower cortical F-actin levels in stretching cells is compounded by lower levels of zonula adherens proteins (Dawes-Hoang et al., 2005; Kolsch et al., 2007; Weng and Wieschaus, 2016), both of which could promote the ability of these cells to remodel and stretch in response to stress. In contrast, the neighboring ectoderm and the medial ventral cells have high F-actin and adherens junction density, which may help those cells maintain their shape or even constrict under stress. Importantly, the effect of Snail on F-actin is independent of functional adherens junction assembly, suggesting that Snail targets several cellular processes in parallel to promote mesoderm cell remodeling.
Alternatively, it was possible that cell strain/stretching or constriction contributed to decreased F-actin density (Latorre et al., 2018). However, the fact that F-actin depletion occurs before stretching and is not disrupted when cells are prevented from constricting and stretching their neighbors suggests that F-actin depletion is not driven by cell shape change. In fact, the transcription factor Snail appears to uniformly deplete F-actin prior to cell shape change. The mechanism by which Snail decreases F-actin is unknown and will be an area of future study. Snail could possibly regulate F-actin assembly via Diaphanous or Enabled (Fox and Peifer, 2007; Homem and Peifer, 2008), or F-actin disassembly via regulation of Cofilin (Jodoin et al., 2015).

**Twist and RhoA signaling create an actomyosin gradient within the Snail domain**

Nested within this zone of Snail mediated F-actin depletion, Twist activity causes actomyosin accumulation via graded RhoA activation. This combination of two overlapping transcription patterns allows for domains of different cell behaviors and mechanical properties within a tissue of uniform cell fate (mesoderm). We further found that patterning of constricting and stretching domains is tightly regulated by the RhoA regulators RhoGEF2 and C-GAP; disrupting that pattern even slightly causes changes to the shape of the resulting fold. RhoGTPases are regulated by complex interactions between their (activating) GEFs and (inhibiting) GAPs in many contexts (Denk-Lobnig and Martin, 2019); in the ventral furrow specifically, interactions between C-GAP and RhoGEF2 tune sub-cellular localization and dynamics of the contractile apparatus during folding (Mason et al., 2016). Here, we found that small changes to C-GAP or RhoGEF2 levels, which do not strongly disrupt cell-level organization, change the pattern of actomyosin levels across the ventral domain. Specifically, this property is dependent on the ratio of RhoGEF2 and C-GAP, or net RhoA activation: C-GAP RNAi and RhoGEF2 O/E both widen the domain of high actomyosin, and create a uniform high-contractility domain around the ventral midline. Conversely, RhoGEF2 RNAi narrows the F-actin gradient and furrow, and creates a wider domain of F-actin-depleted cells.
Our data suggests a system with a graded activator (RhoGEF2) and a uniform inhibitor (C-GAP), in which the inhibitor buffers overall activation and creates an activation threshold (Figure 7 E). We propose that the activation threshold regulates the width of the actomyosin gradient: the gradient widens if overall activator levels increase or inhibitor levels decrease; it narrows if the activator level is decreased. A widened central region of uniform contractility can emerge if some part of the system is saturated at sufficient RhoA activation levels, consistent with our measurements of the contractile gradient in RhoGEF2 O/E and C-GAP-RNAi embryos. If RhoA activation is decreased, such as in RhoGEF2 RNAi embryos, this mechanistic model predicts a narrower gradient. This appears to be true for the F-actin pattern.

Myosin levels in RhoGEF2-RNAi were decreased overall and fewer cells accumulated myosin, but the myosin gradient width was the same as in wild-type. F-actin and myosin may respond to low RhoA activity because they have different thresholds for accumulation, possibly because they are controlled by different regulators downstream of RhoA or. In addition, expression of fluorescently tagged Utrophin actin-binding domain, which disrupts F-actin dynamics (Spracklen et al., 2014), appears to interact with RhoA disruptions, amplifying the phenotype of high RhoA activation and reducing the cell-level effects of RhoGEF2 RNAi. This is consistent with the notion that RhoA activation promotes F-actin assembly across the tissue.

A prediction of this model is that the upstream activator RhoGEF2 should be graded over a wider region in wild-type than the downstream myosin. Our measurements of RhoGEF2::GFP show a gradient of similar width as myosin, however the small signal-to-noise ratio of the RhoGEF2::GFP probe could obscure the lateral tails of that gradient. The upstream twist mRNA and T48 protein gradient is several cells wider than the myosin gradient (Heer et al., 2017), consistent with a possibly wider activation gradient of RhoGEF2.
Our data show that the gradient shape is tuned by a RhoA activator-inhibitor pair. There are other points in the pathway where balance between inhibition and activation is likely to contribute to tissue-wide patterning. In particular, GPRK2, an inhibitor of GPCR signaling, affects myosin organization and cell behaviors (Fuse et al., 2013; Jha et al., 2018). In GPRK2 mutant embryos, apical constriction is expanded, such that lateral mesoderm cells that normally stretch accumulate myosin and constrict (Fuse et al., 2013). This is consistent with a potential role for GPRK2 restricting the contractile gradient. Further downstream, myosin activity is regulated directly by the balance of ROCK and myosin phosphatase (Munjal et al., 2015; Vasquez et al., 2014) and several actin regulators tune the balance of F-actin assembly and disassembly in ventral furrow formation (Fox and Peifer, 2007; Homem and Peifer, 2008; Jodoin et al., 2015). Control of tissue-wide properties by overlapping patterns of regulators, such as F-actin regulation by Snail and Twist and actomyosin gradient regulation by C-GAP and RhoGEF2, appears to be a powerful mechanism to create complex spatial patterns of cell behavior.

**Fold curvature is tuned by tissue-wide actomyosin patterning**

Our disruptions of the actomyosin gradient demonstrate that tissue curvature is sensitive to tissue-wide contractile patterns. The width and position of the contractile gradient is important for the shape of the resulting fold. We showed that adjusting the width of actomyosin gradient by modulating the levels of RhoGEF2 or C-GAP influences tissue curvature. Elevating RhoA activity creates a wider gradient and results in C-shaped furrows with low curvature. By using inherent patterning variability in wild-type and extended variability in genetic disruptions, we showed that the contractile pattern of an individual embryo is correlated with subsequent folding and post-fold shape, suggesting that it would be possible to partially predict tissue curvature based on the observed myosin pattern of an embryo. This is consistent with past work suggesting that broadening contractility lowers tissue curvature. For example, depletion of the ventral fate inhibitor, Serpin27A, caused a wider mesoderm, to form an abnormally shallow furrow (Heer et al., 2017). Similarly, in
GPRK2 mutants, which do not affect the width of the mesoderm, all cells within the mesoderm (~18 cells) constrict and create a U-shaped furrow that often fails to close (Fuse et al., 2013). How F-actin patterning is affected in either of those cases is not known.

That increased contractility in the tissue decreases fold curvature may seem counter-intuitive, but computational modeling shows that graded contractility creates a sharper fold than uniformly high contractility (Heer et al. 2017). Force balance between neighboring high-contractility cells prevents efficient constriction in the uniform case, whereas in the graded case, central high-contractility cells can constrict efficiently against their lower-contractility neighbors, causing a sharp hinge. Considering that successful constriction of cells is dependent on the mechanics of their neighbors, it is plausible that the stretching lateral mesoderm domain can further promote higher curvature folds (Ko et al., 2020; Perez-Mockus et al., 2017; Sui et al., 2018). While we were not able to specifically change F-actin levels because of pleiotropic effects of modulating F-actin dynamics (Jodoin et al., 2015), we speculate that changes to the width of the “soft”, F-actin depleted domain, concomitant with changing gradient width, likely contributed to our observed folding phenotypes. Ventral furrow formation is an embryo-wide mechanical process: it depends on embryo shape (Chanet et al., 2017) and relates to cell shape changes on the opposite, dorsal side of the embryo (Rauzi et al., 2015). Our results further emphasize the importance of understanding tissue-wide mechanics during folding.

Development generates a multitude of tissue curvatures in different contexts. We showed that folding is sensitive to small patterning changes of actomyosin. Our discovery suggests a mechanism by which actomyosin and mechanical cell properties might be patterned in other tissues to generate distinct curvatures.
Materials and Methods

Fly stocks and crosses

For a list of fly stocks and crosses used in this study, see Supplemental Table S1.

An N-terminal GFP tag was inserted at the endogenous C-GAP locus using CRISPR-Cas9 as previously described (Gratz et al., 2015). Coding sequences for two 15 base pair (bp) gRNAs targeting neighboring sites 5’ of the rhoGAP71E gene start codon were cloned into the pU6-BbsI plasmid using the CRISPR Optimal Target Finder (Gratz et al., 2014; Iseli et al., 2007). The donor template plasmid for homology directed repair was generated using Exponential Megapriming PCR (Ulrich et al., 2012). A plasmid backbone (from pHD scarless DS Red) containing an ampicillin resistance gene and an origin of replication was combined with two homology arms (1219 bp and 1119bp, respectively) homologous to the region around the rhoGAP71E gene start codon, flanking a GFP encoding DNA sequence (kindly provided by Iain Cheeseman) with an N-terminal 4 amino acid-encoding linker region (Ser-Gly-Gly-Ser). Both plasmids were injected into nanos>Cas9 expressing embryos. Surviving adults were crossed to y,w; +;+ flies and then screened for mosaic GFP insertion by PCR. Progeny of GFP-positive injected flies were crossed to y,w;+;Dr/TM3 flies and then screened by PCR for the GFP insertion. Successful insertions were further analyzed by sequencing. The fly stock established from their offspring was later back-crossed once to OreR flies in order to eliminate potential off-target mutations. Homozygous C-GAP-GFP flies were viable, suggesting that the GFP-tagged C-GAP is functional.

Live and fixed imaging

Embryos were prepared for live imaging as previously described (Martin et al., 2009). Embryos were collected in plastic cups covered with apple-juice plates. Flies were allowed to lay eggs for 2–4 h at 25 °C (or temperature as noted in Table S1). The plate was removed and the embryos immersed in Halocarbon 27 oil for staging. Embryos undergoing
Cellularization were collected. Embryos were dechorionated with 50% bleach, washed with water, and then mounted on a slide with embryo glue (Scotch tape resuspended in heptane), with the ventral side facing upwards. A chamber was made with two no. 1.5 coverslips as spacers and was filled with Halocarbon 27 oil for imaging. Images were acquired on a Zeiss 710 microscope with an Apochromat 40×/1.2 numerical aperture W Korr M27 objective. Channels were excited and detected concurrently.

Immuno- and phalloidin staining was performed using standard methods (Martin et al., 2009). Embryos were fixed with 8% paraformaldehyde in PO₄ buffer and heptane (3:4) for 30 min, devitellinized manually, stained with phalloidin, primary antibodies and appropriate fluorescently tagged secondary antibodies, and mounted in AquaPolymount (Polysciences, Inc.). Anti-snail (rabbit, 1:100; formaldehyde-hand peeled fixation; gift from M. Biggin), anti-armadillo (mouse, 1:500; heat-methanol fixation; DSHB), anti-GFP (rabbit, 1:500; formaldehyde-hand peeled fixation; Abcam, ab290) and anti-E-cadherin (rat, 1:50; formaldehyde-hand peeled fixation; DHSB) antibodies and AlexaFluor568 phalloidin (Invitrogen) were used. All imaging was carried out on a Zeiss 710 confocal microscope with a Plan-Apochromat 40×/1.2 numerical aperture W Korr M27 objective. Channels were excited and detected concurrently.

For imaging settings for each fluorescent marker, refer to Supplemental table S2.

Gradient analysis

Analysis was done as described in Heer et al., 2017. All image analysis was performed in Fiji (http://fiji.sc) (Schindelin et al., 2012) and MATLAB (MathWorks). Custom software for image processing is available upon request.

1) Definition of developmental timing
Wild-type embryos were staged based on time of folding. The accuracy of this method was confirmed by comparing constricted areas per bin at the selected time point. For embryos with disrupted constriction and folding, a time point was chosen relative to the beginning of myosin/fluorescence accumulation.

2) Shell projection and thresholding to measure apical fluorescence intensity

Shell projections of the apical surface were conducted as previously described (Heer et al., 2017). First, cytoplasmic background signal (defined as the mean cytoplasmic signal plus 2.5 standard deviations) was subtracted from the myosin channel (Martin et al., 2009; Vasquez et al., 2014). For non-myosin fluorescent signal (Figure 3), the cytoplasmic background subtraction was adjusted to account for differences in signal-to-noise ratio for different fluorescent markers (RhoGEF2::GFP: mean + 2 standard deviations (SDs); aniRBD::GFP: mean + 2 SDs, rok::GFP: mean + 2.5 to 3 SDs, GFP::C-GAP: no background subtraction).

The maximum myosin (or other apically enriched fluorescent) signal intensity in the z-plane was used to generate a rough map of the embryo surface. A Fourier transform was used to generate a smooth continuous surface. Myosin signal was averaged over the 4 µm above the surface and membrane signal was the sum of the signal from 1-2 µm below the surface. A gaussian blur filter (radius 1 pixel for fluorescent signal, 0.7-1 membranes) was applied after shell projection to reduce noise.

Shell projections from live and immunostained images were then segmented using an existing MATLAB package, Embryo Development Geometry Explorer (EDGE) (Gelbart et al., 2012). Membrane signal (Gap43::mCherry) or cortical F-actin (phalloidin or Utr::GFP) projections were used to detect cell boundaries (and track cells for live images). Myosin (or equivalent signal) shell projection was used to measure apical signal accumulation.
Segmentation errors in were corrected manually. Our segmentation algorithm was used to determine centroid position, cell diameter, cell area, cell perimeter as well as total myosin/fluorescence signal per cell.

3) Cell bins

For all image quantification, data was segregated into 'cell bins' i.e. cell rows relative to the ventral midline (Heer et al. 2017). Cells were assigned to bins based on the ventral-lateral position of the cell centroid. The ventral midline was defined as the position at which the furrow closes. In fixed images or for embryos that did not fold (or rotated while folding), the position of the VM was determined by symmetry of the fluorescent signal and constriction. Live images were binned based on initial position of the cell centroid before tissue contraction and folding and the boundaries of the bins were set by the average cell diameter along the ventral-lateral axis. For images in which cells had already begun to constrict, the width of each bin was set manually (but still relative to average cell diameter) to approximate the width of cells at that ventral-lateral position. We used MATLAB to generate box-and-whiskers plots depicting the distribution of data, overlaid with the mean of each bin. For boxplots, bottom and top sides of the box represent 25th and 75th percentile of cells, respectively. Outliers were defined as values 1.5 times bigger than the interquartile range. Fluorescent signal was normalized by dividing by the mean of the first bin to adjust for variability in imaging conditions.

Cortical F-actin quantification

For apical projections of phalloidin staining (Figure 1A, 2A and 4B), embryos were shell-projected and cells tracked the same way as for myosin and other markers (see above), except that no background subtraction was applied. To measure cortical F-actin intensity, we integrated the signal intensity of pixels within 2 µm of the identified cell
boundaries. This total junctional F-actin signal was divided by the cell perimeter to obtain junctional F-actin density.

Because mesodermal F-actin depletion was most obvious subapically, not apically, with Utrophin::GFP markers in live embryos (Figures 2B, 5C and 7C), those movies were “shell-projected” in Fiji by generating a Z-reslice (1 µm per slice) and then creating a second reslice along a manually drawn subapical line that followed the ventral-lateral curvature of the embryo surface at about 10-15 µm below the apical surface (Supplement to Figure 2). This allowed us to correct for specific ventral-lateral curvature and get a subapical projection of constant z-depth from the embryo surface. The center part along the anterior-posterior (AP) axis of the shell-projection, where AP curvature is small, was used to quantify F-actin (Utrrophin::mCH or ::GFP) intensity along the ventral-lateral axis. We used the FIJI “Plot profile” command, averaging intensity across the AP length of the selection (consistent between embryos) for each pixel row. Intensity profiles were exported, aligned based on position of the ventral midline, smoothed with moving average smoothing (window-size 20) and plotted in Matlab R2019a. Fluorescence signal was normalized by subtracting the mean intensity, to adjust for variability in imaging conditions.

Cell rows depleted of F-actin (Figure 5D, Supplement to Figure 5C) were counted manually on the subapical projections used for intensity profiles. If depleted regions on both sides of an embryo were visible, both were counted and the average used. Differences in depleted cell rows between genotypes were represented in a box plot using Matlab and statistically compared using the non-parametric two sample Kolmogorov–Smirnov test.

**Pulsing analysis**

Images of live embryos with myosin and membrane markers during folding were obtained as above, but with faster scan speed and smaller z-depth, to obtain time intervals of 9 to 11 seconds between stacks, which is sufficient to capture typical myosin and area pulsing behavior (Martin et al., 2009). Cells across the mesoderm were tracked over time
using EDGE, and cell area and myosin intensity were exported. Using Matlab 2019a, we
detected peaks within individual cells of maximal myosin intensity increase, smoothing with a
moving average filter and then detecting local maxima. The cell behavior about 100 seconds
before and after each maximum was saved as a trace. Pulse traces were averaged for each
cell bin to identify average dynamics based on cell position. Persistence of myosin pulses
was defined as the minimum myosin intensity within each trace after a peak minus minimum
myosin intensity before the same peak. Persistence of area constriction was defined as the
maximum cell area after a myosin peak minus maximum cell area before the same peak.
Each trace was normalized to the center of the pulse. Persistence values were averaged by
bin and plotted in Matlab for an individual embryo.

To analyze further the relationship between area and myosin behaviors, we cross-
correlated rates of myosin and area change (as described in Martin et al., 2009). We used
the xcorr function in Matlab to cross-correlate the change in myosin intensity (myosin
intensity at a timepoint minus myosin intensity at the previous time point) with constriction
change (cell area at a timepoint minus cell area at the next time point, i.e. cross- positive
value if constricting, negative if stretching) for each cell trace. Cross-correlations for each
cell were averaged by bin (distance from the midline) and plotted.

**Curvature analysis**

Movies of the embryos for which we had measured the multicellular myosin gradient
(see above) (with at least 20 µm z-depth) were resliced in FIJI to create 1 µm thick cross
section images of the ventral furrow at the middle of the AP axis of the embryo. Apically
enriched myosin signal was used to trace the surface of the folding tissue with the freehand
line tool in Fiji. The fold was traced for a cross section at the center of the AP axis of the
embryo as well as for cross-sections 20µm anterior and posterior, respectively. The
timepoint of measurement was chosen as when the invagination depth was ~ 10 µm. The
XY coordinates of each trace were imported into Matlab R2019a. After manually determining
the position of the ventral midline, a circle fit (Nikolai Chernov (2020). Circle Fit (Taubin method) https://www.mathworks.com/matlabcentral/fileexchange/22678-circle-fit-taubin-method), MATLAB Central File Exchange. Retrieved February 5, 2020) was applied to the central part of the trace, 2.5 µm width around the midline. The inverse of the fitted circle radius was defined as the fold curvature. The three traces taken at different AP positions were averaged to obtain the fold curvature value for the embryo. Local curvature as displayed in Figure 7 A and B was generated using LineCurvature2D (Dirk-Jan Kroon (2020). 2D Line Curvature and Normals https://www.mathworks.com/matlabcentral/fileexchange/32696-2d-line-curvature-and-normals), MATLAB Central File Exchange. Retrieved March 16, 2020.) in Matlab. Post-fold lumen sizes were measured from central cross-sections of the same embryos whose curvature had been analysed, for a single AP position. Lumen area was measured by manually fitting an ellipse to the lumen in each cross-section in Fiji and measuring its area. Curvature and lumen measurements were compared between genotypes using the non-parametric two sample Kolmogorov–Smirnov test.

Regression analysis

All linear regression fits (Figures 1C, 7D, S7C) were performed in Matlab R2019a using the fitlm command. The original data as well as the best fit line were plotted and the R-squared value and best fit slope were reported.

For myosin gradients, the position of the most lateral bin with myosin levels above the half-maximal myosin intensity was used to define gradient width. This bin position was then compared to central curvature measurements of the same embryo at a later timepoint (at ~10 µm invagination depth, analyzed with circlefit). For Utrophin::GFP embryos, cell rows with depleted F-actin were counted manually and compared to central curvature of the same embryo at a later timepoint (at ~10 µm invagination depth).
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Figure 1. Tissue-wide mesodermal F-actin distribution is distinct from Myosin. (A) Myosin gradient extends 6 cells from the ventral midline (VM) and remains low. Images are apical surface view of embryo labeled with sqh::GFP (Myosin) and Gap43::mCherry (membrane) and segmented example of embryo with cell rows highlighted in different colors. Scale bar = 10 µm. Colors in image correspond to bins in adjacent plots; showing Myosin and apical area distributions in a representative wild-type embryo. Data is represented by box-and-whisker plot, where bottom and top sides of the box represent 25th and 75th percentile of cells, respectively. Midline is the median and red ‘+’ are outliers. Box-and-whisker plot is shown for one representative embryo (corresponds to image on left) with at least 4 cells per bin analyzed (median 52.5 cells). (B) Left: F-actin is depleted at the mesoderm-ectoderm
Images are apical shell projection (top) or cross-section (bottom) images of phalloidin stained embryos imaged before furrow formation. Scale bar = 10 µm. Right: Plot showing F-actin depletion in the mesoderm. Mean cortical F-actin intensity was calculated from segmented cells. Cortical F-actin density was calculated by integrating F-actin intensity around the cell periphery and dividing by cell perimeter. Cortical F-actin density was normalized to mean of first cell row. Data is represented by box-and-whisker plot, where bottom and top sides of the box represent 25th and 75th percentile of cells, respectively. Midline is the median and red ‘+’ are outliers. Box-and-whisker plot is shown for one representative embryo (corresponds to image on left) with at least 23 cells per bin analyzed (median 52 cells). (C) Left: F-actin accumulates in a gradient across the ventral mesoderm. Images are apical shell projection (top) or cross-section (bottom) images of phalloidin stained embryos imaged during furrow formation. Scale bar = 10 µm. Right: Plot showing F-actin depletion in the mesoderm. Mean cortical F-actin intensity was calculated and data represented the same as (B). Box-and-whisker plot is shown for one representative embryo (corresponds to image on left) with at least 15 cells per bin analyzed (median 73 cells). (D) Mean F-actin density traces for 3 embryos corresponding to (B) (pre-fold F-actin) and (C) (F-actin during folding), normalized by mean of 9th and 1st cell row, respectively. (E) Apical area is inversely correlated with cortical F-actin levels. Quantification of cortical F-actin density per cell (cortical F-actin intensity divided by perimeter) for the embryo shown in (C) was plotted as a function of apical area. Color of data points represents physical distance from ventral midline in µm. Average pre-fold cell area for fixed embryos (~16 µm² due to shrinkage during the fixation process) is indicated with dotted grey line. Note that cells towards the margin of the mesoderm have the largest area and lowest F-actin and cells farther out (i.e., ectoderm) have an area centered at ~16 µm² and higher F-actin levels.
Supplement to Figure 1. F-actin is depleted in the mesoderm during cellularization. (A) F-actin depletion in the mesoderm is not present in early cellularization, but appears towards the end of cellularization. Images are subapical shell projections (top) and cross-sections (bottom) of wild-type embryos at different stages stained with phalloidin (red) and anti-snail antibody (cyan). Scale bar = 10 µm. Bending of tissue during late cellularization in cross-
section is a fixation artefact. (B) F-actin marked by the fluorescently marked Utrophin::GFP (cyan) is depleted in the marginal mesoderm, but a general membrane marker, Gap43::mCherry (red), is not. Images show both markers in apical (top) and subapical (middle) shell projections and cross-sections. Scale bar = 10 µm. (C) Quantification by bin of apical Utrophin::GFP (top) and Gap43::mCherry (bottom) junctional fluorescent intensity, divided by perimeter. Although less pronounced, apical Utrophin::GFP shows a similar pattern to phalloidin, with low F-actin density in lateral mesoderm cells compared to ectoderm cells. Data is represented by box-and-whisker plots where each bin is a cell row at a given distance from the ventral midline (at least 67 cells per row analyzed, median 72 cells). Bottom and top sides of the box represent 25th and 75th percentile junctional intensity per cell, respectively. Midline is the median and red ‘+’ are outliers.
Figure 2. Snail and Twist distinctly regulate the tissue-wide F-actin distribution. (A) Snail expression boundary corresponds to F-actin depletion boundary. Images are from phalloidin-stained embryo co-stained with anti-Snail. Scale bar = 10 µm. (B) The snail mutant disrupts mesodermal F-actin depletion. Images are from representative, live homozygous snail mutant and normal sibling embryo expressing Utrrophin::GFP (F-actin). The snail mutant shows a lack of F-actin patterning. Scale bar = 20 µm. (C) Quantification of tissue-wide F-actin intensity profiles from 4 snail mutant and 4 normal sibling embryos (See Methods and Supplement to Figure 2). All snail mutants lack F-actin patterning. The decreased F-actin intensity at the edges of the ventral-lateral axis reflects the curvature of the tissue, which...
causes more tissue to scatter light between objective and more lateral parts of the projected layers. (D) The twist mutant exhibits F-actin depletion, but lacks F-actin elevation around midline. Images are from 6 representative, live homozygous twist mutant and 6 normal sibling embryos expressing Utrophin::GFP (F-actin). The twist mutant exhibits F-actin depletion. Scale bar = 20 µm. (E) Quantification of tissue-wide F-actin intensity profiles from multiple twist mutant and normal sibling embryos. All twist mutants exhibit F-actin depletion, but not the increase around the midline.
Supplement to Figure 2. Workflow for subapical shell projections in FIJI. Confocal image stacks are resliced along the ventral-lateral axis to get a cross-sectional view. A subapical trace is then drawn manually about 10-15 µm below the apical surface and the embryo is
resliced a second time along the trace. The central part of the embryo along the AP axis is then used to quantify intensity along the ventral-lateral axis.

**Figure 3.** RhoA activation occurs in a gradient. (A) Simplified diagram of signaling downstream of Twist, focused on RhoA regulation. (B) Image of RhoGEF2::GFP (orange) and Gap43::mCherry (magenta). Ventral midline on left. Scale bar = 10 µm. (D) Image of Anillin Rho-binding domain::GFP (yellow) and Gap43::mCherry (magenta). Ventral midline on left. (F) Image of Rok::GFP (cyan) and Gap43::mCherry (magenta). Ventral midline on left. (C, E, G) Apical RhoGEF2::GFP intensity (orange, C), Anillin Rho-binding domain::GFP intensity (yellow, E), and Rok::GFP intensity (cyan, G) as a function of distance from the ventral midline for one representative embryo, respectively. Data is represented by box-and-whisker plots where each bin is a cell row at a given distance from the ventral midline. Bottom and top sides of the box represent 25th and 75th percentile, respectively. Midline is the median and red points are outliers. At least 32 cells (RhoGEF2, median 85 cells), 58 cells (Active RhoA, median 76.5 cells), or 51 cells (ROCK, median 68.5 cells) were analyzed for each cell row, respectively. Insets shows average cell behavior from 5 embryos per condition.
Supplement to Figure 3. C-GAP is uniformly cytoplasmic across the ventral furrow. Apical shell projection of an embryo expressing GFP::C-GAP (green) and gap43::mCherry (membranes, magenta). White arrow indicates apical nucleus with no GFP::C-GAP signal. Scale bar = 10 µm.
Figure 4. Myosin gradient and uniform F-actin depletion do not require intercellular coupling.

(A) Images (apical shell projections) of control (Rh3-RNAi) and α-catenin-RNAi embryos expressing sqh::GFP (Myosin, green) and Gap43::mCherry (Membranes, magenta). Scale bar = 10 µm. (B) and (C) Quantification of apical area (magenta, B) and normalized apical, active myosin (green, C) as a function of distance from ventral midline. Data is represented by box-and-whisker plots where each bin is a cell row at a given distance from the ventral midline (at least 41 (control) or 44 (α-catenin-RNAi) cells per row analyzed, respectively; median 72 (control) or 107 (α-catenin-RNAi) cells). Bottom and top sides of the box represent 25th and 75th percentile, respectively. Midline is the median and red points are
outliers. (D) Left: Images of phalloidin-stained control (Rh3-RNAi, top) and α-catenin-RNAi (bottom) embryos focused on the mesoderm-ectoderm boundary. Scale bar = 10 µm. Right:
Quantification of normalized cortical F-actin levels in rows of cells along the ventral-lateral axis (at least 52 (control) or 8 (α-catenin-RNAi) cells per row analyzed, respectively; median 78 (control) or 40.5 (α-catenin-RNAi) cells). Data is represented by box-and-whisker plots where each bin is a cell row at a given distance from the ventral midline. Bottom and top sides of the box represent 25th and 75th percentile, respectively. Midline is the median and red points are outliers. (E) Individual mean traces for multiple control and α-catenin-RNAi embryos. Top: Average apical area behavior from 4 (control) and 5 (and α-catenin-RNAi) different embryos, respectively. Mid: Average active myosin behavior from 4 (control) and 5 (and α-catenin-RNAi) different embryos, respectively. Bottom: Average cortical actin behavior from 3 different embryos, respectively
Supplement to Figure 4: F-actin is colocalized with junctions in wild-type and with medioapical myosin spots in α-catenin-RNAi embryos. (A) Top: Apical shell projection and cross-section (maximum intensity projection of 20 µm) images of fixed control (Rh3-RNAi) embryo stained with phalloidin (red) and anti-E-cadherin antibody (cyan). Bottom: Cross-section images of control (Rh3-RNAi) embryo stained with anti-armadillo (β-catenin) antibody (red) and anti-Snail antibody (cyan, marking the mesoderm). Note that transition from low to high F-actin at ectoderm boundary matches adherens junction intensity transition. Scale bars = 10 µm. (B) Images of ventral apical surface of fixed embryo expressing sqh::GFP (Myosin light chain marker), stained with phalloidin (red) and anti-GFP (cyan) antibody. White arrows indicate medioapical spots in which myosin and F-actin are colocalized. Scale bar = 10 µm.
**Figure 5:** RhoA overactivation widens actomyosin gradient and narrows F-actin depleted zone. (A) Images (apical shell projections) of control (Rh3-RNAi), C-GAP-RNAi and RhoGEF2 O/E embryos expressing sqh::GFP (Myosin, green) and Gap43::mCherry (Membranes, magenta). Scale bar = 10 µm. (B) and (C) Quantification of normalized apical, active myosin (green, B) and apical area (magenta, C) as a function of distance from ventral
midline. Data is represented by box-and-whisker plot where each bin is a cell row at a given distance from the ventral midline (at least 27 (control), 50 (C-GAP-RNAi) or 23 (RhoGEF2 O/E) cells per row analyzed, respectively; median 85 (control), 108.5 (C-GAP-RNAi) or 85 (RhoGEF2 O/E) cells). Bottom and top sides of the box represent 25th and 75th percentile, respectively. Midline is the median and red points are outliers. Insets show 4 embryos for each condition. (D) Width of F-actin depleted zone is narrower in C-GAP-RNAi and RhoGEF2 O/E embryos. Left: Images (Top, subapical shell projection, and Bottom, 20 µm maximum intensity projection of cross-section) of control (Rh3-RNAi), C-GAP-RNAi and RhoGEF2 O/E embryos expressing Utrophin::GFP. Scale bars = 20 µm. Right: Quantification of cortical F-actin levels by physical distance along the ventral-lateral axis. Data is represented as mean intensity for one representative embryo. (E) Width of F-actin depleted zone is wider in RhoGEF2-RNAi embryo. Top: Images (subapical shell projection (top), and 20 µm maximum intensity projection of cross-section (below)) of RhoGEF2-RNAi embryo expressing Utrophin::GFP. Scale bar = 20 µm. Bottom: Quantification of cortical F-actin levels by physical distance along the ventral-lateral axis for one RhoGEF2-RNAi embryo. (F) Width of F-actin depleted zone in cell rows for RhoGEF2-RNAi, control (Rh3-RNAi), C-GAP-RNAi and RhoGEF2 O/E embryos. Data is represented by box-and-whisker plot overlaid with data points for each analyzed embryo. Bottom and top sides of the box represent 25th and 75th percentile, respectively. Midline is the median and red ‘+’ are outliers.
Supplement to Figure 5: RhoA and actomyosin gradient width, but not mesoderm width, are affected by RhoA regulators. (A) Images (apical shell projection) of control (Rh3-RNAi) and C-GAP-RNAi embryos expressing Anillin Rho-binding domain::GFP (active RhoA, yellow) and Gap43::mCherry (Membranes, magenta). Scale bar = 10 µm. (B) Subapical shell projections of embryos expressing Utrophin::GFP, indicating number of cell rows from the ventral midline to the mesoderm border (as visualized by change in F-actin level) for different genotypes (~9 cell rows). Cell rows with blue numbers are within the F-actin depleted zone and cell rows with orange numbers are within the F-actin gradient around the ventral midline. Scale bars = 20 µm. (C) Top: Image (apical shell projection) of RhoGEF2-RNAi embryo expressing sqh::GFP (Myosin, green) and Gap43::mCherry (Membranes, magenta). Scale bar = 10 µm. Bottom: Quantification of apical area (magenta) and normalized apical, active myosin (green) as a function of distance from ventral midline (at least 23 cells per cell row were analyzed; median 38 cells). Data is represented by box-and-whisker plot where each bin is a cell row at a given distance from the ventral midline. Bottom, and top, sides of the box represent 25th and 75th percentile of cells, respectively.
Midline is the median and red ‘+’ are outliers. Inset shows average cell behavior from 3 different embryos.
**Figure 6:** Contractile dynamics vary with ventral-lateral cell position. (A) There are distinct types of dynamic cell behaviors during ventral furrow formation. Top: When RhoA activity is high, cells preferably undergo “ratcheted” myosin pulses after which myosin increases and apical area is sustained. Middle: At moderate RhoA activity, cells undergo myosin pulses in which myosin and apical area reverse after contraction. Bottom: In marginal mesoderm cells, myosin pulses occur while the apical cell surface stretches, but the relationship of myosin and stretching dynamics was not understood. (B) Myosin persistence is highest close to the ventral midline. Average and standard error of myosin levels during and after a myosin pulse (local maxima in myosin accumulation rate) for cell row 1 at the midline (light green), intermediate cell row 4 (mint) and marginal mesoderm cell row 7 (dark green) shown for one representative embryo. 106 pulses were analyzed for cell row 1; 192 pulses were analyzed for cell row 4; 335 pulses were analyzed for cell row 7. (C) Myosin pulses away from the midline are associated with stretching. Average and standard error of apical area dynamics around pulses (local maxima in constriction rate) for cell row 1 at the midline (light magenta),
intermediate cell row 4 (maroon) and marginal mesoderm cell row 7 (dark magenta) shown for one representative embryo. 106 pulses were analyzed for cell row 1; 192 pulses were analyzed for cell row 4; 335 pulses were analyzed for cell row 7. (D) Average and standard error for persistence of myosin (minimum myosin 0-100 s after pulse - minimum myosin 0-100 s before pulse) and area (maximum area 0-100 s after pulse - maximum area 0-100 s before pulse) by bin (distance from the midline) for three embryos. At least 83 pulses were analyzed for each cell row in each embryo. Median 192, 238 and 343 pulses analyzed per cell row for the three embryos, respectively. (E)-(F) Top: Cross-correlation between the rate of change in apical myosin intensity (normalized to apical area) and constriction rate averaged by cell bin; split up by ventral mesoderm (cell rows 1-5, E), marginal mesoderm (cell rows 7-9, F) and ectoderm (cell rows 11 and 12, G). At least 21 cells per cell row were analyzed, median 32 cells per cell row. Bottom: Myosin (green) and apical area (magenta) traces (normalized to average) and images of representative individual cells during a myosin pulse, for each region. Scale bars = 2 µm.
Figure 7: The contractile pattern determines fold curvature and post-fold shape. (A) Subapical shell projection and later cross-sections during and after folding of a single control (Rh3-RNAi) embryo expressing sqh::GFP (myosin) and gap43::mCh (membranes). In control embryos, in which the actomyosin gradient extends from cells 2-5, the ventral-lateral cross-section shows a narrow, v-shaped fold with high curvature at the center (local curvature is color-coded on the surface of the fold). The cross-sectional view of this embryo after folding shows a tube with a very small lumen. Scale bars = 10 µm. (B) Subapical shell projections and cross-sections during and after folding of a single C-GAP-RNAi embryo (top) and a single RhoGEF2 O/E embryo (bottom), expressing sqh::GFP (myosin) and gap43::mCh (membranes). C-GAP-RNAi and RhoGEF2 O/E embryos, which have a widened gradient with uniform myosin at the ventral midline display lower central fold curvature (local curvature is color-coded on the surface of the fold). At later timepoints, they often have an enlarged tube lumen as outlined by myosin signal in cross-section. (C)
Quantification of curvature at the center of the fold (measured by fitting a circle, 3 measurements at different AP positions averaged per embryo) (top) and lumen size (measured by fitting an ellipse) (bottom) for control (Rh3-RNAi), C-GAP-RNAi and RhoGEF2 O/E embryos. Data is represented by box-and-whisker plot overlaid with data points representing each quantified embryo. Bottom and top sides of the box represent 25th and 75th percentile of embryos, respectively. Midline is the median and red ‘+’ are outliers. (D) Regression analysis of the relationship between myosin gradient width and curvature for control, C-GAP-RNAi and RhoGEF2 O/E embryos. Gradient width was measured as the cell row bin that was just above the half-maximal intensity of the myosin gradient. Curvature was measured as in (C). (E) Model for the regulation of tissue-wide patterning in the ventral furrow. Uniform snail expression causes uniform F-actin depletion in the mesoderm. An overlapping pattern of graded twist expression acts via tightly tuned RhoA activation to generate an actomyosin gradient. Folding is driven by the combination of ventral midline cell actomyosin contractility and stretching of marginal mesoderm cells, which are depleted of F-actin.
Supplement to Figure 7: The width of the F-actin depletion zone is correlated with fold curvature. (A) Subapical shell projections during early folding (left) and cross-sections (right) later during folding for single RhoGEF2-RNAi, control (Rh3-RNAi), C-GAP-RNAi and RhoGEF2 O/E embryos expressing Utrophin-GFP. In cross-section, local curvature is color-coded on the surface of the fold. Scale bars = 10 µm. (B) Quantification of curvature at the center of the fold (measured by fitting a circle, three measurements averaged per embryo) for RhoGEF2-RNAi, control (Rh3-RNAi), C-GAP-RNAi and RhoGEF2 O/E embryos. Data is represented by box-and-whisker plot overlaid with data points from all quantified embryos. Bottom, and top sides, of the box represent 25th and 75th percentile, respectively. Midline is the median and black ‘+’ are outliers. (C) Regression analysis of the relationship between F-actin depleted region width and curvature for RhoGEF2-RNAi, control, C-GAP-RNAi and RhoGEF2 O/E embryos. Depleted F-actin region width was determined by the number of cell rows with low F-actin intensity. Curvature was measured as in (B).
Supplemental Movie S1 (related to Figure 4). Shell-projection (with background subtraction and gaussian blur) of control (Rh3-RNAi) embryo expressing sqh::GFP (myosin, green) and gap43::mCh (membranes, magenta) during ventral furrow formation. t=0 corresponds to the timepoint analyzed in Figure 4. Time step = 43.29 s, Frame rate = 2.5/s.

Supplemental Movie S2 (related to Figure 4). Shell-projection (with background subtraction and gaussian blur) of α-catenin-RNAi embryo expressing sqh::GFP (myosin, green) and gap43::mCh (membranes, magenta) trying to initiate ventral furrow formation. t=0 corresponds to the timepoint analyzed in Figure 4. Time step = 21.94 s, Frame rate = 5/s.

Supplemental Movie S3 (related to Figure 7). Maximum intensity projection of control (Rh3-RNAi) embryo expressing sqh::GFP (myosin, green) and gap43::mCh (membranes, magenta), during and after ventral furrow formation (gaussian blur radius =1). Time step = 53.653 s, Frame rate = 3.1/s.

Supplemental Movie S4 (related to Figure 7). Maximum intensity projection of RhoGEF2-RNAi embryo expressing sqh::GFP (myosin, green) and gap43::mCh (membranes, magenta), during and after ventral furrow formation (gaussian blur radius =1). Note divisions of mitotic domain 10 visible at the embryo surface after folding. Time step = 73.643 s, Frame rate = 4/s.
Supplemental Table S1. Fly Stocks and crosses used in this study:

<table>
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<th>Stock</th>
<th>Genotype</th>
<th>Source/Reference</th>
<th>Figure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>w, Gap43::mCherry(Gfp40); Ssoh::GFP</td>
<td>Bloomingston Drosophila Stock Center</td>
<td></td>
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<td>2</td>
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<td>CnsR</td>
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<td>6</td>
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<td>Rausch et al., 2019</td>
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<td>7</td>
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<td>Barde et al., 2013</td>
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This study (original Ubi-AnRBD-GFP stock: Munjal et al., 2015).

Supplemental Table S2. Imaging settings used in this study:

- F2 embryos imaged from these crosses, using above stock numbers/genotypes. Non-balancer females were used for cages.
- Stock #1 x 2 (Virgin x males)
  - Stock A: 1A, 6
  - Stock B: 4A, 5A
  - Stock C: 6x, 5x
  - Stock D: 8x, 8x
  - Stock E: 1x, 1x
  - Stock F: 2x, 2x
  - Stock G: 3x, 3x
  - Stock H: 4x, 4x
  - Stock I: 5x, 5x
  - Stock J: 6x, 6x
  - Stock K: 7x, 7x
  - Stock L: 8x, 8x
  - Stock M: 9x, 9x
  - Stock N: 10x, 10x
  - Stock O: 11x, 11x
  - Stock P: 12x, 12x

References:


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<th>Figures</th>
<th>Excitation laser wavelength</th>
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<th>Detector end wavelength (nm)</th>
<th>Detector Gain (a.u.)</th>
<th>Laser power (%)</th>
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References


