Dynamic actin-based epithelial adhesion and cell matching during *Drosophila* dorsal closure

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Background: The adhesion of two epithelial sheets is a fundamental process that occurs throughout embryogenesis and during wound repair. Sealing of the dorsal epidermis along the midline of the *Drosophila* embryo provides a genetically tractable model to analyse the closure of such holes. Several studies indicate that the actin cytoskeleton plays a critical role in dorsal closure. Although many components of the signalling cascade directing this process have been identified, the precise cell-biological events upon which these signals act remain poorly described.

Results: By confocal imaging of living fly embryos expressing green fluorescent protein (GFP)-tagged actin, we found that dorsal closure relies on the activity of dynamic filopodia and lamellipodia that extend from front-row cells to actively zipper the epithelial sheets together. As these epithelial fronts approach one another, we observed long, thin filopodia, apparently 'sampling' cells on the opposing face. When the assembly of these actin-based protrusions was blocked (by interfering with the activities of Cdc42 and Jun N-terminal kinase signalling), the adhesion and fusion of opposing epithelial cells was prevented and their ability to 'sense' correct partners was also blocked, leading to segment misalignment along the midline seam.

Conclusions: Dynamic, actin-based protrusions (filopodia and lamellae) are critical, both in the mechanics of epithelial adhesion during dorsal closure and in the correct 'matching' of opposing cells along the fusion seam.

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Background

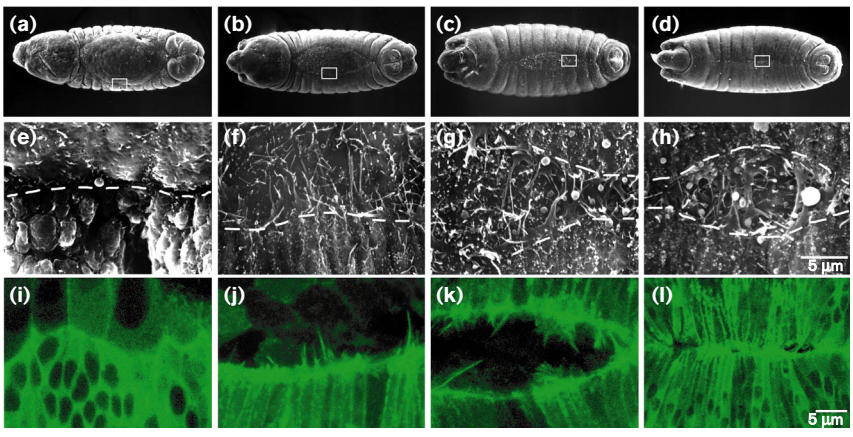
Dorsal closure in *Drosophila* begins about 11 hours after egg laying when the completion of germ-band retraction reveals an exposed, pavement-like amnioserosa, surrounded by lateral epidermis. From this moment on, the opposing epithelial fronts move towards one another until they meet and fuse to form a precise, segmentally aligned midline seam, about 2 hours later (see Figure 1a–d, reviewed in [1]). Several studies indicate that the actin cytoskeleton plays a critical role in dorsal closure [2,3]. Although many components of the signalling cascade directing this process have been identified [4–18], the precise cell-biological events upon which these signals act remain poorly de-scribed. The best clue from fixed embryos as to the cytoskeletal machinery driving dorsal closure is a cable of actin and associated myosin running around the circumference of the leading epithelial margin [2], analogous to the actinomyosin cable found during purse-string re-epithelialisation of embryonic skin and tissue-culture wounds [19–21]. However, contraction of this cable cannot be the only provider of motive force for dorsal closure as cutting it with a laser does not stop epithelial forward movement [22]. Further support for believing that more than just an actin cable might be necessary for this epithelial movement comes from genetic analysis of small GTPase function during dorsal closure [5,13–16]. These studies show that not only Rho, which mediates assembly of cable-like stress-fibre bundles in fibroblasts [23], but also Cdc42 and Rac, which are required for filopodia and lamellipodia extension in fi-broblasts [24,25], all play a role in dorsal closure. Here, we report that filopodia and lamellipodia are dynamic and key players in the final stages of dorsal closure when the two epithelial faces zipper together. We found that blocking the assembly of these actin-based protrusions prevents adhesion of the epithelial fronts along the fusing seam and also prevents the appropriate cell–cell matching necessary to bring opposing segments into correct alignment.

Results and discussion

Leading-edge epithelial cells extend dynamic filopodia and lamellipodia

We performed a time-lapse confocal analysis of actin cytoskeletal activity in leading-edge epithelial cells during dorsal closure in living *Drosophila* embryos expressing a green fluorescent protein (GFP)–actin fusion protein [26] under the control of the Gal4–UAS system [27]. Expression

Figure 1



Filopodia and lamellipodia on leading-edge cells during dorsal closure. **(a–d)** Scanning electron micrographs of embryos: (a) just before the onset of dorsal closure; (b) at early dorsal closure; (c) during the zippering stage; and (d) at the final stage of dorsal closure. **(e–h)** High-magnification scanning electron micrographs of the corresponding boxed regions indicated in (a–d): (e) smooth leading edge without filopodia; (f) filopodial extensions up to 10 μm in length; (g) filopodia accompanied by lamellae at the zipper front where opposing epithelial edges meet; and (h) final lamellar ruffling as the dorsal hole is about to close. The white dotted line indicates the leading edge. **(i–l)** Still images from GFP–actin videos parallelling our scanning electron microscopy (SEM) observations: (i) at this early stage, the leading edge appears scalloped, with elongated front-row cells that do not yet express a strong actin cable or filopodia; (j) approximately 1 h later, there is a clear actin cable and now each cell has several filopodial protrusions; (k) the zippering front expresses lamellae that reach out across the exposed amnioserosa; (l) as the fronts meet, the opposing epithelial faces fuse together.

of GFP–actin, driven in the epidermis by the epithelial driver *e22c–Gal4* or by *en–Gal4* [27], revealed not only the presence of an actin cable but also dynamic protrusions from front-row cells as they sweep forwards towards the midline (Figures 1i–l and 2a–d; see also Movie 1 in Supplementary material). Most of these protrusions are filopodia and almost certainly correspond to the small microspikes occasionally ob-served in fixed, phalloidin-stained embryos [28]. Filopodia were first apparent from stage 14 and could also be seen by SEM (Figure 1e–h); they were up to 10 μm in length and those that we captured in the process of protrusion extended at about 1 μm/minute (Figure 2a–d), which is comparable to the rate of filopodial extension in tissue culture of Cdc42-activated fibroblasts [25]. Some filopodia appeared as stiff struts contacting the amnioserosa, whereas others exhibited random waving movements with their tips touching down on the amnioserosa only for brief moments (Figure 2a–d; see Movie 1 in Supplementary material). At these early stages, we saw only very occasional small membrane ruffles or lamellipodia spreading between two adjacent filopodia, but as dorsal closure progressed and the two epithelial faces met and began to zipper up, highly motile lamellipodia were frequently visible in cells close to the zippering fronts (Figure 2b; see Movie 2 in Supplementary material).

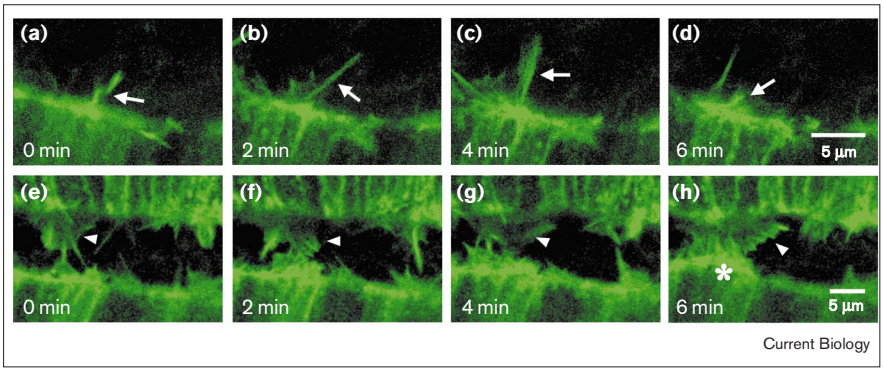
Filopodia and lamellipodia appear to drag the opposing epithelial fronts together

From the zippering stage of dorsal closure onwards, these actin-rich protrusions appear to play a key role in drawing together the two epithelial edges. At the zipper front, the filopodia and lamellipodia on opposing epithelial faces contact and engage, and then tug towards one another at a rate that exceeds the previous forward movement of the epithelium. Prior to engagement, at the end of stage 14, the fronts move towards one another at a rate of 0.11 ± 0.02 μm/minute (mean ± SD, $n = 40$) but this forward movement accelerates up to 0.24 ± 0.07 μm/minute ($n = 40$) after lamellipodial engagement (Figure 2e–h; see Movie 2 in Supplementary material). At these sites of tugging, we observed that the actin cable was kinked towards the opposite epithelial leading edge (Figure 2f–h), confirming that it is traction of the lamellae, rather than of the cable, that drives this final forward movement of the epithelium.

Long filopodia extend from leading-edge cells and 'sample' the opposing epithelial front

Frequently, we observed that the initial engagement of cell protrusions from opposite sides of the gap is not with nearest neighbours. Rather, we saw long thin filopodia resembling the cytonemes of imaginal disc cells [29], apparently ignoring the closest opposite cell and extending up to four or more cell diameters anteriorly or posteriorly to make contact with a cell or filopodial protrusion, as though searching out a 'correct' partner. This filopodial 'sampling' of the opposing epithelial front was most dramatically observed in embryos in which we used the *en–Gal4* driver to express GFP–actin in four-cell-wide on/off stripes (Figure 3; see Movie 3 in Supplementary material). In such embryos, long, actin-rich filopodia were seen to extend from one *engrailed* (*en*)-positive domain, across the adjacent *en*-negative domain to 'sample' in the next-most anterior *en*-positive domain (Figure 3c,d; see Movie 3 in Supplementary material).

Figure 2



Confocal time-lapse images of dynamic filopodia and lamellae during dorsal closure, as revealed by GFP–actin. **(a–d)** Sequence of images showing (a to b) extension, (b to c) waving movement, and (c to d) subsequent retraction of a filopodium (arrows) expressed by a leading-edge cell during early dorsal closure. **(e–h)** Similar sequence at zipper stage showing (e to f) initial ruffling of a lamella (arrowhead), (f to g) contact and fusion with the opposing front, and (g to h) subsequent tugging of epithelial fronts towards one another at the zone of contact. Note the cable drawn towards the opposing leading edge (asterisk). In both sequences, the images were samples taken at 2 min intervals.

Filopodial interdigitation precedes, and may drive, epithelial fusion

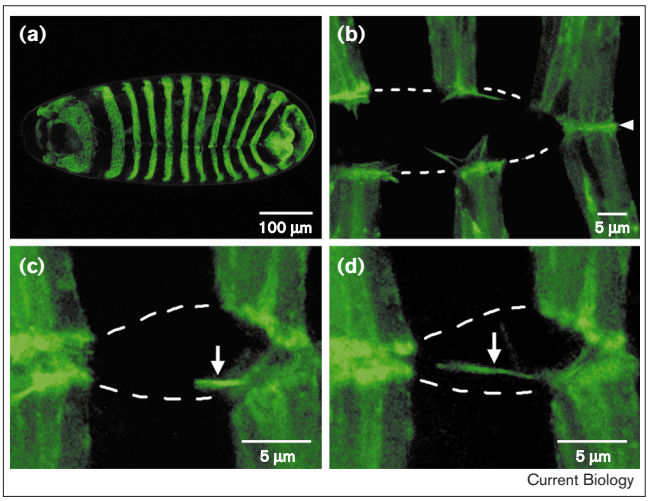
The process of seam formation at the zipper front may be analogous to cell–cell adhesion between primary mouse keratinocytes stimulated by exposure to Ca^{2+} and during ventral enclosure in *Caenorhabditis elegans* embryos. A recent *in vitro* study showed how these adhesions develop from, and are dependent on, filopodial projection into pockets in a neighbouring cell's plasma membrane, resulting in a tramline pairing of α-catenin staining where keratinocytes contact one another [30]. Filopodial priming seems also to play a key role in the formation of adherens junctions and seaming together of epithelial sheets after ventral enclosure in the *C. elegans* embryo [31]. Our studies of dorsal closure in living flies expressing a GFP–α-catenin fusion protein [32] and transmission electron microscopy (TEM) studies of wild-type embryos suggest that a similar process may be occurring at sites of cell–cell adhesion as the dorsal epithelium zippers up. We saw large α-catenin-rich junctions linking intracellular segments of the actin cable in the free epithelial margin (Figure 4a), but where the epithelial fronts make contact we observed opposing pairs of junctions, 2–4 µm apart from one another, in a pattern resembling that seen when keratinocytes first engage, suggesting that plasma membrane protrusion into an opposing cell may also be important here (Figure 4a). Four or five cell diameters into the zipper seam, these junction pairs appear to merge into a single cell–cell junction linking the two fused epithelial fronts. TEM sections at different points along the zipper front indicated, more clearly, how filopodia from opposing cells may interact in order to bring about fusion (Figure 4b–e). At the free epithelial edge, before cell–cell contact, filopodia tips generally terminate above the amnioserosa but do occasionally make contacts with the amnioserosa ahead of them (Figure 4b). Where opposite cells first confront one another, we saw filopodia and lamellipodia interweaving at the cell–cell interface (Figure 4c). Subsequently, the filopodia appear to interdigitate and penetrate into the plasma membrane of the opposite cell (Figure 4d), and further back still from the zipper front these interdigitations resolve and become shorter, as opposite cells form mature adherens junctions with one another (Figure 4e).

Expression of dominant-negative Cdc42 blocks filopodial extension and epithelial adhesion

Studies in fibroblasts in tissue culture have revealed that the actin-based assembly of filopodia and lamellipodia is triggered by activation of the small GTPase Cdc42, which in turn activates Rac [25], and, as genetic studies in *Drosophila* have shown that both of these molecular switches play some role in dorsal closure [5,13,14,16], it made good sense to assume that the actin-based protrusions that we observed during dorsal closure are triggered by, and are dependent on, Cdc42 activation. The expression of dominant-negative Cdc42 throughout the embryonic epithelium using the *e22c–Gal4* driver causes developmental arrest during germ-band retraction before initiation of dorsal closure, at the same stage as in Cdc42 mutants ([16], and A.J., unpublished observations). However, using the *en–Gal4* driver to give four-cell-wide on/off epithelial stripes of expression of both GFP–actin and mutant Cdc42 [14,33,34], we were able to test Cdc42 function beyond the germ-band retraction stage. In these embryos, the expressing cells extend only the most rudimentary filopodia and lamellipodia (Figure 5a–c), which may reflect residual activity of endogenous Cdc42. Subcellular localisation of GFP–actin in these cells suggests that actin cable assembly is maintained, but we were unable to determine whether its function remains as in the wild type.

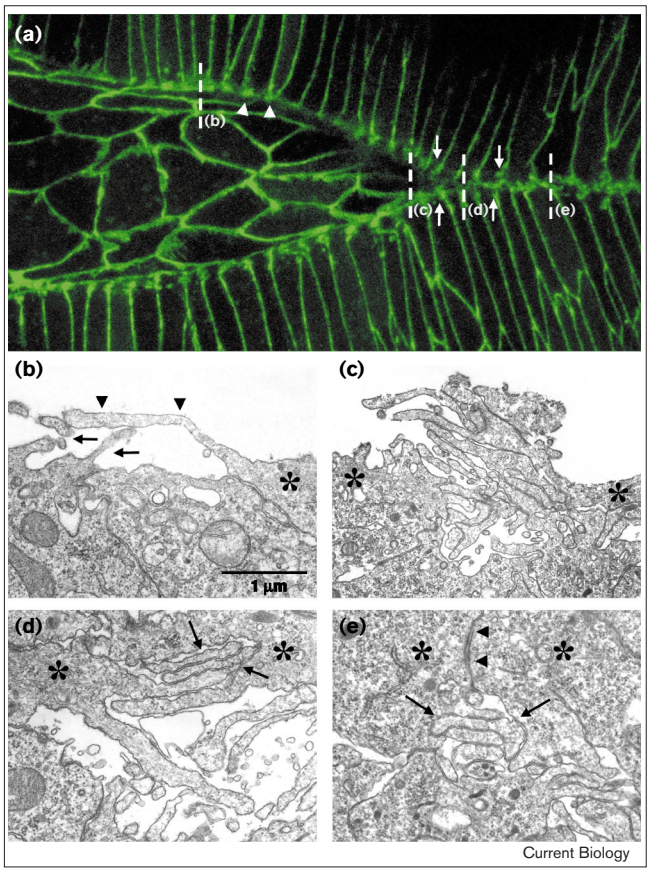
Consequently, where opposite faces expressing the dominant-negative Cdc42 meet, they now fail to engage and are not actively drawn towards one another. Subsequently, these opposing cells do not fuse properly, and we generally

Figure 3



Expression of *UAS–GFP–actin* using the *en–Gal4* driver. **(a)** Confocal image of an almost-closed embryo expressing *UAS–GFP–actin* driven by *en–Gal4*, showing stripes of GFP-positive cells (green) adjacent to stripes of wild-type tissue that are not stained. All stripes are aligned perfectly across the just-closed seam. **(b)** Zippering-stage embryo expressing GFP–actin in *en–Gal4* stripes as in (a). Leading-edge cells protrude extensive filopodia and lamellae. Note how GFP–actin boundaries are in exact alignment (arrowhead) where opposing cells have made contact. The white dotted line indicates the leading edge. **(c,d)** Two higher magnification images, 4 min apart, from a slightly later stage *en–Gal4/UAS–GFP–actin* embryo, revealing a long thin filopodia extending four or more cell diameters (the width of a GFP-negative stripe) as it senses out the correct opposite epithelial partner.

Figure 4



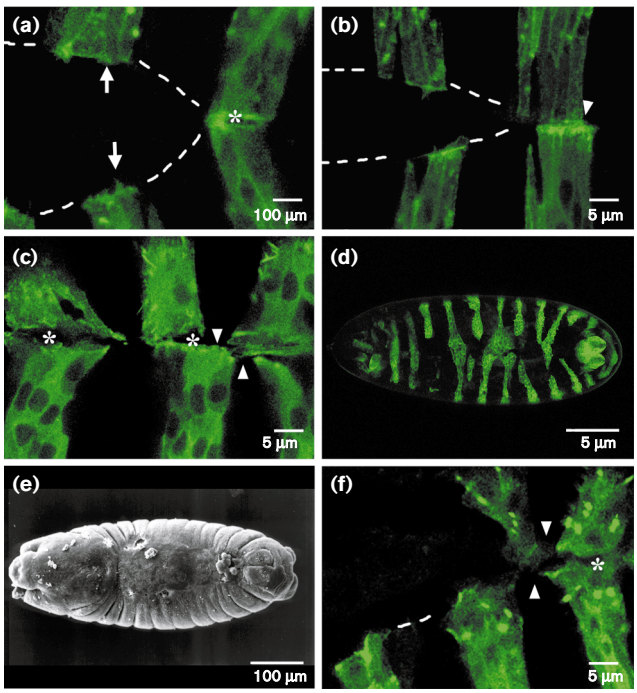
Adhesion of the zippering epithelial fronts as seen by confocal analysis of embryos expressing GFP–α-catenin, and by TEM. **(a)** Confocal image of the fusing epithelial fronts of a zippering-stage embryo expressing GFP–α-catenin. Along the free margin are substantial adherens junctions (arrowheads) linking leading-edge cells to their neighbours. For several cell diameters back from where the two epithelial faces meet, paired junctions persist (arrows), but further back the junctions resolve into a single seam line of junctions where the two fronts have fused. **(b)** TEM section at the level indicated by dotted line in (a), showing filopodia (arrowheads) from a leading epithelial cell (asterisk). Note also the membrane protrusions (arrows) from the exposed amnioserosa. **(c)** TEM section at the zipper front as indicated in (a), showing interaction between filopodia and lamellae of opposing epithelial fronts (asterisks). **(d)** TEM section from the fused seam at the level indicated in (a); basal filopodia are seen to be protruding into pockets (arrows) within adjacent epithelial cells (asterisks). **(e)** TEM section at the level indicated in (a) to show the near-final resolution of filopodial interdigitations (arrows) as leading-edge cells (asterisks) form mature adherens junctions (arrowheads).

saw gaps between cells that should have formed a tight seam (Figure 5a,c). Even given that half of the epithelial leading-edge cells — those that are *en*-negative — are not expressing the mutant Cdc42 transgene, and thus are competent to extend filopodia, these embryos generally fail to complete dorsal closure. Embryos expressing the weaker dominant-negative $Cdc42^{S89}$ occasionally did close completely to give a wild-type dorsal cuticle (6 of 33 embryos analysed), but expression of $Cdc42^{N17}$, a stronger dominant-negative form of Cdc42, always resulted in dorsal-closure defects.

In the absence of filopodia, cell–cell matching fails along the midline seam

Our observations of filopodial behaviour at the zipper front (Figure 3c,d; see also Movie 3 in Supplementary material) suggests that these processes may play a role in the correct matching of opposite cells along the zippering seam. To test this, we compared alignment of *en–Gal4/UAS–GFP–actin* stripes in wild-type embryos versus embryos in which alternate (*en*-positive) stripes of leading-edge cells are unable to produce filopodia because of the expression of *UAS–*$Cdc42^{S89}$ or *UAS–*$Cdc42^{N17}$. As expected, GFP–actin stripes in otherwise wild-type embryos show perfect cell alignment along the seam where the opposing epithelial fronts have met (Figure 3a,b). By contrast, those embryos also expressing dominant-negative Cdc42 frequently (18 of 33 *en–Gal4/UAS–*$Cdc42^{S89}$ embryos, and all of the embryos expressing $Cdc42^{N17}$) fail to close fully and reveal a clear misalignment of one or two cells during the closure process such that some *en*-positive cells now confront *en*-negative cells across the midline seam (Figure 5b). The failure to globally align the segments correctly was

Figure 5



Reduced filopodia and segment misalignment in embryos that lack Cdc42 or Hep activity. **(a)** Embryo at a stage equivalent to that in Figure 3b, expressing dominant-negative Cdc42^{S89} as well as GFP–actin using the *en–Gal4* driver. Expressing cells have only rudimentary protrusions (arrows) and, where the zipper fronts meet, they do not form a fused seam (asterisk). **(b)** Another *en–Gal4/Cdc42^{S89}* embryo, as in (a), showing how segmental stripes typically misalign by one or more cells (arrowhead). The white dotted lines in (a,b) indicate the leading edge. **(c)** A more severe failure to seam together (asterisks) and misalignment of segmental stripes (arrowheads) is revealed in a late-stage embryo expressing dominant-negative Cdc42^{N17} as well as GFP–actin in *en* stripes. **(d)** Embryo expressing Cdc42S89 using the *en–Gal4* driver. Note that in this example of a milder phenotype, where dorsal closure had completed, several GFP–actin stripes align with inappropriate segments on the opposing epithelial front. **(e)** SEM of an embryo mutant for *hep* reveals the stage at which dorsal closure aborts. These embryos show no sign of actin-based protrusions from leading-edge cells at any stage during dorsal closure. **(f)** In embryos mutant for *hep* and expressing GFP–actin driven by *en–Gal4*, fusion of opposing fronts also fails (asterisk) and segmental stripes are misaligned (arrowheads).

especially striking in some of the *en–Gal4/UAS–Cdc42^{S89}* embryos that successfully progress all the way through dorsal closure. In such embryos (9 of 33), the dorsal hole is closed but some of the *en–Gal4/UAS–GFP–actin* stripes do not match the correct opposing segment; instead, they fuse with more anterior or posterior segments (Figure 5d).

Blocking Jun N-terminal kinase signalling also prevents filopodia formation

The relatively late stage at which filopodia on opposite epithelial faces first interact suggests that these protrusions may only be needed for the final zippering or adhesion stages of dorsal closure. Interestingly, this coincides with the stage at which embryos mutant for *hemipterous* (*hep*), which encodes a Jun N-terminal kinase (JNK) kinase, begin to deviate from the wild type and abort dorsal closure ([4]; A.M-A., unpublished observations). For this reason, we looked at the dynamics of actin in *hep* mutant embryos and found that they did not exhibit filopodia or lamellipodia. In these embryos, none of the leading-edge cells are able to protrude filopodia (Figure 5e,f) and, wherever they meet (usually at the posterior end of the embryo), we saw even more extreme misalignment of GFP–actin-expressing segments (Figure 5f), suggesting that the JNK pathway is involved in the regulation of filopodial extension either by a feedback mechanism that re-activates Cdc42 [8], or by regulating Cdc42 through the Decapentaplegic (Dpp) signalling cascade [13,16].

Our data suggest that actin protrusions are required to facilitate correct segmental alignment at the zipper seam and that filopodia may function as sense organs, identifying correct cell contacts in a way analogous to how filopodia operate when a growth cone searches for target cues in the developing nervous system [35]. If this is the case, then it becomes important to determine what cell–cell adhesion molecules or receptors are being presented on the long filopodia and used to identify matching cells on the opposite epithelial front. Almost certainly, these molecules will mirror those being used by the embryonic and later imaginal disc cells to sort and maintain compartment boundaries [1,36].

What is the function of leading-edge filopodia during the early stages of dorsal closure and how is the epithelium drawn forwards before the stage when filopodial-dependent zippering begins? At these earlier stages, we occasionally saw filopodia that adhere down to the amnioserosa and shorten as the epithelial sheet moves forward, but the majority of filopodia are not obviously being used to tug the epithelium forward. We cannot rule out a guidance or crawling role for filopodia at these early stages but, in the absence of filopodia, for example, as in *hep* mutants, the epithelium still sweeps forwards and only fails at the onset of zippering (Figure 5e). Quite possibly, this early epithelial movement is driven by a combination of extension of leading edge and lateral cells, and forces exerted by the actin cable, and by the exposed amnioserosa which may also provide some contractile force [22]. Scanning and transmission electron micrographs reveal that although at early stages the amnioserosa cells have a smooth apical surface (Figure 1a,e), as dorsal closure proceeds they too express large apical lamellipodia, which first appear adjacent to the zippering epithelial fronts and then spread to cover all the exposed amnioserosa (Figures 1b,g and 4b). This wave of membrane ruffling by amnioserosa cells is coincident with a change in their cell shape, which may

play some role in bringing the epithelial fronts together before zippering. Indeed, focal laser ablation studies of patches of amnioserosa show that releasing tension within this tissue results in short-term gaping of the advancing epithelium [22]. It seems likely that, just as in repair of a skin wound [37], several tissue movements in sequence and in concert may be responsible for closure of the hole.

Conclusions

Our imaging analysis of live wild-type and mutant flies reveals for the first time the full repertoire of dynamic cytoskeletal machinery underlying dorsal closure, and shows how both the small GTPase Cdc42 and Jun kinase signalling regulate this adhesion and cell-matching apparatus. To preserve global patterning within the epithelium during closure of a hole, it is vital that the free epithelial edges are brought together in a coordinated way. Our studies of flies expressing dominant-negative Cdc42 or mutant *hep* suggest that actin protrusions (filopodia and lamellae) may not only act as mechanical zippers allowing the epithelial fronts to adhere and fuse, but that specialised filopodia may also play a critical role as sensors, allowing cells to search out their correct partners, and thus keep segments aligned along the closing seam. These findings have implications for the many other occasions during embryogenesis and wound repair when epithelial sheets must precisely zipper up a hole or fuse together two edges to form a neat closure seam.

Materials and methods

Fly stocks

GFP–actin, α-catenin–GFP and dominant-negative forms of Cdc42 were expressed in the embryonic epidermis using the Gal4–UAS system [27]; *w^{1118}; UAS–GFP–actin* [26] or *w^{1118}; UAS–α-catenin–GFP* [32] stocks were crossed to *yw^{1118}; e22c–Gal4* [27] to express the GFP-tagged proteins throughout the epidermis, and *w^{1118}; UAS–GFP–actin* was crossed to *yw^{1118}; en–Gal4* [27] to express GFP–actin in *en* stripes. Coexpression of Cdc42 mutants and GFP–actin was achieved by crossing *w^{1118}; UAS–cdc42^{S89}* [34] or *w^{1118}; UAS–cdc42^{N17}* [33] flies to a stock carrying both *en–Gal4* and *UAS–GFP–actin* in a recombined chromosome. The *hep* mutant embryos were obtained by crossing semi-viable *yw^{1118} hep^{r75}/hep^{1}* females to *hep^{1}/Y; en–Gal4, UAS–GFP–actin/CyO* males. All embryos resulting from this cross died during dorsal closure and showed a dorsal open phenotype due to the lack of maternal or paternal wild-type *hep* RNA. *Or^{R}* stocks were used for SEM and TEM.

SEM

Embryos for SEM were collected at dorsal closure stages (11–13 h AEL), dechorionated in 50% bleach for 5 min, washed in H_2O and fixed overnight at room temperature in a 1:1 mix of heptane and fixative A (1.6% formaldehyde and 2.5% glutaraldehyde in PBS). After fixation, embryos were hand-devitellinised in PBS, post-fixed in 1% osmium tetroxide in PBS and rinsed further in PBS. After dehydration in a graded ethanol series, specimens were critical point-dried and sputter-coated with gold before viewing on a Jeol 5410 scanning electron microscope.

TEM

Embryos for TEM were collected and dechorionated as before but were fixed in 1:1 mix of heptane and fixative B (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer) for 30 min before being hand-devitellinised and refixed in fixative B for 1 h at 4°C. After fixation, embryos were rinsed in 0.1 M sodium cacodylate, post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate, rinsed in H_2O and then incubated in 2% uranyl acetate in H_2O for 15 min. Specimens were further washed in H_2O before being dehydrated through a graded ethanol series. Embryos were embedded in Agar Resin mix and ultrathin sections were cut. Sections were examined using a Jeol 1010 transmission electron microscope.

Time-lapse confocal microscopy

Embryos expressing GFP–actin and about to start dorsal closure were hand-dechorionated with forceps, mounted in Voltalef oil under a coverslip and imaged live using a Leica TCS SP confocal system. Images compiled from two or four confocal optical sections (each averaged two times) were collected every 30 or 60 sec. The time-lapse series were assembled and analysed using NIH image software.

Supplementary material

Three videos showing the movement of filopodia and lamellae during dorsal closure are available at http://current-biology.com/supmat/supmatin.htm.

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