Dynamic analysis of filopodial interactions during the zippering phase of *Drosophila* dorsal closure

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Dorsal closure is a paradigm epithelial fusion episode that occurs late in *Drosophila* embryogenesis and leads to sealing of a midline hole by bonding of two opposing epithelial sheets. The leading edge epithelial cells express filopodia and fusion is dependent on interdigitation of these filopodia to prime formation of adhesions. Since the opposing epithelia are molecularly patterned there must exist some mechanism for accurately aligning the two sheets across this fusion seam. To address this, we generated a fly in which RFP-Moesin and GFP-Moesin are expressed in mutually exclusive stripes within each segment using the *engrailed* and *patched* promoters. We observe mutually exclusive interactions between the filopodia of *engrailed* and *patched* cells. Interactions between filopodia from matching cells leads to formation of tethers between them, and these tethers can pull misaligned epithelial sheets into alignment. Filopodial matching also occurs during repair of laser wounds in the ventral epithelium, and so this behaviour is not restricted to leading edge cells during dorsal closure. Finally, we characterise the behaviour of a *patched*-expressing cell that we observe within the *engrailed* region of segments A1-A5, and provide evidence that this cell contributes to cell matching.

INTRODUCTION

The fusion of sheets of epithelial cells is a common event during embryonic development and also occurs during the process of wound healing (Martin and Parkhurst, 2004). Failure of epithelial fusions during human embryonic development gives rise to a spectrum of birth defects including spina bifida and cleft palate. A widely used model of epithelial fusion is the process of dorsal closure (DC), which occurs during *Drosophila* embryogenesis (Harden, 2002; Kiehart, 1999). During DC, two epithelial sheets sweep towards one another over the surface of the embryo and fuse at the dorsal midline to form a continuous epidermis. Live imaging studies have revealed that dynamic needle-like protrusions called filopodia project beyond the leading edges of the epithelial sheets during DC (Jacinto et al., 2000). When filopodia from the two epithelial sheets meet one another, they interdigitate in a process known as 'zippering'. Suppressing filopodia formation during DC by expressing dominant-negative Cdc42 or disassembling microtubules leads to a failure of fusion, suggesting that zippering is an essential part of the fusion process (Jacinto et al., 2000; Jankovics and Brunner, 2006). Filopodial zippering has also been observed in other systems, including cultured keratinocytes and in the embryonic mouse eyelid, suggesting that it is a universal mechanism for epithelial fusion (Vasioukhin et al., 2000; Zenz et al., 2003).

Embryonic epithelial fusions must occur in a precise fashion when the fusing sheets are patterned, as is the case for neural tube closure in vertebrates and DC in flies. The epithelium of the *Drosophila* embryo is finely patterned prior to DC and imprecise fusion would disrupt this patterning. Early in development, the embryo is divided into a series of repeating units called parasegments, with the boundary between parasegments forming anterior to stripes of cells expressing the transcription factor Engrailed (Lawrence and Struhl, 1996). Later in embryogenesis, visible segment boundaries form posterior to the *engrailed* stripes (Larsen et al., 2003). Thus, by DC, the embryo is patterned into segments, with each segment being divided into an anterior (A) and a posterior (P) compartment by the parasegment boundary. Engrailed and Hedgehog are expressed exclusively in P compartments (Dahmann and Basler, 2000), whereas the Hedgehog receptor Patched is expressed exclusively in A compartments (Nakano et al., 1989).

DC occurs with remarkable accuracy, such that patterning is perfectly maintained across the fusion seam at single-cell resolution. In order to achieve this level of accuracy, each cell in the leading edge must be able to identify, and specifically fuse with, its matching cell in the opposing epithelial sheet. Interestingly, cell-cell matching is perturbed by genetic interventions that abolish filopodia formation (dominant-negative Cdc42, Ena sequestration), suggesting that, in addition to mechanical zippering, filopodia might also play a role in the cell-cell matching that occurs during epithelial fusion (Gates et al., 2007; Jacinto et al., 2000).

Filopodia are widely observed in biology and they often appear to be sensory structures, allowing a cell to explore its environment, searching for guidance cues, other cells or suitable sites for attachment (Gerhardt et al., 2003; Ribeiro et al., 2002; Ritzenthaler et al., 2000; Zheng et al., 1996). However, although there is much circumstantial evidence that filopodia perform a sensory function, in most cases it is not possible to directly observe this occurring in the living embryo.

We have performed experiments to gain a clearer understanding of how cell-cell matching is achieved during DC. We demonstrate how filopodia contribute to matching and provide clear evidence of sensory and motile functions of filopodia in a live organism. This work also provides new insight into the organisation of the dorsal epithelium in the fly embryo.

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MATERIALS AND METHODS

Plasmids

RFP-Moesin-pUASp

DNA encoding mCherry and the actin-binding domain of *Drosophila* Moesin (C-terminal 137 residues) were cloned by PCR and inserted into pUASp as *Kpn*I-*Not*I and *Not*I-*Bam*HI fragments, respectively (Edwards et al., 1997; Rorth, 1998; Shaner et al., 2004). Expression of this construct is non-toxic and co-localisation with GFP-actin confirmed that it effectively labels the actin cytoskeleton (data not shown).

patched-GFP-Moesin expression cassette

DNA encoding the actin-binding domain of *Drosophila* Moesin and the SV40 terminator sequence were cloned by PCR and inserted into *Not*I-*Bgl*II and *Bgl*II-*Eco*RI sites of pCASPER2, respectively. The 1160 bp of *Drosophila* genomic DNA immediately upstream of the *patched* coding region (Forbes et al., 1993) was fused to DNA encoding eGFP by PCR and the resulting fragment was cloned into pCR4TOPO (Invitrogen) according to the manufacturer's instructions. Genomic DNA from 11,200 to 1160 bp upstream of *patched* was then inserted upstream of this as a *Bam*HI-*Nde*I fragment. Finally, the complete *patched*-GFP fragment was inserted into pCASPER2 upstream of *Moesin* as a *Bam*HI-*Not*I fragment.

Fly lines

UAS-RFP-Moesin and *patched*-GFP-Moesin transgenic lines were generated in a *w* background by *P*-element-mediated germline transformation. UAS-RFP-Moesin and *patched*-GFP-Moesin insertions on chromosome 2 were recombined with *engrailed*-Gal4 (Bloomington Stock Center) (Brand and Perrimon, 1993). sGMCA (constitutively expressing GFP-Moesin) (obtained from Daniel Kiehart, Duke University, Durham, NC) on chromosome 2 was recombined with *engrailed*-Gal4 and UAS-RFP-Moesin (Kiehart et al., 2000).

Imaging

Embryos were dechorionated in bleach then mounted in Voltalef oil under a coverslip. Images were collected on a Leica SP2 or SP5 confocal microscope and processed using ImageJ, Photoshop (Adobe) and Volocity (Improvision). Wounding was performed using a Spectra-Physics nitrogen laser.

RESULTS AND DISCUSSION

Expression pattern of *ptc*-GFP-Moesin and *en*-RFP-Moesin

In order to directly observe cell matching occurring during DC we generated a fly line in which two distinct populations of epithelial cells were labelled with different fluorescent proteins, enabling us to compare interactions between like and unlike cells during zippering. The strategy we used for this is illustrated in Fig. 1A. P compartments were labelled red by expressing the F-actin-binding domain of Moesin fused to the red fluorescent protein mCherry (henceforth RFP-Moesin) under the control of the *engrailed* (*en*) promoter using the UAS-Gal4 system (Brand and Perrimon, 1993; Edwards et al., 1997). Alongside this, A compartments were labelled green by expressing GFP-Moesin directly under the control of 11,200 bp of sequence upstream of the *patched* (*ptc*) coding region. Consistent with the known expression pattern of *ptc*, the *ptc*-GFP-Moesin transgene was expressed in a 1- to 2-cell-wide stripe at either end of A compartments during DC, thus flanking each *en*-RFP-Moesin stripe (Fig. 1B) (Nakano et al., 1989). Expression of RFP-Moesin and GFP-Moesin were strictly segregated by parasegment boundaries; however, expression of both fluorophores was sporadically observed in individual cells abutting segment boundaries. The expression patterns of RFP- and GFP-Moesin were maintained through DC and perfect matching of red and green stripes along the fusion seam was observed as zippering proceeded (Fig. 1Bi-iii).

Complex patterning of the DC leading edge

Examination of the leading edge cells during and after DC in *en*-RFP-Moesin/*ptc*-GFP-Moesin embryos revealed an unexpected but reproducible aberration in patterning. Isolated cells expressing *ptc*-GFP-Moesin rather than *en*-RFP-Moesin were frequently present within the *en* domain (Fig. 1C). These misplaced *ptc* cells were found in a conserved location, towards the posterior end of the *en* domain and exclusively at the leading edge. On zippering, the misplaced *ptc* cells fused with their matching counterpart in the opposing sheet, thus forming an island of two *ptc*-GFP-Moesin-expressing cells within the *en* domain (Fig. 1Ciii). The segmental distribution of the misplaced *ptc* cells was also well conserved; they were reproducibly present in segments A1-A5, but rarely in other segments (Fig. 1D). In order to identify the origin of the misplaced *ptc* cells, we used live imaging, commencing at the start of DC. As DC proceeded, a single *ptc* cell became isolated from the anterior edge of the A compartment by dorsalward migration of *en* cells to the leading edge (Fig. 1F; see Movie 1 in the supplementary material). Following DC, the pairs of misplaced *ptc* cells remained within the *en* stripe. Thus, the misplaced *ptc* cells derive from the A compartment, but ultimately reside in the P compartment owing to an epithelial rearrangement. This rearrangement is surprising because differential adhesion characteristics should prevent mixing of A and P cells.

The arrangement of trichomes on late embryos has been widely used as a morphological readout of epithelial patterning and we were interested to establish the trichome characteristics of the misplaced *ptc* cells (Payre, 2004). In order to visualise the trichome pattern, GFP-Moesin was expressed constitutively, alongside RFP-Moesin in *en* stripes as a positional marker. The trichome pattern of each segment of the dorsal epithelium consists of a broad band of cells possessing trichomes alongside a narrow band with naked cuticle, corresponding to the anterior end of the A compartment (illustrated in Fig. 1A) (Bokor and DiNardo, 1996). Imaging revealed that, in common with the anterior-most cells of the A compartment, the misplaced *ptc* cells were naked (Fig. 1E). By contrast, the *en* cells that surround the misplaced *ptc* cells all possessed trichomes. The misplaced *ptc* cells therefore share the morphological characteristics of the A compartment, despite residing within the P compartment.

Filopodia can recognise matching cells

Having characterised the dorsal epithelium in *en*-RFP-Moesin/*ptc*-GFP-Moesin embryos, we then used this fly line to investigate cell matching during DC in live embryos. In these embryos, we can clearly see red and green filopodia and observe their behaviour during zippering. Filopodia appeared to scan the opposing epithelium and interactions were only observed between filopodia of the same colour (Fig. 2A; see Movie 2 in the supplementary material). When filopodia of differing colours came into close proximity with one another, no observable interaction took place. Notably, both red-to-red and green-to-green interactions were observed, indicating that both A and P compartments are actively involved in the matching process. Thus, our data suggest that at least two distinct recognition mechanisms mediate cell matching during DC. The misplaced *ptc* cells within the *en* domain described above are consistently able to recognise and specifically fuse with one another, indicating that they have recognition properties distinct from their neighbouring *en* cells.

Filopodial tethers can realign mismatched epithelia

Often, the two epithelial sheets are poorly aligned immediately prior to zippering and mismatched fusion appears likely. Under these conditions, we observed that filopodia could identify and
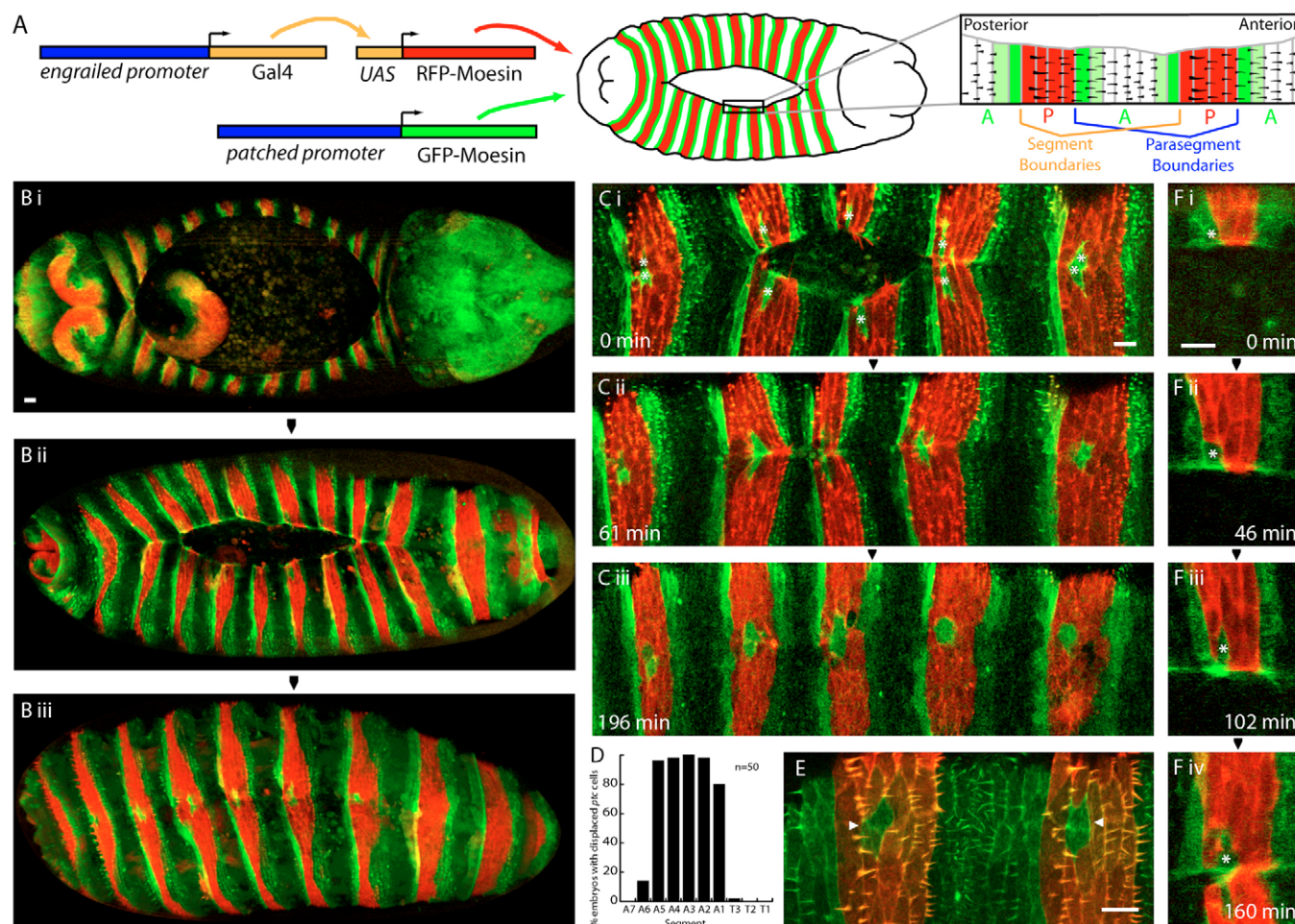
Fig. 1. Expression pattern of *en*-RFP-Moesin and *ptc*-GFP-Moesin in the dorsal epithelium. (**A**) Schematic illustrating construction of the fly line, transgene expression pattern and dorsal trichome pattern. (B,C,F) Images of embryos expressing *en*-RFP-Moesin (red) and *ptc*-GFP-Moesin (green). (**Bi-iii**) Dorsal view of embryos at the start (i), midway through (ii), and shortly after completion (iii) of DC in a live embryo. (**Ci-iii**) Time course of the latter stages of DC. Misplaced *ptc*-GFP-Moesin-expressing cells (asterisks in i) are present at a conserved position at the leading edge of the *en* domains of all segments shown. (**D**) Bar chart showing proportion of embryos with misplaced *ptc*-GFP-Moesin-expressing cells in each segment. (**E**) Dorsal view of stage 16 embryo expressing GFP-Moesin (green) constitutively and RFP-Moesin (red) under control of *en*-Gal4. Pairs of cells in the P compartment not expressing RFP-Moesin are indicated by arrowheads. (**Fi-iv**) Images from Movie 1 (see Movie 1 in the supplementary material) showing cell rearrangements during DC that lead to the presence of the misplaced *ptc* cell (asterisk) in the *en* domain. Scale bars: 10 μm.

bind to matching cells several cell-diameters distant in the opposing epithelial sheet (Fig. 2B; see Movie 3 in the supplementary material). Having made contact with the appropriate partner, the filopodia form tethers, linking the matching cells together. Contraction of these filopodial tethers then draws matching cells towards one another, correcting the misalignment. These filopodial tethers thus appear to be able to exert sufficient contractile force to drag the entire epithelial sheet. Our data therefore suggest that, in addition to acting as sensory devices, filopodia also play an active role in motility, pulling the cell towards its point of attachment. This is consistent with recent in vitro studies demonstrating that filopodia can exert significant pulling forces (Kress et al., 2007). Realignments such as that shown in Fig. 2B are common, with 44% of stripes observed (*n*=63) exhibiting an adjustment of one cell width or more during zippering. Notably, the filopodial interactions occurring during DC do not necessarily become permanent adhesions: we noted at least one tether break during zippering of 42% of the stripes observed (*n*=74) (see Fig. 2Bi; for an example, see Movie 3 in the

supplementary material). These breakages occur when a tether forms in isolation, unsupported by other tethers pulling in the same direction.

Matching in embryos with leading edge asymmetries

Our data show that filopodia are able to identify matching cells and pull misaligned epithelia into the correct alignment; however, this is under normal conditions in which there is a relatively high level of symmetry in the patterning of the two opposing epithelia. We were curious to know what would happen if the patterning of the two epithelia was asymmetric. We observed that asymmetries occasionally occurred spontaneously, and we imaged DC in several such asymmetric embryos. The embryo shown in Fig. 3A (see also Movie 4 in the supplementary material) has an extra misplaced *ptc* cell within an *en* domain. This extra *ptc* cell fused with the neighbouring A compartment and this subsequently pushed the remainder of the segment out of alignment. This resulted in *en* cells being brought into close proximity to the
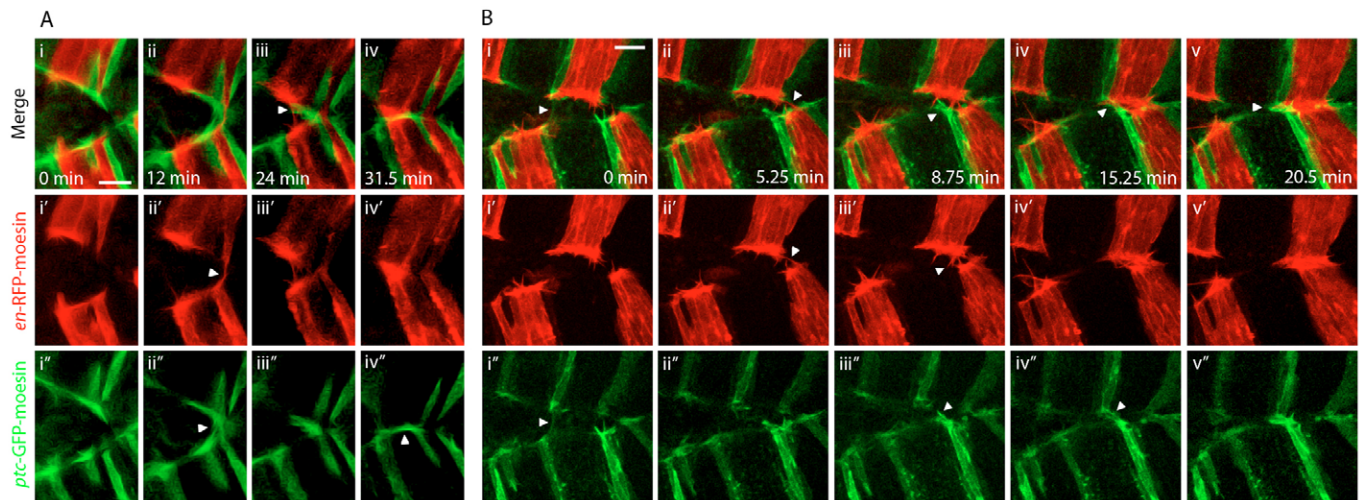
Fig. 2. Cell matching and realignment during DC is mediated by filopodia. Zippering in *Drosophila* embryos expressing *en*-RFP-Moesin (red) and *ptc*-GFP-Moesin (green), as shown merged (top row) and in isolation (middle and bottom rows, respectively). (**Ai-iv″**) Images from Movie 2 (see Movie 2 in the supplementary material) showing filopodial matching. (i) Red and green filopodia protrude from leading edge cells. (ii) Contacts are made between red filopodia from opposing epithelia, while at the same time separate contacts are made between green filopodia. (iii) Further contacts are made between red filopodia; however, green filopodia in close proximity to these red filopodial contacts do not interact. (iv) Green filopodia transiently form contacts between *ptc*-GFP-Moesin cells over the top of the fused red cells. (**Bi-v″**) Images from Movie 3 (see Movie 3 in the supplementary material) showing realignment of misaligned epithelial sheets by filopodial searching and pulling. (i) The two epithelial sheets are initially poorly aligned. A filopodial tether transiently exists between green cells but later breaks. (ii) Contacts form between filopodia from *en*-RFP-Moesin-expressing cells. (iii,iv) Green filopodia in the lower sheet do not interact with nearby red filopodia and ultimately form contacts with green cells some distance away in the opposing sheet. (v) The tethers that result from the contacts made by red and green filopodia pull the sheets into alignment. The described filopodial interactions are indicated by arrowheads. Scale bars: 10 μm.

opposing *en* stripe of the neighbouring segment and, interestingly, fusion then occurred between these *en* cells, despite the fact that they reside in different segments. DC subsequently continued as normal, but one segment out of register. We observed a similar sequence of events in three other asymmetric embryos. These observations demonstrate that although matching is specific to the compartment, it is not specific to the segment. Therefore, asymmetries that are sufficiently great that filopodia can reach the equivalent compartment of a neighbouring segment have the potential to result in mismatch.
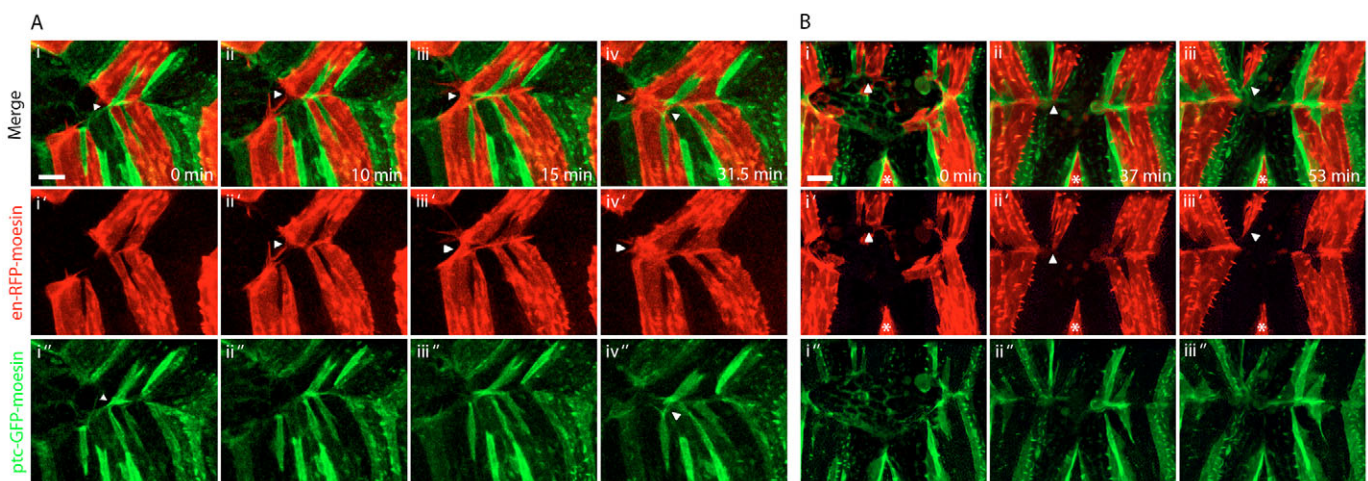
Fig. 3. Matching in embryos with asymmetries between the opposing epithelial sheets. DC zippering in *Drosophila* embryos with asymmetries as revealed by *en*-RFP-Moesin (red) and *ptc*-GFP-Moesin (green) expression, shown merged (top row) and in isolation (middle and bottom rows, respectively). (**Ai-iv″**) Images from Movie 4 (see Movie 4 in the supplementary material) showing zippering in an embryo with a spontaneous asymmetry. (i) The upper epithelial sheet has two rather than one misplaced *ptc* cell in the *en* domain and one of these has associated with the A compartment of the lower sheet blocking matching of *en* cells. (ii) As a result, the blocked *en* cells make filopodial contacts with *en* cells of the neighbouring segment. (iii) These develop into permanent contacts. (iv) Cells that are not associated with matching partners continue to produce filopodia. (**Bi-iii″**) Zippering in an embryo with a laser-induced asymmetry. (i) An *en* stripe has been removed from the leading edge of the lower epithelial sheet by laser ablation (asterisk), whereas the opposing *en* stripe (arrow) is normal. (ii,iii) On contact with the opposing leading edge, the unpartnered *en* stripe constricts and then withdraws completely from the leading edge. The described filopodial interactions are indicated by arrowheads. Scale bars: 10 μm.
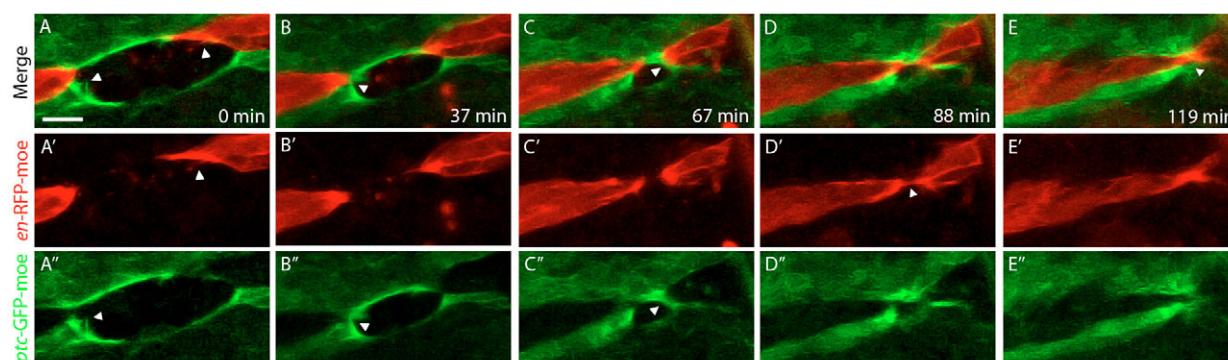
Fig. 4. Filopodial matching occurs during healing of a wound in the ventral epithelium. Images from Movie 5 (see Movie 5 in the supplementary material) showing healing of a wound across an *en* stripe in the epithelium of an *en*-RFP-Moesin (red), *ptc*-GFP-Moesin (green) *Drosophila* embryo. (**A-A″**) Filopodia are produced by wound edge cells. (**B-C″**) Interactions occur between *ptc*-GFP-Moesin cells across the wound. (**D-E″**) Eventually a filopodial tether forms between *en*-RFP-Moesin cells on either side of the wound and this tether rapidly leads to fusion of the two *en* cells. Notably, the last part of the wound to heal is at the junction between *en* and *ptc* cells. The described filopodial interactions are indicated by arrowheads. Scale bar: 10 μm.

We next used laser ablation of leading edge cells to assess the effect of inducing asymmetry in symmetrically patterned embryos. We were able to specifically ablate a single *en* stripe of leading edge cells from one of the epithelial sheets. The corresponding *en* stripe of the opposing epithelial sheet no longer had matching cells with which to fuse, and we focused on the behaviour of this unpartnered *en* stripe upon confrontation with the opposing epithelial sheet. The most common outcome (11 of 13 embryos) was that this *en* stripe became constricted and was effectively extruded from the leading edge, such that the *en* cells did not participate in zippering (Fig. 2B). In the remaining cases, the unpartnered *en* stripe partially constricted, then fused with the opposing *en* stripe of the neighbouring segment, as observed in Fig. 2A.

These data demonstrate that when faced with asymmetries that cannot be resolved by filopodial searching and tethering, zippering occurs in such a way that contact between the mismatched cells is minimised.

Matching during epithelial wound healing

What is the molecular basis of cell matching? The data above are consistent with matching being based on just two sets of molecular interactions, one allowing A compartment cells to recognise one another and the other performing the same function for P compartment cells. An obvious possibility is that the molecules that mediate cell matching during DC are the same as those that maintain the integrity of these compartments throughout the epithelium. Alternatively, there could be a different set of recognition molecules present exclusively at the leading edge to mediate cell-cell matching. Filopodia are also observed during the healing of wounds in the ventral epithelium and we reasoned that these wound filopodia should exhibit matching behaviour if the molecules that mediate matching are present throughout the epithelium (Wood et al., 2002). Laser wounds were made to the ventral epithelium across *en* stripes such that the wound edge consisted of both *en*-RFP-Moesin and *ptc*-GFP-Moesin cells. On healing of these wounds, we observed repeated interactions between the filopodia of matching cells, but not between mismatching cells. In the example shown in Fig. 4, a number of filopodial tethers formed between *ptc* cells on opposite sides of the wound as closure proceeded. Near the end of closure, a filopodial tether formed between *en* cells on opposite sides of the wound, leading to fusion of these cells and regeneration of the *en* stripe (Fig. 4; see Movie 5 in the supplementary material). This

sequence of events was observed in six out of ten similar wounds. In the remaining wounds, the tethers between *ptc* cells became permanent adhesions before the *en* cells were close enough to form tethers and hence the *en* stripe was not regenerated. These data suggest that both A and P compartment cells away from the leading edge can carry out filopodial matching analogous to that occurring during DC, and hence the adhesion molecules that mediate the process are not leading edge specific.

The data presented here demonstrate that specific recognition events ensure the accuracy of fusion during DC. Filopodia facilitate matching by allowing a cell to search for its match and also to pull misaligned sheets into alignment. This explains why genetic interventions that abolish filopodia lead to an increase in mismatching (Gates et al., 2007; Jacinto et al., 2000). It appears that at least two recognition processes act during DC, one for P compartments and one for A compartment cells, but these recognition events are not segment-specific, as fusions can occur between matching compartments from different segments. Filopodial matching is also observed during healing of wounds in the ventral epithelium, suggesting that the molecules mediating recognition are found throughout the epithelium. These data are consistent with the notion that the adhesion molecules that mediate filopodial matching during DC are the same as those that ensure compartment integrity throughout the epithelium; however, the identity of these molecules is currently unknown. Experimental and modelling studies have shown that cells can sort based on differential levels of just one adhesion molecule, and it has been hypothesised that a single adhesion molecule might be responsible for compartmental segregation (Dahmann and Basler, 2000). Our data suggest that, at least during filopodial matching, this is not the case, as we observe specific recognition events for both A and P compartments and neither compartment is obviously dominant in the matching process. It is of course possible that multiple mechanisms contribute to cell matching and segregation, perhaps with different adhesion molecules governing the rapid, transient associations between filopodia and the long-lived adhesions that hold cells together permanently. Whereas segregation between leading edge A and P compartment cells is absolute at the parasegment boundary, as discussed above we reproducibly see a single A compartment *ptc* cell move into the P compartment at the segment boundary. This might suggest that differences in adhesive properties between cells either side of the segment boundary are

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small, permitting a degree of mixing. However, during DC, the misplaced *ptc* cells are consistently able to recognise and specifically fuse with matching cells in the opposing epithelial sheet, indicating adhesive properties distinct from their neighbours. When the arrangement of the misplaced *ptc* cells is disrupted, it can result in severe mismatches; therefore, correct positioning of these cells is clearly important in epithelial sheet alignment. These cells occupy a unique and defined position in each segment and might assist the matching process by acting as a 'keystone' that helps to ensure precise alignment within the segment.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/4/621/DC1

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