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REVIEW

Small GTPases modulate intrinsic and extrinsic forces that control epithelial folding in *Drosophila* embryos

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ABSTRACT

Epithelial folding is a common means to execute morphogenetic movements. The gastrulating Drosophila embryo offers many examples of epithelial folding events, including the ventral, cephalic, and dorsal furrows. Each of these folding events is associated with changes in intracellular contractility and/or cytoskeleton structures that autonomously promote epithelial folding. Here, we review accumulating evidence that suggests the progression and final form of ventral, cephalic, and dorsal furrows are also influenced by the behaviour of cells neighbouring these folds. We further discuss the prevalence and importance of junctional rearrangements during epithelial folding events, suggesting adherens junction components are prime candidates to modulate the transmission of the intercellular forces that influence folding events. Finally, we discuss how recently developed methods that enable precise spatial and/or temporal control of protein activity allow direct testing of molecular models of morphogenesis in vivo.

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Introduction

Morphogenetic movements convert simple embryonic body plans into multilayered embryos that are the precursors of complex adult organisms. Epithelial folding is an elemental morphogenetic movement which underlies several important developmental events, including gastrulation and neurulation. Tissue folding events involve changes in cell shape that are driven by cytoskeletal rearrangements and reorganization of cell-cell junctions, which are regulated by several small GTPases. These cell shape changes result from the interplay between intrinsic forces generated by the cells participating in folding events and tensile or compressive forces generated by neighbouring cells.

The early *Drosophila* embryo has emerged as one of the most well-studied models of epithelial folding. The earliest stages of Drosophila development take place in a syncytium, a contiguous cytoplasm with multiple nuclei undergoing synchronous divisions. Three hours post egg laying, during the 14th nuclear division, cellularization occurs, and membranes encapsulate individual nuclei, generating a simple columnar epithelium that surrounds the yolk and is surrounded by a vitelline membrane [1]. The elongated 14th nuclear division cycle is also remarkable for the pervasive and highly patterned transcription of the zygotic genome [2,3].

Several tissue folding events immediately follow cellularization, including ventral, cephalic, and dorsal furrowing. The tissue folding events in this system are genetically tractable, and the underlying cellular behaviours are amenable to high-resolution imaging. Ventral furrow formation, the first step in *Drosophila* gastrulation, has been particularly deeply analysed. During this process, approximately 1000 ventrally located epithelial cells constrict their apical surfaces and invaginate (Figure 1) [4–6]. This morphogenetic movement initiates shortly after zygotic transcription begins and cellularization completes. Ventral furrowing proceeds rapidly, completing in about 15 minutes [5]. Proper execution of ventral furrow formation is critical for development as it internalizes the cells of the embryonic mesoderm. Once internalized by the ventral furrow, mesodermal precursors undergo an epithelial to mesenchymal transition, losing their cell-cell adhesions and eventually migrating dorsally [7,8]. The information gleaned from extensive genetic and cell biological experiments has led to the development of feasible, coarse-grained molecular models of ventral furrow formation.

The ventral furrow is not the only example of epithelial folding during early *Drosophila* embryogenesis. Concomitant with ventral furrow formation, the

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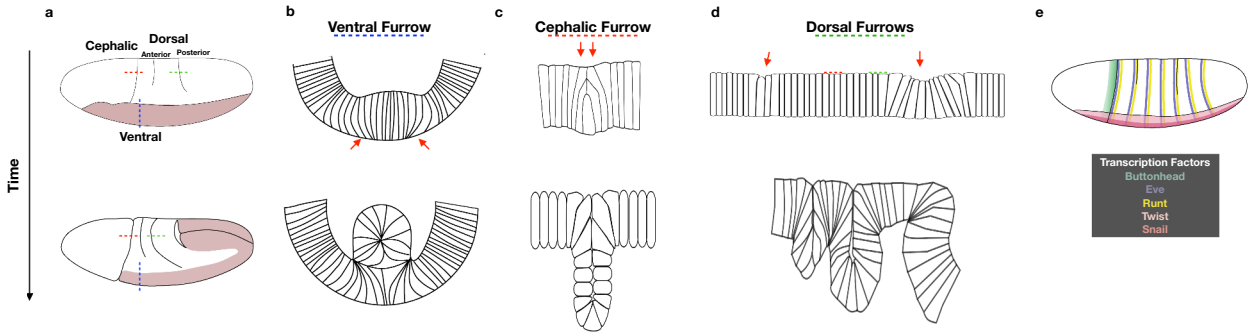

Figure 1. Overview of ventral, cephalic, and dorsal folding events that occur in the early *Drosophila* epithelium.
a) Schematic of *Drosophila* embryo at late stage 6 (top) and stage 8 (bottom) with positions of ventral, cephalic, and dorsal furrows indicated. Dashed lines indicate the orientation of the cross-sections shown in b-d. Pink shading indicates internalized mesoderm.
b-d) Cross-sectional views of cellular behaviours during ventral (b), cephalic (c), and dorsal (d) folding events. Red arrows indicate bending of neighbouring cells towards furrows.
e) Schematic of transcriptional patterning associated with folding events.

cephalic furrow appears in the anterior region of the gastrulating embryo, and, shortly thereafter, a pair of transverse folds forms in the dorsal epithelium (Figure 1) [6,9]. Unlike the ventral furrow, the cephalic and dorsal folds eventually regress. Though each of these processes result in an epithelial fold, the cell shape changes and molecular events underpinning each are distinct. Comparing and contrasting these folding events, which all occur within the continuous *Drosophila* embryonic epithelium, provides a useful lens through which to elucidate some general principles of morphogenesis.

This review integrates a set of results that indicate that the nature of a fold in a given region of the *Drosophila* embryonic epithelium depends not only on the organization and remodelling of intracellular structural components, such as the contractile cytoskeleton, but also on the generation and transmission of tensile and compressive forces throughout the embryo. We further discuss the role of junctional proteins in transmitting these forces. In particular, we will discuss how three GTPases from the Ras superfamily, RhoA/Rho1, Rap1, and Rab35, contribute to the formation of epithelial folds in the *Drosophila* embryo. Each of these GTPases undergoes activation into the GTP-bound state by guanine nucleotide exchange factors (GEFs) on the plasma or other internal membranes. Active GTPases then recruit and/or activate a cognate set of effector proteins that trigger myosin-dependent contractility (Rho1), alteration of cell-cell or cell-substrate adhesion (Rap1), or plasma membrane remodelling (Rab35) [10–13]. Subsequently, GTPase activating proteins (GAPs) induce the GTPases to self-inactivate by hydrolysing their bound nucleotide (GTP). Each of these cellular events is spatiotemporally regulated in large part through control of GEF and GAP activity. The effectors of these GTPases are understood to various degrees. In the case of Rho1, these include a subset of f-actin nucleators of the formin family, the kinase ROCK, and the scaffold protein anillin [14]. A number of effectors of Rap1 and Rab35 has been identified, such as the Rap1 effectors Canoe and Raf as well as the Rab35 effector OCRL, a PIP2 lipid phosphatase [15,16]. The complete set of relevant effectors in the context of morphogenesis remains to be fully elucidated [17,18].

Specification and execution of cell shape changes during furrow formation

We begin by briefly summarizing the cellular and molecular events that regulate and execute the formation of the ventral, cephalic, and dorsal folds (Table 1). Ventral furrow formation has been extensively studied, and we refer interested readers to several recent reviews that focused on this morphogenetic event [19,20]. Formation of the cephalic and dorsal folds are less well understood.

Cells of the ventral furrow are specified to undergo furrowing by a maternally contributed, extracellular protease cascade that specifically acts in the ventral extra-embryonic perivitelline space to activate a ligand, Spätzle, of the Toll receptor (Figure 2) [21–23]. Once activated, Toll induces a gradient of nuclear entry of the transcription factor Dorsal along the dorsal-ventral axis, with the nuclei of the ventral-most cells

Table 1. Summary of *Drosophila* embryonic epithelial folding events.

Folding Event	GTPase(s)	Apical Narrowing	Actomyosin Contractility	Adherens Junction Remodelling
Ventral Furrow	Rho1, Rap1, Rab35	Myosin directed	Apical & lateral	Apical shift
Cephalic Furrow	Rho1	Yes	Lateral	Apical shift
Dorsal Furrows	Rap1	Yes	Not detected	Basal shift

accumulating the highest amounts of active Dorsal protein [24–26]. In these cells, Dorsal drives the expression of two additional transcription factors, Snail and Twist, which are the primary transcription factors that regulate ventral furrow formation [27,28].

Snail and Twist, through the intracellular signalling cascade described below, induce cell shape changes that drive ventral furrowing, beginning with the flattening of ventral cells' apical surfaces. Subsequently, the apical surfaces of these cells begin to constrict sporadically throughout the ventral epithelium [5,29,30]. These constrictions become more widespread, involving approximately 12 rows of ventral cells along the anterior-posterior axis [5,31]. Volume is conserved in these cells during apical constriction, generating wedge-shaped cells with broader basal domains (Figure 3b) [32]. The apical constriction of ventral cells is anisotropic, deforming more along the dorsal-ventral axis than the anterior-posterior axis. Collectively, these cell shape changes lead to the formation of a shallow invagination that gradually deepens (Figure 1b) [4,5]. The apical regions of several rows of cells lateral to the invaginating cells deform towards the invagination, while their basal surfaces largely remain stationary, resulting in cell bending (Figure 1b) [4,5]. The apical surfaces of these bent cells eventually meet across the midline and form new junctions, severing the tube of invaginated cells from the ectoderm (Figure 1b, bottom) [33].

Like the ventral furrow, transcriptional patterning also defines the cephalic furrow. Specifically, the overlapping expression of Buttonhead and stripe 1 of Even-skipped (Eve), two transcription factors acting downstream of the anterior morphogen Bicoid, specify the initiating cells of the cephalic furrow (Figure 1e) [34]. This furrow initiates in the lateral epithelium on each side of the embryo and extends bidirectionally, ultimately around the entire embryo (Figure 1a) [6,35]. Furrowing initiates with lateral shortening of one or two cells, drawing and bending the neighbouring cells towards the initiating cells, similar to the bending of the cells flanking the ventral furrow (Figure 1c) [35,36].

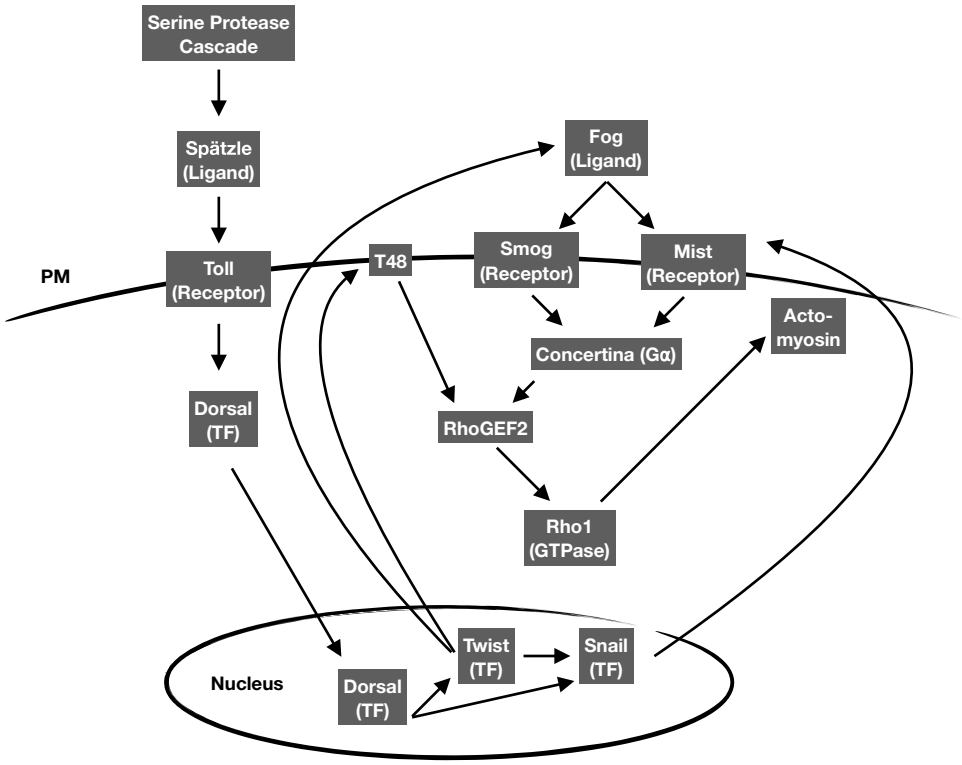

Figure 2. Genetic logic of ventral furrow formation. TF: transcription factor. PM: Plasma membrane.

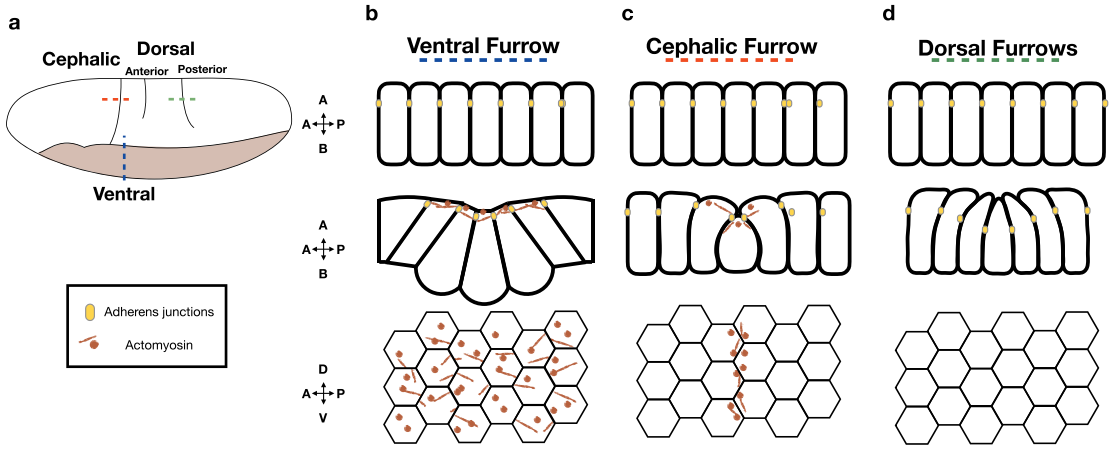

Figure 3. Cytoskeletal and junctional changes at furrow initiation.
a) Schematic of furrowing events in a late stage 6 *Drosophila* embryo. Dashed lines indicate position and orientation of cross-sections shown in b-d. Pink shading indicates internalized mesoderm.
b-d) Cross-sections (top & middle) of ventral (b), cephalic (c), and dorsal (d) furrows before (top) and during (middle) furrow formation schematize the rearrangements of adherens junctions components (yellow) during all three folding events as well as the accumulation of actomyosin in the ventral and cephalic furrows. En face views (bottom) demonstrate that actomyosin accumulates throughout apical regions of invaginating ventral furrow cells (b, bottom). In contrast, apical and lateral actomyosin accumulate preferentially along the dorsal-ventral axis of cephalic furrow cells (c, bottom).

The initiating cells, despite apically narrowing, do not exhibit apical flattening [36]. Subsequently, this furrow deepens, involving approximately 13–15 rows of cells along the anterior-poster axis (Figure 1c) [36]. This deep furrow brings the cell rows just outside the furrow into contact with one another, but new junctions do not form.

The transcriptional programme that specifies the two dorsal folds is not known. These folds form coincident with the second and fifth stripes of Runt expression, but Runt is not required for dorsal fold formation [9]. Dorsal furrowing initiates around the same time as ventral furrow formation initiation, with each central, initiating cell undergoing isotropic apical narrowing and lateral shortening, much like the cells in the cephalic furrow (Figure 1d) [37]. As the folds develop, initiating cells expand their basal volume and become wedge shaped (Figure 1d) [9]. These shape changes in the initiator cell appear to induce the flanking cells to bend towards the initiator, forming the dorsal transverse folds (Figure 1d) [9]. The anterior dorsal fold contains 5–7 rows of cells along the anterior-posterior axis, whereas the more deeply invaginating posterior dorsal fold contains 19–21 rows of cells (Figure 1) [9, 2013]. These folds propagate across the dorsal and lateral surfaces of the embryo but do not reach the ventral surface [9].

Importantly, ventral, cephalic, and dorsal furrows form alongside other morphogenetic movements. For example, shortly after the initiation of the ventral and cephalic furrows, the germ band begins to elongate. During this morphogenetic process, lateral ectoderm cells converge along the dorsal-ventral axis, elongating the ectoderm along the anterior-posterior axis and causing it to wrap around the posterior end of the embryo (Figure 1a, bottom). Subsequently, the dorsal and then the cephalic folds regress, well before germ band retraction [6,37].

Each of these folds initiate via intrinsic, or cell autonomous, regulation. However, the final forms of these folds are subject to extrinsic regulation, resulting from tension and/or compression exerted by cells neighbouring the cells within folds [37]. In the following sections, we will review what is known about these intrinsic and extrinsic forces.

Cellular mechanisms that induce furrow formation

Actomyosin contractility promotes ventral and cephalic furrowing

The intrinsic mechanism that drives cell shape changes and epithelial folding is best understood in the context of ventral furrowing, where the aforementioned transcriptional network involving Twist and Snail indirectly promote Rho1 GTPase activation in a subset of ventral cells (Figure 2). Twist promotes the expression of T48, a transmembrane protein, and Fog, a secreted ligand

[38,39]. Snail promotes the expression of a G protein coupled receptor (GPCR), Mist [40]. The ligand Fog binds Mist and activates Concertina, a maternally contributed Gα protein [40–42]. Activated Concertina, T48, and an additional, maternally contributed GPCR, Smog, cooperate to apically localize and activate RhoGEF2, a guanine nucleotide exchange factor that promotes Rho1 activity and actomyosin accumulation [10,11,39,40,43,44].

Once activated, Rho1 drives the formation of apical, contractile actomyosin networks in individual ventral cells [45,46]. This apical actomyosin network drives the apical constriction and cellular wedging discussed in the previous section (Figure 3b). These intracellular actomyosin networks within cells are coupled via adherens junctions, generating a supracellular actomyosin network that promotes robust ventral furrow formation [47,48]. Detailed analysis of the subcellular dynamics reveals that Rho1 activity and myosin accumulate in a pulsatile manner at the apical surface of each cell and that these pulses of Rho1 and myosin translate into pulsed apical constrictions [46,49]. Initially, the pulses of apical constriction are not ratcheted and relax to their pre-constriction state following dissolution of actomyosin punctae. Ventral cells eventually transition to a phase of ratcheted constriction, where actomyosin-driven cell shape changes are stabilized via a Twist-dependent ratcheting mechanism [30,46]. The current model of ventral furrow formation posits that pulsed actomyosin contractions enable step-wise intracellular rearrangements and are essential for ventral furrow formation.

Much focus has been placed on the role of apical-medial actomyosin during ventral furrow formation, but recent work suggests that actomyosin cables running along the apico-basal axis might also contribute to this morphogenetic movement [50]. These apico-basal cables of myosin in the presumptive mesoderm are under tension but they are not essential for ventral furrowing. However, these apico-basal cables are required for ventral furrow formation in embryos in which the ventral domain is isolated from the poles by laser microsurgery. Thus, these apico-basal myosin cables may contribute to the robustness of ventral furrow formation and/or act as a redundant mechanism for internalizing the mesoderm.

The intrinsic molecular underpinnings of cephalic furrow formation are less well understood, but, like the ventral furrow, they appear to involve actomyosin-mediated contractility. Myosin accumulates in the apical medial region as well as along the lateral cortex of the initiator cells of the cephalic furrow [35,36]. The accumulation of lateral myosin occurs preferentially along the dorsal-ventral axis (Figure 3c, bottom) [35]. The intracellular signalling cascade that drives this myosin accumulation is not known. Optogenetic recruitment of dominant negative Rho1 to the plasma membrane of initiator cells delays and compromises cephalic furrowing, suggesting Rho1 mediates this myosin accumulation [35].

Rearrangement of polarity proteins is required for dorsal furrowing

The earliest detected molecular event prior to the formation of the dorsal transverse folds is the shifting of Bazooka, a canonical polarity factor, to a more basal position in the lateral membrane (Figure 3d) [9]. This shift of Bazooka in dorsal fold cells relative to neighbouring cells is required for dorsal fold formation [9]. As in cephalic furrowing, the apical surfaces of cells within the dorsal folds retain their dome shapes during dorsal fold formation [9]. Reorganization of an apical microtubule network within dorsal fold cells is required to reduce the height of cells in the dorsal fold [51].

In contrast to the ventral and cephalic furrows, myosin accumulation is not detected prior to the formation of the dorsal transverse folds [9], suggesting that actomyosin-mediated contractility does not drive these cell shape changes. However, these folds form in an epithelium with low levels of cortical actin and myosin [52], so rather modest changes in intrinsic contractility, which evaded detection by the methods used thus far, may be sufficient to drive these cell shape changes. Additionally, external forces may contribute to the cell shape changes that occur during dorsal transverse fold formation (see below).

Folding events are associated with junctional rearrangements

Cells within each of the epithelial furrows discussed here are connected to each other and to neighbouring cells outside of the furrows by adherens junctions, composed of cadherin proteins. These cell-cell junctions undergo dynamic rearrangements during all of the tissue folding events reviewed here. These rearrangements both precede and follow furrow initiation.

Prior to ventral furrow formation, sub-apically localized adherens junctions undergo partial disassembly specifically in cells of the presumptive ventral furrow [53]. This disassembly requires the expression of the transcription factor Snail [53]. The polarity protein Baz also dissociates from the membrane following the disassembly of junctions [54]. Just a few minutes later, junctional proteins reassemble into dense, apically located structures (Figure 3b) [39,53,55]. This process requires myosin-mediated contractility [53] and wild-

type levels of Traf4, a protein family best known for its association with tumour necrosis factor (TNF) receptor [56]. While the functional consequences of these junctional rearrangements remain to be determined, these rearrangements may influence or even enable the cell shape changes that underlie ventral furrow formation.

The mechanism underlying these junctional rearrangements in ventral cells during ventral furrow formation is incompletely understood. Interestingly, Rap1, a GTPase known to regulate both adherens junctions and focal adhesions, is required for proper ventral furrow formation: Rap1 deficient embryos exhibit uncoordinated apical constriction and delayed ventral furrow formation [13]. Rap1 activity may be specifically modulated in cells of the ventral furrow and, thus, directly promote the aforementioned junctional rearrangements. Alternatively, Rap1 may affect ventral furrow formation indirectly by modulating junctions in cells neighbouring the ventral furrow. Sensors that map the activity pattern of Rap1 during early embryogenesis will help distinguish between these two possibilities.

Endocytosis of junctional proteins may also play a role in the cell shape changes that occur during ventral furrowing. Rab35 and its GEF, Sbf (SET binding factor), which are involved in regulating plasma membrane remodelling, likely via endocytosis, are required for apical constriction and ventral furrowing [12,56]. Endocytosis could contribute to ratcheted apical constriction by remodelling apical cell membranes following actomyosin-mediated constriction and/or altering the amount or the stability of junctional E-Cadherin.

The importance of adherens junctions during ventral furrowing is highlighted by the fact that this morphogenetic movement is particularly sensitive to the perturbation of E-cadherin function. A form of E-Cadherin that lacks the extracellular, juxtamembrane domain does not support successful ventral furrowing. Embryos expressing only this mutant version of E-cadherin undergo an aberrant, non-uniform, less anisotropic apical constriction, and the mesoderm fails to fully invaginate [57]. In principle, these results could be explained by this mutant E-cadherin functioning as a hypomorph. However, partial depletion of junctional components by RNAi results in epithelial tears [47], which are not observed in embryos expressing the E-Cadherin variant that lacks its extracellular juxtamembrane domain [57]. Furthermore, this E-cadherin variant is sufficient for cell adhesion in a number of other developmental contexts [57]. These results suggest that this juxtamembrane region of E-cadherin may perform a ventral furrow-specific function and that ventral junctions may have distinct requirements beyond simple cell-cell adhesion.

This ventral-specific requirement for full E-cadherin function may be due to the higher forces experienced by ventral cells than lateral or dorsal cells during ventral furrowing, resulting from the intracellular actomyosin contractility in ventral cells. Consistent with this, Ajuba, a LIM domain containing protein, preferentially accumulates at junctions of ventral cells during the early stages of gastrulation [58]. Recent data indicate that a subset of LIM domain proteins bind to actin filaments under tension [59,60]. However, a functional role of Ajuba in ventral furrowing or other folding events has not yet been described.

As in the ventral furrow, adherens junctions shift apically during cephalic furrow formation (Figure 3c) [36]. However, in contrast to ventral furrow formation, cephalic furrow initiation precedes the apical shift of junctional proteins. The biological implications of the junctional shift during cephalic furrowing have not been shown nor has the mechanism that drives this reorganization in cephalic furrow cells been elucidated. However, given that myosin promotes the shift of junctional proteins in ventral furrow cells and that myosin accumulates in cells of the cephalic fold, it is possible that the apical shift of junctional proteins in these cells is also driven by myosin contractility.

Unlike the ventral and cephalic furrows, junctional proteins in cells of the dorsal transverse folds shift basally (Figure 3d). As noted above, this junctional shift is critical to the formation of these folds. Canonical polarity proteins regulate junctional shifts during dorsal fold formation: Embryos deficient in the polarity protein Bazooka fail to shift junctions in the dorsal epithelium, whereas embryos deficient in Par1 kinase shift junctions throughout the dorsal epithelium [9]. As in ventral furrow formation, the GTPase Rap1 regulates dorsal fold formation. The overexpression of constitutively active or dominant negative Rap1 limits the invagination of dorsal folds, suggesting that the level of Rap1 activity is important for the formation of these folds [61].

Thus, though distinct intracellular mechanisms appear to drive ventral, cephalic, and dorsal furrowing, junctional rearrangements occur in each of these morphogenetic events. Moreover, in the case of ventral and dorsal furrows, these junctional rearrangements are required for proper morphogenesis. We speculate that these junctional rearrangements promote these morphogenetic events by facilitating rapid and efficient cell shape changes as well as altering the site of force transmission.

Extrinsic forces, likely mediated by adherens junctions, influence furrow formation

The ventral, cephalic, and dorsal furrows form in the continuous embryonic epithelium, albeit in distinct regions of this epithelium. This raises the possibility that the shapes and behaviours of cells outside of furrows are influenced by these morphogenetic events. Consistent with this hypothesis, cells adjacent to the constricting cells of the ventral furrow undergo dramatic shape changes: Their apical surfaces move towards the invaginating ventral furrow while their basal surfaces remain in place, resulting in these ventro-lateral cells exhibiting a profoundly bent cell shape along the apicobasal axis (Figure 1b) [4,5,38,52,62]. Ventrolateral cells do not exhibit this bending in embryos that fail to form a ventral furrow, such as those lacking Snail and/or Twist [4], and recent optogenetic experiments show that this bending is driven, at least in part, by Rho1 activation within ventral cells [63]. Bending of neighbouring cells towards the cephalic and dorsal furrows is also observed (Figure 1c,d) [9,34,36]. Collectively, these results support the idea that cells within the furrows of the *Drosophila* embryonic epithelium influence the behaviour of adjacent, non-ingressing cells.

In addition to being influenced by cells of invaginating furrows, cells neighbouring the ventral, cephalic, and dorsal furrows influence, either passively or actively, the progression of these furrows. For example, lateral ectoderm cells move towards the invaginating ventral furrow, and this collective cell movement is essential for full invagination of the mesoderm (Figure 4) [52]. Remarkably, the movement of lateral cells is not induced by the contractility of ventral cells: Ventral cells in embryos lacking Snail and Twist fail to initiate contractility, but lateral cells in these embryos still move towards the ventral midline, though at severely reduced rates [52]. In support of a model whereby neighbouring ectodermal cells influence this morphogenetic event, ventral furrows formed in embryos exhibiting elevated lateral ectodermal contractility fail to stabilize and complete ventral furrow invagination [64]. Furthermore, recent optogenetic experiments reveal that once the furrow has partially invaginated, myosin contractility in the furrowing mesoderm is dispensable for full furrow invagination due to compressive forces from the lateral ectoderm [65]. Thus, although actomyosin-based contractility is central to ventral furrowing, this morphogenetic movement also relies on the compliance of the lateral epithelium and the external compressive forces it produces as it moves.

Similarly, the progression of dorsal transverse folding appears to be influenced by germ band extension. Nascent dorsal folds are apparent before germ band extension, suggesting that germ band extension is not required for the initiation of these folds [37,66]. However, strongly reducing the rate of germ band extension, by eliminating torso-like, a secreted protein that promotes terminal patterning [67], slows the rate of dorsal fold ingression [61]. The dorsal folds in these mutant embryos eventually reach comparable depths as those in wild-type embryos, but the slowed rate of ingression in embryos lacking torso-like suggests that this folding in the dorsal epithelium is influenced by forces generated by cells in other regions of the epithelium [9, 38].

There is also strong evidence that the progression of furrows within the dorsal embryonic epithelium are affected by the level of contractility in cells between the two dorsal transverse folds, which express low levels of Fog, an upstream activator of Rho1: Embryos lacking Fog exhibit deeper dorsal furrows [54]. Conversely, the overexpression of Fog in an ectopic region of the embryonic epithelium eliminates dorsal transverse fold formation altogether [54]. These observations suggest that dorsal transverse furrow formation requires inhomogeneity in contractility in the furrowing cells relative to the surrounding epithelium.

The results discussed above provide strong evidence that furrow formation in the *Drosophila* embryo is influenced by the structure and behaviour of cells outside of the furrow. Adherens junctions, which couple

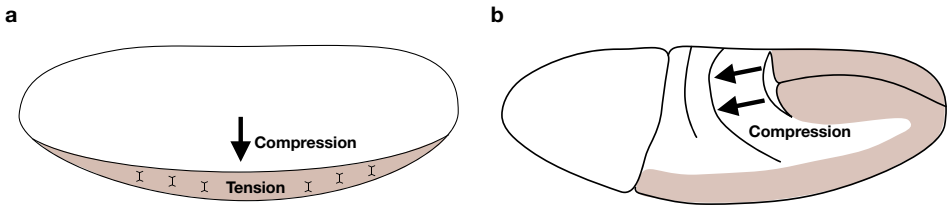

Figure 4. External forces acting upon the ventral and dorsal furrows. a) Schematic depicting the external forces that promote successful invagination of the ventral furrow. b) Schematic depicting the external forces that influence dorsal folding. though germ band extension is not essential for dorsal furrowing, it accelerates the process. Pink shading indicates internalized mesoderm.

cells within and outside of deformations together, and their associated proteins, such as catenins which couple junctions to the cytoskeleton, are prime candidates for modulating these intercellular influences. In support of this hypothesis, modulating alpha-catenin, a protein known to link beta-catenin with f-actin, activity affects furrow formation. Specifically, while the posterior dorsal furrow is deeper than the anterior dorsal furrow in wild-type embryos, depletion of alpha-catenin results in the anterior dorsal fold invaginating to a similar depth as the posterior dorsal fold [61]. Similarly, alpha-catenin depletion results in more deeply invaginated cephalic furrows [35]. One explanation of these phenotypes is that the tension mediated by junctions and associated proteins, including alpha-catenin, in the invaginating cells and their neighbours limits the ingression of epithelial folds.

Upstream of alpha-catenin, Rap1, a small GTPase, has emerged as a regulator of this junction-associated protein during furrow formation in the *Drosophila* embryo. The deeper invagination of the posterior dorsal fold relative to the anterior dorsal fold requires negative regulation of Rap1 [61]. Furthermore, as mentioned above, dorsal folding is impaired when Rap1 activity is altered by the expression of constitutively active or dominant negative Rap1 [61]. Importantly, the inhibitory effect of activated Rap1 on dorsal fold formation can be suppressed by depletion of alpha-catenin [61], suggesting that Rap1 regulates the activity of alpha-catenin during dorsal fold formation, perhaps by enhancing the molecular linkage between junctions and the actin cytoskeleton. While these results are consistent with Rap1 overexpression affecting the behaviour of the dorsal fold cells directly, these phenotypes may result from alterations in the external forces that modulate dorsal folding. A role for Rap1 in the cephalic furrow has not been demonstrated to date, but Rap1 and its GEF, Dizzy, are required for proper ventral furrow formation [13]. Thus, Rap1 and its regulation of junctional complexes via alpha-catenin may play a conserved role in modulating intercellular transmission of forces during furrow formation in the *Drosophila* embryo.

Testing models of morphogenesis

Furrow formation in early *Drosophila* embryos results from a combination of intrinsic and extrinsic forces. To what extent do we fully understand the intra- and intercellular events that underlie furrow formation in the *Drosophila* embryo? One way to assess the completeness of molecular models of ventral, cephalic, or dorsal furrowing is to attempt to recapitulate each of these furrowing events in an ectopic location in the embryonic epithelium by providing an exogenous molecular cue.

Towards this end, Bazooka over-expression throughout early embryos induces additional folds to form specifically in the dorsal epithelium [9]. These folds overlap with additional sites of Runt expression, which do not normally undergo folding. This result suggests that Bazooka is limiting for the formation of dorsal folds. However, Bazooka is not strictly sufficient to induce furrow formation as the ectopic folds form exclusively in the dorsal epithelium and at sites of Runt expression that do not normally furrow [9], suggesting that Bazooka cooperates with other factors which are also required for dorsal folding.

In the context of the ventral furrow, embryos defective in production of mature Spätzle fail to pattern the dorsal-ventral axis of the embryo and do not initiate ventral furrow formation [68]. This mutant phenotype can be rescued by injection of purified or recombinant Spätzle into the perivitelline (extraembryonic) space in either the dorsal or ventral epithelium [22,69]. Remarkably, a complete ventral furrow forms near the site of Spätzle injection, proving that the entire embryonic epithelium is competent to receive and interpret the ligand Spätzle. Similarly, the local expression of activated alleles of Toll can ventralize dorsal tissue [70].

Although ectopic Spätzle is sufficient to induce the signalling events that drive ventral furrow formation, overexpression of neither Fog nor Snail, which lie genetically downstream of Spätzle, throughout the entire embryonic epithelium, is sufficient to induce ectopic ventral furrow formation [53]. Ectopic Snail expression does induce junctional disassembly throughout the embryonic epithelium but does not recapitulate ventral cell behaviour, such as apical constriction or apical flattening [53]. Despite the strong ventral expression of the receptor Mist [40], Fog overexpression throughout the embryonic epithelium is sufficient to drive ectopic flattening of apical cell surfaces and induce junctions to shift apically, two hallmarks of ventral furrow formation [53,71]. However, even when Fog is ectopically expressed in a restricted region of the embryo via the Krüppel promoter, where it could induce symmetry breaking, it is not sufficient to induce furrow formation [53, 55]. Thus, while overexpression of Fog induces myosin accumulation and overexpression of Fog or Snail induces apical shifting of junctions, neither perturbation is sufficient to induce tissue folding.

The primary function of Fog in the context of ventral furrow formation is to induce the activation of

RhoGEF2, via a GPCR cascade [38]. RhoGEF2 is also directly activated in ventral cells by T48, a transmembrane protein that directly recruits RhoGEF2 via its PDZ domain [39,43]. If both Fog and T48 only activate RhoGEF2, and, if Rho1 activation was the sole requirement for ventral furrow formation, one would expect Fog overexpression to recapitulate ventral furrow formation. The fact that Fog over expression does not recapitulate ventral furrow formation suggests that Rho1 activation is not the only important cellular event downstream of Toll receptor activation. Spätzle activation of Toll induces Twist and Snail expression, and these transcription factors have additional targets beyond those, such as Fog, that are involved in Rho1 activation. Alternatively, these Fog overexpression experiments may fail to recapitulate ventral furrow formation because they do not generate an appropriate zone, level, and/or asymmetry of Rho1 activation.

Directly testing the Rho1-driven model of ventral furrow formation requires generating an asymmetric zone of Rho1 and determining whether it recapitulates the cell- and tissue-level behaviours observed during endogenous ventral furrow formation [72]. Two recent studies have directly tested this Rho1-centric model using optogenetics, a technique that readily enables precise spatiotemporal control of Rho1. Using two distinct optogenetic approaches that locally activate Rho1, via different recruitable Rho1 GEFs, both studies demonstrate that Rho1 activity is sufficient to induce ectopic furrowing in the dorsal *Drosophila* embryonic epithelium [63,73]. However, although these perturbations induce furrowing, the more recent of these studies demonstrates that local actomyosin contractility alone is insufficient to drive all the tissue-level shape changes that are characteristic of ventral furrowing. Specifically, Rho1 activation in the ventral epithelium can induce precocious furrows that recapitulate the hallmarks of ventral furrowing, including anisotropic apical constriction and bending of the flanking cells towards the contracting cells. Rho1 activation in the dorsal epithelium does not result in these behaviours [63].

The simplest explanation for the ability of Rho1 activation to induce anisotropic apical constriction and deformation of neighbouring cells in the ventral but not the dorsal epithelium is that the ventral epithelium has distinct properties as a consequence of ventral-specific gene expression. For example, ventral cells are thought to uniquely contain active Neuralized, a ubiquitin E3 ligase, although the relevant targets of this ubiquitin ligase are not yet known [64]. Additionally, there are notable differences in the cytoskeleton and junctions in the dorsal and ventral epithelia that could result in a different pattern of force transmission within the ventral epithelium [52]. Consistent with this hypothesis, additional optogenetic experiments on *Drosophila* embryos during cellularization have shown that distinct cytoskeletal network arrangements determine whether identical inputs of Rho1 activation induce contraction of an existing actin network [74]. Collectively, this series of optogenetic experiments demonstrate the power of this technique to evaluate existing molecular models and probe the underlying architecture of tissues [63,74].

Notably, earlier published optogenetic work, which activated Rho1 via light-dependent recruitment of RhoGEF2, found that an asymmetric zone of Rho1 activation was sufficient to induce anisotropic apical constriction in the dorsal epithelium [73]. This contrasts with the later published work discussed above, in which light-dependent recruitment of the LARG, a distinct Rho GEF, did not induce anisotropic apical constriction in the dorsal epithelium [63]. As we have highlighted throughout, the outcome of a contractile event depends on the overall tissue context. Forces outside of a particular region can alter the consequences of force generation within a region. We suggest that such considerations, arising from optogenetic probe design, may underlie this difference between these two optogenetic studies [63,73]. Specifically, while both studies activated Rho1 using portions of Dbl family Rho GEFs, there are significant differences between the recruited GEF domains. Dbl family proteins activate GTPases through their Dbl homology (DH) domain, which promotes the exchange of GDP for GTP and generates active, GTP-bound Rho1/RhoA. While both studies involved a recruited DH domain, only the earlier work, which recruited RhoGEF2, also included a pleckstrin homology (PH) domain [73]. Biochemical studies have indicated that PH domains from the PDZ RhoGEF family, which includes RhoGEF2, enable membrane recruitment of GEFs via binding to active Rho1 at the membrane [75,76]. Thus, it is plausible that inclusion of the PH domain in recruitable RhoGEF2(DHPH) enables it to bind endogenously activated Rho1-GTP even in the absence of optogenetic activation, which might elevate cellular contractility throughout the epithelium and impact the outcome of local Rho1 activation (Figure 5); this conjecture is supported by circumstantial evidence [63]. Thus, when designing optogenetic probes to interrogate existing molecular models of cellular and developmental processes, the impact of the probes on the resting state of cells and tissues should be carefully considered.

In addition to providing exceptional spatial control over the activation pattern, many optogenetic tools also

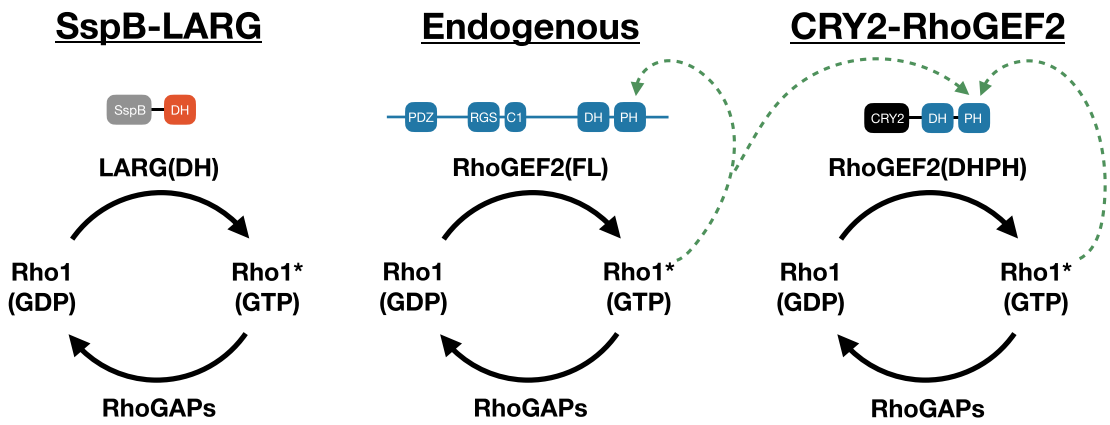

Figure 5. Comparison of endogenous and optogenetic Rho1 GEFs used to activate Rho1 during early *Drosophila* development. Dashed green lines indicate potential for positive feedback.

provide a high degree of temporal control. Thus, this class of approaches also provides an opportunity to evaluate the importance of the pulsed myosin accumulation, which is seen in numerous morphogenetic processes, including ventral furrow formation, germband extension, and dorsal closure [46,77–79]. Live imaging of each of these processes convincingly demonstrates that such pulses induce transient deformations. Moreover, in the context of germ band extension, laser ablation of a pulse results in relaxation of the apical surface [78]. However, these actomyosin pulses are also thought to induce junctional rearrangements that stabilize these transient deformations. Thus, genetic experiments that eliminate actomyosin pulses do not distinguish between the role of pulses in driving deformation versus stabilization. Rapidly reversible optogenetic tools afford the ability to test whether alternating cycles of constriction and relaxation are required for these morphogenetic processes or whether these can be driven with similar efficiency by continuous constriction. Junctional activation experiments in stable epithelial monolayers indicate that pulsing can facilitate constriction [80], though the situation may differ in – even among – actively rearranging tissues.

Closing thoughts

The results summarized here indicate that there are several pathways that can drive the folding of epithelial sheet. Some epithelial folds are triggered by local induction of contractility. The forces driving these folds can be generated intrinsically by Rho1-mediated contractility in the cells directly involved in the furrow. However, external forces, such as those derived from movement of cells outside the furrow, can also make important contributions to and/or modulate the response to internally generated contractility. Additionally, other folds may be triggered primarily by a local increase in deformability in response to external forces. In each case, force transmission is mediated by adherens junctions and their connections to the cytoskeleton, and the behaviour of these assemblies are subject to essential regulation by other small GTPases, including Rap1 and Rab35. This likely reflects a requirement for dynamic rearrangement of adherens junctions that accommodate or direct changes in cell shape. It will be fascinating to determine whether some degree of coordinate regulation or crosstalk exists between Rho1, Rap1, and/or Rab35. To date, due to limitations of the tools used, many genetic perturbations of the early embryos impact all cells in a tissue and, therefore, affect both intrinsic and extrinsic forces that underlie cell shape changes, complicating the interpretations of these experiments. To fully understand the mechanisms of the complex multicellular rearrangements that drive epithelial folding, as well as morphogenesis broadly, it will be crucial to build probes that can dynamically report on the behaviour of key molecules as well as probes that allow spatially and temporally controlled perturbations of specific molecules. Light-regulated probes, including optogenetic and optochemical approaches, are well suited to the latter task; they allow molecular-level perturbations at high spatiotemporal resolution [81,82]. Additional methods, including lasers, magnets, microneedles, and atomic force microscopy can induce, alter, and/or measure forces with high spatiotemporal precision; though these methods lack molecular specificity, in some instances, they can induce forces of known magnitude [52,83–85]. Likewise, significant insights have been

obtained using extracellular matrix substrates with specifically defined compliances [86]. Through these diverse approaches, we will continue to sharpen our understanding of epithelial folding and other related morphogenetic events.

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