During tissue morphogenesis, simple epithelial sheets undergo folding to form complex structures. The prevailing model underlying epithelial folding involves cell shape changes driven by myosin-dependent apical constriction. Here we describe an alternative mechanism that requires differential positioning of adherens junctions controlled by modulation of epithelial apical–basal polarity. Using live embryo imaging, we show that before the initiation of dorsal transverse folds during Drosophila gastrulation, adherens junctions shift basally in the initiating cells, but maintain their original subapical positioning in the neighbouring cells. Junctional positioning in the dorsal epithelium depends on the polarity proteins Bazooka and Par-1. In particular, the basal shift that occurs in the initiating cells is associated with a progressive decrease in Par-1 levels. We show that uniform reduction of the activity of Bazooka or Par-1 results in uniform apical or lateral positioning of junctions and in each case dorsal fold initiation is abolished. In addition, an increase in the Bazooka/Par-1 ratio causes formation of ectopic dorsal folds. The basal shift of junctions not only alters the apical shape of the initiating cells, but also forces the lateral membrane of the adjacent cells to bend towards the initiating cells, thereby facilitating tissue deformation. Our data thus establish a direct link between modification of epithelial polarity and initiation of epithelial folding.

The anterior and posterior dorsal transverse folds, or the dorsal folds, are epithelial folds that form on the dorsal side of the gastrulating Drosophila embryo at stereotypical locations coincident with the second and fifth stripes of the Runt expression (Fig. 1a–f, Supplementary Movie 1 and Supplementary Fig. 1a). Whereas the anterior fold is eventually shallow and the posterior fold deep, the initial cell shape changes are similar in both and the underlying mechanisms appear to be cell-autonomous (Supplementary Movies 2, 3 and Supplementary Fig. 1b, c).

We monitored cell shape changes using two-photon laser scanning microscopy in live embryos that express a membrane marker conjugated with the green fluorescent protein (Resille–GFP, also known as P[PTT-un1]CG86685-17-2). Optical sectioning of embryos at the mid-sagittal plane reveals that two stripes of dorsal cells, each three to seven cells wide, narrow their apices and shorten cell length during early gastrulation, producing two clefts on the dorsal surface that represent the first morphological signs of dorsal fold formation (Supplementary Fig. 2a and Supplementary Movie 4, see also Fig. 4b for measurements of shortening). Cells that undergo apical narrowing retain dome-like apices (Supplementary Fig. 2b), contrasting with the flattened apical surface caused by apical constriction during Drosophila ventral furrow formation.

We sought to identify dynamic cellular processes that precede cell shape changes. Unlike the canonical mode of epithelial folding in which spatially restricted activation of the molecular motor, myosin II (encoded by spaghetti squash), drives localized apical constriction to initiate tissue deformation12, the basal levels of apical myosin remain low and constant across the dorsal epithelium throughout the course of dorsal fold initiation with infrequent bursts of myosin activity that do not differ between the initiating and neighbouring cells (Supplementary Movie 5 and Supplementary Fig. 3a, b). These results indicate that the initiation of dorsal fold formation is not associated with differential myosin contractility.

In contrast, E-Cadherin (encoded by shotgun), the core component of adherens junctions, shows a cell-type-specific change in its positioning; in the initiating cells, junctions shift basally from the subapical regions where they are originally assembled, whereas in the neighbouring cells junctions maintain their original subapical positioning (Fig. 1g, Supplementary Movies 6 and 7 and Supplementary Fig. 3c). Simultaneous imaging of E-Cadherin–GFP and Resille–GFP reveals that basal shift of junctions can be observed as early as 300 s before the onset of gastrulation during the last phase of cellularization, which precedes the apical narrowing and cell shortening that occur 100–200 s after the onset of gastrulation (Fig. 1h and Supplementary Movie 8). During this seven-minute interval, junctions in the initiating cells shift approximately 10 μm basally to lie at 34 ± 5% (n = 18) below the apical surface, whereas junctions in the neighbouring cells show only a slight shift (≈3 μm) to lie at 15 ± 4% (n = 27) below the apical surface (Supplementary Fig. 4). The basal shift of junctions in the initiating cells increases the asymmetry in the junctional positioning on the opposite sides of the neighbouring cells that immediately flank the initiating cells. The lateral membrane of these cells becomes increasingly curved, correlating with the increased junctional asymmetry (Supplementary Fig. 5).

If the apparent basal shift of E-Cadherin positioning reflects an actual movement or remodelling of the junctions, it should be associated with an increase in the volume and surface area above the junctions. To test this hypothesis, we measured the two-dimensional parameters of area and perimeter of the apical domain in the living embryos. As the junctions shift basally in the initiating cells, both of these parameters increase, consistent with a basal movement of the junctions within the cells (Supplementary Fig. 6). We corroborated these observations by developing computer software that reconstructs and quantifies three-dimensional cell shape in fixed embryos (Fig. 1i). As the cell length increases during the last phase of cellularization, the length, volume and surface area of the apical domain in the initiating cells all increase significantly more than they do in the neighbouring cells (Fig. 1j–l), indicating that the junctional shift is accompanied by an expansion of the apical domain and that mobility of the E-Cadherin complex underlies the apparent basal shift of the junctions.

Adherens junctions are positioned to the subapical regions of the polarized epithelial cells by the concerted action of the scaffolding protein Par-3 (encoded by bazooka in Drosophila), the atypical protein kinase C (aPKC) and the MARK family kinase Par-1: apically localized aPKC and basolaterally localized Par-1 restrict Par-3 to the subapical regions, where it directs junctional assembly10–12. We found that the...
levels of Bazooka and aPKC are not differentially regulated across the dorsal epithelium and thus do not account for the observed junctional shift (Fig. 2a, Supplementary Movie 9 and Supplementary Fig. 7). In contrast, live imaging of Par-1–GFP shows that the levels of Par-1 in the presumptive initiating cells, although initially similar (~95%) to those in the neighbouring cells before the onset of junctional shift, reduce progressively during the last phase of cellularization to reach approximately 80% of its levels in the neighbouring cells as gastrulation commences (Fig. 2b, c and Supplementary Movie 10, n = 7). This differential modulation of Par-1 levels seems to require the anterior–posterior patterning system (Supplementary Fig. 8 and Supplementary Movie 11). To ask whether the reduction in Par-1 levels in the initiating cells correlate temporally with the junctional shift, we quantified the levels of Par-1 in fixed embryos and determined the position of junctions using Bazooka staining. As Bazooka becomes more basally positioned in the initiating cells, their Par-1 levels also become lower, whereas the Bazooka levels remain constant (Fig. 2d). These analyses confirm our live imaging data and establish a correlation between the position of junctions and the ratio of Bazooka/Par-1 (Fig. 2e).

This correlation suggests that Par-1 downregulation allows Bazooka to gradually localize more basally, which in turn directs basal repositioning of junctions. To test this hypothesis, we altered the levels of Bazooka and Par-1 to investigate the function of junctional positioning during the formation of dorsal folds. Uniform reduction of Bazooka activity by RNA interference (RNAi) causes accumulation of E-Cadherin–GFP at the edges between apical and lateral surfaces, resulting in an extreme apical positioning of junctions across the epithelium (Fig. 3a and Supplementary Movie 13), similar to embryos produced by the germline clones of a strong loss-of-function allele of bazooka (Supplementary Fig. 9). Conversely, in par-1 RNAi embryos, junctions are located in the lateral regions of all dorsal cells at an average position of 39 ± 8% below the surface, slightly more basal than the junctions in the initiating cells in the wild-type (Fig. 3b and Supplementary Movie 14, 30 cells from 3 embryos). Importantly, in both bazooka and par-1 RNAi embryos, the junctional positioning is uniform across the entire dorsal epithelium and in each case, the initiation of dorsal folds is abolished despite the normal appearance of junction and epithelial structure (75% for bazooka RNAi, n = 8; 70% for par-1 RNAi, n = 10). Thus, dorsal fold formation seems to

Figure 2 | The dynamics of Bazooka and Par-1 during dorsal fold initiation. a, b, Two-photon time-lapse mid-sagittal section of Bazooka–GFP (a) or Par-1–GFP (b). Arrows and brackets, anterior (pink) and posterior (cyan) fold-initiating cells. Scale bars, 10 μm. c, A time-course analysis of Par-1–GFP levels in the initiating cells relative to those in the neighbouring cells (n = 7). d, A scatter plot of the average Bazooka positioning in the initiating cells normalized by that in the neighbouring cells against the average levels of Bazooka or Par-1 relative to their respective levels in the neighbouring cells with the corresponding trend lines. e, A scatter plot of the average Bazooka positioning along the apical–basal axis against the average Bazooka/Par-1 ratio within individual cells. Error bars indicate s.d.
require a differential positioning of junctions between the initiating cells and their neighbours.

Par-1 phosphorylates and thereby excludes Bazooka from the basal-lateral regions of a polarized epithelial cell. We examined the behaviour of BazookaS151A, S1085A, a mutant form of Bazooka that cannot be phosphorylated by the Par-1 kinase4. When the endogenous Bazooka is present, the GFP-tagged BazookaS151A, S1085A shows a subapical (junctional) distribution similar to the GFP-tagged wild-type form (Fig. 3c and Supplementary Movie 15). However, when we knocked down the endogenous Bazooka using RNAi, Bazooka initially shows a broad distribution along the apical–basal axis and eventually coalesces in the lateral regions of all dorsal cells (Fig. 3d and Supplementary Movie 16). A similar localization was observed for wild-type Bazooka–GFP in par-1 RNAi embryos (Supplementary Movie 17) and in both cases, dorsal fold formation is blocked. These results indicate that serine 151 and 1085 of Bazooka are two main substrates of Par-1 during dorsal fold initiation, whose differential phosphorylation determines the heterogeneous positioning of Bazooka across the dorsal epithelium and is critical for dorsal fold initiation.

When we altered the ratio of Bazooka/Par-1 by a uniform increase in Bazooka levels throughout the epithelium, we saw shifts of junctions that lead to eventual formation of epithelial folds in regions that are outside the sites of anterior and posterior folds and typically near the third and seventh stripes of Runt expression (Fig. 3e, Supplementary Movie 18 and Supplementary Fig. 10). An increase in the Bazooka phosphorylation determines the heterogeneous positioning of Bazooka across the dorsal epithelium and is critical for dorsal fold initiation.

In most epithelia, aPKC phosphorylates Bazooka and becomes segregated to establish the apical domain above the junctions7,9,10. We asked whether aPKC plays a role during junctional repositioning. In embryos that lack aPKC activity, the basal margin of the junctions shows its characteristic basal shift in the initiating cells, but the apical margin unexpectedly maintains its typical subapical positioning, leading to an abnormally wide junctional domain. In contrast, the width and positioning of the junctions in the neighbouring cells appear normal (Fig. 4a and Supplementary Movie 19). These results indicate that aPKC controls the apical margin to maintain the size of the junctions, but is not required for the basal shift of junctions. These observations also decouple the junctional shift from an increase in the size of the apical membrane. The widening of junctional expanse was also observed in embryos that overexpress BazookaS980A, a mutant form of Bazooka that cannot be phosphorylated by aPKC (Supplementary Fig. 12). It seems that the segregation of aPKC from Bazooka establishes the apical domain, enabling junctional disassembly at the apical margin of the junctions.
Although the basal shift of junctions occurs in the initiating cells in the aPKC mutant embryo, these cells fail to shorten and the dorsal folds do not form properly (Fig. 4b and Supplementary Movie 19). It seems that as the basal margin of the junctions shifts basally in the initiating cells in response to a decrease in Par-1 levels, their apical margin needs to become disassembled in an aPKC-dependent manner so that the subsequent apical cell shape changes could occur.

In this report, we present evidence that dorsal fold initiation requires the establishment of distinct ratios of Bazooka/Par-1 that impose different positions for the adherens junctions in the initiating cells and their neighbours. We propose that the differential positioning of junctions facilitates epithelial folding through two cellular processes (Supplementary Fig. 13). Within the initiating cells, the resultant increase in the non-adherent apical surface after junctional shift may be unstable such that a shrinkage of the apical domain is triggered to restore the balance between cell surface tension and local adhesive forces. The shortened cells thus produced would then create a localized structural inhomogeneity in the epithelium where buckling would preferentially occur. Second, in the immediate flanking cells, a junctional asymmetry is produced because the basal positioning in the initiating cells on one side and the subapical positioning in the neighbouring cells on the other must be accommodated. Because all junctions in an epithelium are mechanically coupled, the asymmetry may cause the lateral surfaces to curve and cells to bend towards the shortened initiating cells. This bending would drive and deepen any buckles or folds initiated in the epithelial sheet.

Directional movement of the cadherin complex along the apical-basal axis has been observed previously in cultured cells in vitro but, to our knowledge, Drosophila gastrulation provides the first case where such movement has been described in an intact developing organism. When the shifts occur in stripes as they do on the dorsal side of the Drosophila embryo, they seem to initiate infolding of the epithelium. In tissues in which the levels of cortical myosin are low and constant, junctional repositioning regulated by Par-1/Bazooka interactions may play a more prominent role in epithelial folding than does differential activation of cortical contractility. Junctional repositioning may also represent an important mechanism in folding events that do not lead to internalization or delamination, or where the integrity of junctions within the epithelia must be maintained. How junctions are repositioned while maintaining junctional integrity is unclear, but in principle the process could involve remodelling via local endocytic trafficking, or lateral movement of the intact junctions in the membrane. Regardless of the mechanism, dorsal fold formation represents an emergent model in which the insights into this alternative mode of epithelial folding could be further analysed.

**METHODS SUMMARY**

Detailed information about reagents and methods used in this paper, including the Drosophila stocks, RNAi, live imaging, immunofluorescence, scanning electron microscopy, image processing, three-dimensional cell boundary reconstruction and image quantification is described in Methods.

**Full Methods** and any associated references are available in the online version of the paper at www.nature.com/nature.

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METHODS

Drosophila genetics. Drosophila stocks used for live imaging were Resille-GFP\(^{13}\), myosin–GFP\(^{14}\), Squash-squash-GFP\(^{15}\), membrane-mCherry\(^{16}\) (also known as P{shg-mCherry membrane}), E-Cadherin-GFP\(^{17}\), Par-1 protein trap\(^{18}\) (also known as P{PTT-GCpar-1}\(^{19}\)) and mat-tub-GFP-Par-1\(^{18}\). UASp-Bazooka-GFP\(^{20}\) was driven maternally by one copy (67C) of the matsub-Gal4VP16 driver in live imaging experiments and one or two copies (67C, 15) in overexpression experiments. UASp-Bazooka-GFP\(^{21}\), Soma-GFP\(^{22}\) and UASP-Bazooka-GFP\(^{23}\) were driven by matsub-Gal4VP16 (67C; 15). Mutant stocks used were: runtL5; torso\(^{24}\)/torsos\(^{25}\), torso-like/torso-like\(^{26}\), bicoid\(^{27}\) nanos\(^{28}\) torso-like. Germline clones of bazooka\(^{29}\) and aPDE\(^{30}\) were generated using the FLP-recombinase/ dominant female sterile system with the o\(^{32}\) FRT\(^{33}\) or FRT\(^{34}\) o\(^{35}\) chromosome (FRT\(^{36}\) and FRT\(^{37}\) are also known as P{fneo(FRT)19A and P{fRT(w)}11G31). RNAi. Double-stranded RNAs were synthesized using a MEGAscript T7 kit (Ambion) from PCR products that contain the T7 promoter sequence (5'-TA ATACGACTCATAAGGTTACT-3') at each end. The PCR products used in in vitro transcription reactions were amplified from 0–4 h embryonic cDNA using the following primer pairs: bazooka, 5'-GAGCGTTTCTTGATCAAGGCG-3', 5'-TTGCGAGTGTAGATGACAC-3'; bazooka 5'UTR (for knockdown of endogenous but not transgenic bazooka), 5'-AACGGCGGGATCATATGAAATACA CAC-3', 5'-AGACGCGCATCATCATCTGTCG-3'; par-1, 5'-CAGGTTCTG CGTGGACCC-3', 5'-GCTTGGACGCTGAAATC-3'. Double-stranded RNAs were injected into the embryos during the syncytial blastoderm stage, typically 3–4 h before imaging.

Live imaging. Immunofluorescence and scanning electron microscopy. Two-photon live embryo imaging was performed on a custom-made system built on an upright Olympus BX51 microscope that is equipped with a Ti:sapphire tunable laser (2 or 3 m). Live imaging, immunofluorescence and scanning electron microscopy (SEM) was performed on a Hitachi TM-1000 system as previously described\(^{32}\). UASp-Bazooka-GFP\(^{33}\) and Resille-GFP\(^{15}\) were the thinned binary image until a Watershed-segmented region reached a volume of less than 640 voxels. Regions smaller than 40 µm\(^3\) were removed as noise. The segmented cell regions were then converted into three-dimensional triangle meshes by the Marching Cubes algorithm\(^{34}\). Lastly, the resulting meshes were adapted to the intensity of the image by a finite difference approximation to an Active Surface\(^{35}\). Image quantification. The frequency of myosin bursts (Supplementary Fig. 3b) was measured in confocal time-lapse images of myosin–GFP and membrane–mCherry. These data sets have a z resolution of 1 µm and cover a 10 µm distance from the apical cortex with a temporal resolution ranging between 15 and 22 s per frame. The intense myosin structures were visually identified from each z slice of the image stacks. The total number of myosin bursts was the sum of myosin bursts from all movies throughout the duration of imaging. The frequency was then calculated by dividing the total number of bursts by the imaging duration and the number of cells in which the bursts were counted. The imaging duration ranges between 436 and 689 s. The numbers of initiating and neighbouring cells that were counted range between 30 to 41 and 51 to 86, respectively.

Time-course analyses of junctional positioning and apical domain size (Supplementary Fig. 4, 6) were performed using two-photon time-lapse images of E-Cadherin–GFP and Resille–GFP. The onset of gastrulation was defined by the onset of anterior cell movement driven by the posterior midgut invagination. For the analysis of apical domain size, the central initiating cell of the anterior and posterior poles that showed partial junctional movement were chosen to represent the initiating and neighbouring cells. The vertical distance between the visually defined centre of the junctional complex and the apex of the cell was measured in Image J to represent the positioning of the junctions. For the analysis of apical domain size, the central initiating cell of the posterior side and a representative neighbouring cell in the region between the anterior and posterior poles was chosen. The apical domain above the junctions was manually outlined based on the membrane fluorescence of Resille–GFP and measured for its area and perimeter in ImageJ.

Correlation analysis between differential junctional displacement and lateral membrane curvature (Supplementary Fig. 5) was performed using two-photon time-lapse images of E-Cadherin–GFP. Cells that are in the immediate flanking regions of the initiating cells and show a marked asymmetry of junctional positioning on the opposite sides were chosen for these measurements. The differential junctional displacement, which defines the extent of junctional asymmetry, was calculated by subtracting the length of the apical domain on the distal side (y) from that on the proximal side (x). The lateral membrane curvature was defined as the ratio between the height (h) and the chord (C) of the arc of lateral membrane on the distal side of the cell.

Three-dimensional cell shape measurements (Fig. 1j–l) were made in image stacks of late cellularization embryos that have been stained for Bazooka and Neurotactin and processed for three-dimensional reconstruction as described above. The position of Bazooka was defined by the Bazooka junctional triangles. Briefly, an average intensity of Bazooka was first assigned for the voxels that intersect with a three-dimensional triangle mesh in the reconstructed cell boundary. The Bazooka junctional triangles were then selected based on an intensity threshold of the 99th percentile of the Bazooka intensity histogram. For each of the centroids of the Bazooka junctional triangles, a three-dimensional principal component analysis (PCA) was performed to determine the Bazooka mean position (a point on a plane) and the eigenvector corresponding to smallest eigenvalue (plane normal). These were then used to define the Bazooka junctional plane that subdivides the cell into the apical and basal domains. The geometric measurements were as follows: three-dimensional PCA was applied to all of the vertices of the triangle mesh and the long direction of the cell was defined using the eigenvector corresponding to the largest eigenvalue. The apical domain length was measured by first creating vectors between the cell centroid and each mesh vertex on the apical side of the Bazooka junctional plane. These vectors were then projected onto the long direction vector of the cell. The length of the longest projected vector was used as the apical domain length. The basal domain length was measured similarly, using triangle mesh vertices on the basal domain of the cell. The total cell length was computed by a sum of the apical and basal length. The apical volume was computed by voxelizing the three-dimensional triangle meshes, and summing the volumes of voxels apical to the Bazooka junctional plane. Similarly, the apical surface area was computed by voxelizing the areas of the mesh triangles for which the triangle centroid falls on the apical side of the Bazooka junctional plane. The initiating cells were selected on the basis of their location and junctional positioning. Approximately 500 dorsal cells in the region between the first and the seventh stripe of Runt were ranked by the apical domain length and the top 150 cells were selected for further analysis. A second selection was performed to isolate those that are in close proximity to the second stripe of Runt. Those cells whose apical domain length was above the average were used for analysis. For the early-stage embryos that showed no junctional shift, only the location-based selection was made. Cells that reside in the region between the anterior and posterior poles with junctional positioning that was below average were used as the neighbouring cells.
Time-course analysis of Par-1 dynamics (Fig. 2c) was performed using two-photon time-lapse images of Par-1–GFP. The average fluorescent intensity of Par-1–GFP in manually selected areas consisting of two anterior or four posterior initiating cells was measured and normalized by that in areas consisting of four neighbouring cells that reside in the regions between the anterior and posterior folds. The onset of gastrulation was defined by the onset of anterior cell movement driven by posterior midgut invagination.

Bazooka and Par-1 immunofluorescence (Fig. 2d, e and Supplementary Fig. 11) was quantified in image stacks of fixed embryos that were triply labelled for Bazooka, Par-1 and Neurotactin and processed for three-dimensional reconstruction as described above to define the Bazooka junctional triangles. Junctional intensity of Bazooka within a cell was measured within and normalized by the Bazooka junctional volume that was defined by the voxelization of Bazooka junctional triangles. The basal–lateral intensity of Par-1 in each cell was measured within and normalized by the volume within a two-voxel distance from the cell boundary basal to the Bazooka junctional plane. For Fig. 2d, e, the anterior and posterior fold-initiating cells were selected on the basis of location and above-average Bazooka positioning, whereas the neighbouring cells were selected from the cells that reside in the region between the anterior and posterior folds with below-average Bazooka positioning. For Supplementary Fig. 11, the wild-type and Bazooka overexpression embryos were fixed, stained and imaged in parallel under identical conditions. Cell selection was performed as in Fig. 2d, e.

Time-course of cell shortening in the initiating cells (Fig. 4b) was analysed by measuring the total cell length of five and four initiating cells each from wild-type mutant embryos in ImageJ. The measurements were normalized by cell length at time zero, which was defined by the onset of anterior cell movement driven by the posterior midgut invagination.

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