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Specialized extraembryonic cells connect embryonic and extraembryonic epidermis in response to Dpp during dorsal closure in *Drosophila*

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Abstract

Dorsal closure in *Drosophila* embryogenesis involves expansion of the dorsal epidermis, followed by closure of the opposite epidermal edges. This process is driven by contractile force generated by an extraembryonic epithelium covering the yolk syncytium known as the amnioserosa. The secreted signaling molecule Dpp is expressed in the leading edge of the dorsal epidermis and is essential for dorsal closure. We found that the outermost row of amnioserosa cells (termed pAS) maintains a tight basolateral cell–cell adhesion interface with the leading edge of dorsal epidermis throughout the dorsal closure process. pAS was subject to altered cell motility in response to Dpp emanating from the dorsal epidermis, and this response was essential for dorsal closure. αPS3 and βPS integrin subunits accumulated in the interface between pAS and dorsal epidermis, and were both required for dorsal closure. Looking at αPS3, type I Dpp receptor, and JNK mutants, we found that pAS cell motility was altered and pAS and dorsal epidermis adhesion failed under the mechanical stress of dorsal closure, suggesting that a Dpp-mediated mechanism connects the squamous pAS to the columnar dorsal epidermis to form a single coherent epithelial layer.

Keywords: Dorsal closure; Amnioserosa; Cell adhesion; Integrin; Dpp; JNK; *Drosophila*

Introduction

The process of animal embryogenesis often proceeds in the form of flat germ-layer sheets flanked by extraembryonic tissues that cover the yolk-rich region of fertilized eggs, and which helps in delivering nutrition to the embryo proper. Completion of embryogenesis involves the closure of the embryonic body wall that is accompanied by a loss of extraembryonic tissues through a combination of absorption and degeneration. A number of genetic and cell biological studies of body wall closure in *Drosophila* embryos have begun to reveal the roles of signaling molecules produced in the epidermis in epithelial sheet movement (reviewed in Kiehart et al., 2000; Martin and Parkhurst, 2004). Exactly how extraembryonic tissues respond to signals emanating from embryonic tissues and how they subsequently contribute to body wall closure, however, remain poorly understood.

Drosophila embryos develop with the dorsal margin of the dorsal epidermis (DE) open. Following completion of germ-band retraction at embryonic stage 13, DE is directly contacted by the amnioserosa (AS), an extraembryonic epithelium covering the yolk syncytium. During the dorsal closure (DC) of the body wall, the DE sheets expand on both sides of the midline. They then meet at the dorsal midline, and the two DE sheets are eventually sealed together by interdigitated cell protrusions that establish cell–cell adhesion in a similar way to the fastening of a zipper. This zippering stage has been shown to be somewhat similar to the wound healing process (Jacinto et al., 2000; Kiehart et al., 2000; Martin and Parkhurst, 2004 for review).

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The epithelial movement of DC involves expansion of the DE accompanied by continuous stretching of cell length along the dorsal–ventral axis, together with AS shrinkage and a stepwise loss of its cells. Previous studies had implicated two types of contractile forces as being involved in DC, one from the AS, and the other from the supracellular F-actin bundles connecting the leading edge cells (LE) of epidermis, known as the purse string (Hutson et al., 2003; Kiehart et al., 2000). The combined force directed toward the dorsal midline is countered by the pulling forces found in the epidermis at the interface of DE and AS. This indicates that the structural integrity of the DE-AS interface is important for coordinating the multiple forces to effect a steady movement of the DE.

Two signaling pathways, Dpp and JNK, play an essential role in controlling the DC. JNK signaling is activated in LE (Glise et al., 1995; Hou et al., 1997; Martin-Blanco et al., 1998; Riesgo-Escovar and Hafen, 1997; Riesgo-Escovar et al., 1996) and stimulates transcription of a number of genes including the actin regulator *chikadee* that is involved in the migrative activities of LE cells (Homsy et al., 2006; Jasper et al., 2001). JNK signaling also activates the expression of Dpp in LE (Hou et al., 1997), and Dpp is subsequently secreted and elicits a variety of responses in neighboring cells in regulating DC (Affolter et al., 1994). Seen in this light, this evidence shows that localized expression of Dpp in the LE plays a key role in coordinating cellular behaviors in DE and AS during DC.

Integrins, which are receptors for extracellular matrix (ECM) and function in cell adhesion, are known to be required for DC (Brown, 1994; Stark et al., 1997). Integrins are noncovalent heterodimers of the single-pass transmembrane subunits α and β (Hynes, 2002) and play a crucial role in cell-ECM adhesion in various kinds of organogenesis (Bokel and Brown, 2002; Brower, 2003). Five α and two β integrin subunits are known to exist in *Drosophila* (Brown et al., 2000). βPS is the major β subunit and associates with at least three α subunits: αPS1, αPS2, and αPS3. Although the βPS and αPS3 integrins are required for DC, βν is not (Brower, 2003; Brown et al., 2000; Devenport and Brown, 2004; Homsy et al., 2006; Hutson et al., 2003). Further studies have suggested integrins as playing a role in the maintenance of the adhesion of the yolk membrane to the AS (Reed et al., 2004); the DE to the AS (Narasimha and Brown, 2004); as well as the sealing of two opposing epidermis at the midline (Hutson et al., 2003). Here we identified a specialized AS cell, termed pAS, that makes direct contact with the DE. Our analyses demonstrated that pAS plays a major role in the integrin-dependent adhesion of AS and DE in response to Dpp signaling.

Materials and methods

Drosophila strains

The following strains were used: $scb^{Vol\text{-}3}$ (Rohrbough et al., 2000), scb^{2} (Stark et al., 1997), tkv^{7} (FBal0016824 = FlyBase identifier), tkv^{8} (FBal0016825), bsk^{1} and bsk^{2} (FBal0001321, FBal0001322), *ush-Gal4* (Hayashi et al., 2002), *btl-Gal4* (Shiga et al., 1996), *en-Gal4* (Bloomington Stock Center), *ptc-Gal4* (ptc559.1; Fbal0042573), *UAS-gfp-moesin* (Chihara et al., 2003), *UAS-E-cadherin-GFP* (Oda and Tsukita, 1999), *UAS-gfp.nls* (Bloomington Stock Center), *UAS-DEcadherin-GFP* (Oda and Tsukita, 1999), $UAS\text{-}tkv^{Q253D}$ (Okano et al., 1994), *UAS-dad*, and *dad-lacZ* (Tsuneizumi et al., 1997). *pAS-Gal4* is an *escargot* enhancer–Gal4 fusion construct (Tanaka-Matakasu, Yagi and Hayashi, unpublished) that is expressed in a row of cells containing large nuclei characteristic of the amnioserosa and which directly flank the leading edge of the DE. We termed those cells the peripheral amnioserosa (pAS). *pAS-Gal4* also showed sporadic expression inner amnioserosa cells. As *esg* was not expressed at a detectable level in the pAS (Fuse et al., 1994), this pAS expression is likely to be a fortuitous effect of the vector construction. *UAS-scb-PA* (Vol-S described by Grotewiel; Grotewiel et al., 1998), *UAS-scb-PB (Vol-L)*, and *UAS-αPS5* were constructed from full-length cDNAs isolated by RT-PCR. The amino acid sequence of *αPS5* used here differs from the published sequence (Q9W1M8) by the addition of an N-terminal MHRLLFLIFLALKYQSNA sequence derived from an upstream exon.

Time-lapse observations

Mutations were recombined into chromosomes carrying the Gal4 drivers or UAS-gfp reporters on the second chromosome. Homozygous mutant embryos were positively identified by the GFP expression from the progenies of the Gal4- and UAS-gfp carrying strains. Embryos at an appropriate developmental stage were collected under fluorescent stereomicroscope (MZFLIII, Leica) and mounted on glass-bottom Petri dishes and covered with distilled water. Time-lapse images were taken by a confocal microscope (Olympus FV300) with PlanApo 60× objective lens NA 1.4 (Olympus, Japan) at 25°C, as reported previously (Kato et al., 2004). Processing of individual images and movies was performed using FLUOVIEW (Olympus). Images were processed with Photoshop (Adobe Systems) and Canvas (ACD Systems). Movies were encoded with QuickTime Pro (Apple Computer).

Immunostaining

The rabbit antibody against αPS3 was produced against the peptide NH_2-C ELDRLVQQNPVEPEAENLNSGGNN-COOH, and was affinity purified for double labeling with mouse anti-βPS (Developmental Studies Hybridoma Bank, University of Iowa). Cy3 conjugated anti-rabbit IgG and Cy5 conjugated anti-mouse IgG (Chemicon) were used as secondary antibodies. Transverse sections of embryos were prepared by refixing prestained whole-mount embryos with 4% paraformaldehyde and cutting with a razor blade in Vectashield fluorescent mountant (Vector Laboratories, Burlingame, CA, USA) essentially as described previously (Narasimha and Brown, 2004). Images were taken using a confocal microscope (FV500) with PlanApo x60 NA 1.4 or UPlanApo 20× NA 0.70 objective lens (Olympus). Images were processed by FLUOVIEW (Olympus).

Transmission electron microscopy

Wild-type and $scb^{Vol\text{-}3}$ embryos were fixed by high-pressure freezing and freeze-substitution as described previously (McDonald, 1994). Samples were embedded in Poly/Bed 812 resin (Polyscience, Inc., PA, USA). Standard protocols were used for sample dissection. 70- to 90-nm sections were collected onto copper grids, double stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope, JEM-1010 (JEOL, Japan), at 100 kV accelerating voltage. Photographs were digitized using a scanner, CanoScan 8400F (Canon, Japan).

Results

Unique cell behavior of the outermost row of amnioserosa

Using time-lapse recording of embryos labeled with the F-actin marker GFP-moesin (Chihara et al., 2003; Figs. 1, 4, 6), E-cadherin-GFP (Oda and Tsukita, 1999; Fig. 1), or nuclear localization signal–tagged GFP (*gpf.nls*, Fig. 5), we set out to examine cell behavior during DC in detail. Through this we found that GFP-moesin expression driven by *ush-Gal4* (*ush>gfp-*

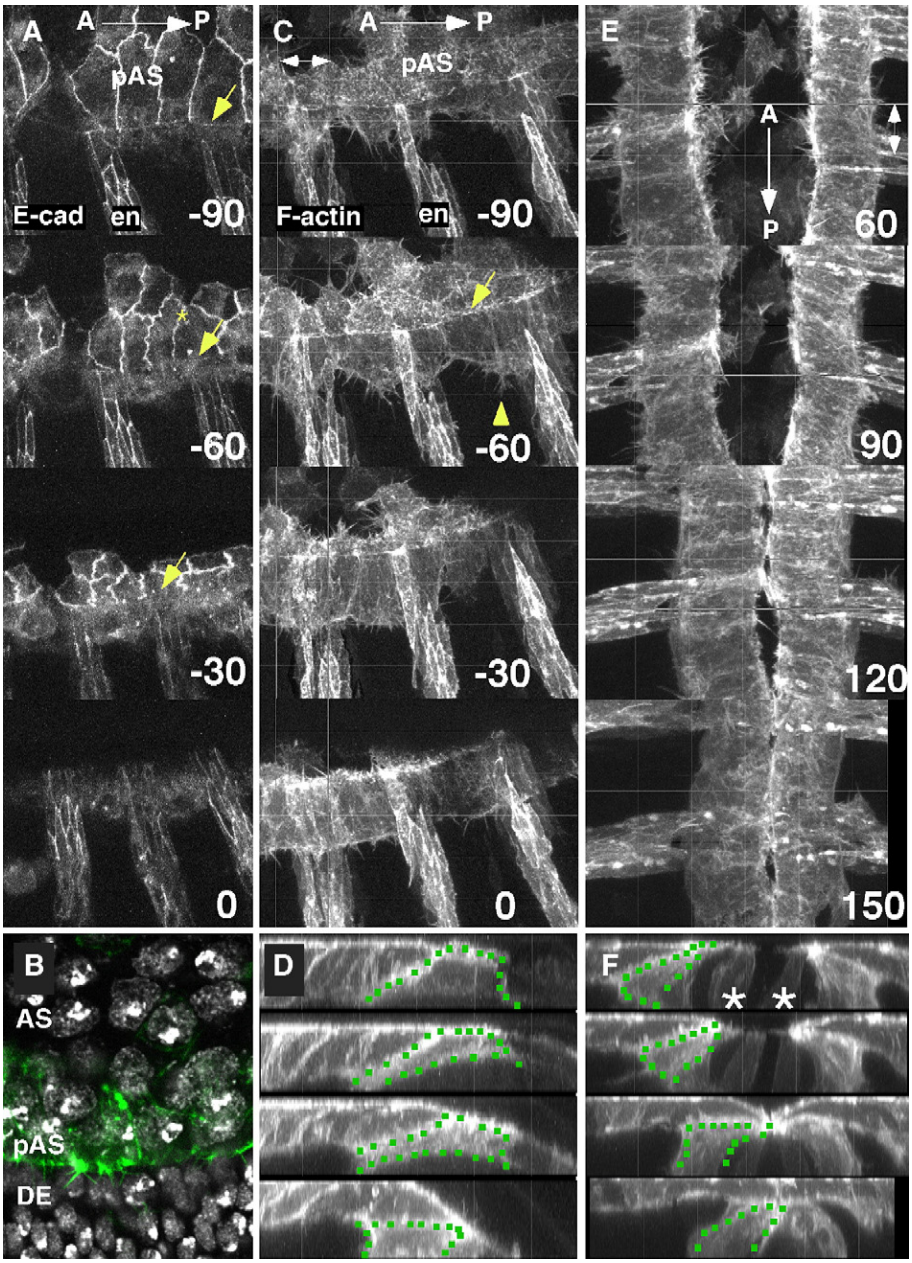

Fig. 1. Tight association of dorsal epidermis and peripheral amnioserosa in dorsal closure. Time-lapse confocal images of dorsal closure in embryos visualized by E-cadherin-GFP (A) or GFP-moesin (C–F) expressed by *pAS-Gal4* (pAS) and *en-Gal4* (en). The onset of DC in stage 13 is set as time 0 (min) and the elapsed time is indicated in each panel. Arrows indicate adherens junction between pAS and DE (A) or F-actin cable in pAS (C) and arrowhead indicates intense filopodia formed in the ventral edge of pAS (C). (A, C, E) *x–y* projection views of 4D time-lapse images. Both apical and basal surfaces of the pAS and LE are included. (D, F) *y–z* or *x–z* stacks of the region indicated by double headed arrows in panels C (time 0) and E (time 60). pAS is indicated by dotted lines. Internal amnioserosa expressing GFP were labeled with asterisks. (B) Labeling of DAPI (gray scale) and pAS > GFP-moesin (green). pAS contained large nuclei typical of extraembryonic tissues.

moesin), which is expressed in the DE and AS (Hayashi et al., 2002), revealed the dynamic cell movement of the leading-edge cells of the DE with a purse-string-like accumulation of F-actin, as reported previously (Kiehart et al., 2000) (Fig. 4A). The same study had also noted that the outer most rows of AS cells were located beneath the LE cells during DC (Kiehart et al., 2000). We actually found a Gal4 driver (*pAS-Gal4*) that was highly expressed in the outermost row of AS cells (Figs. 1B and 6; Materials and methods). Their unique cell behavior and the existence of a cell-specific marker suggested that the outermost row of the AS is a specialized region. We therefore decided to call them peripheral AS (pAS) and studied their cellular behavior using high-resolution time-lapse microscopy.

Dynamic change in cell adhesive behavior of peripheral AS

We compared the cellular behaviors of pAS and DE by labeling them with GFP-moesin or E-cadherin-GFP simultaneously driven by *pAS-Gal4* and *en-Gal4* (Fig. 1, Supplemental movies 1–3). Before the start of DC, pAS and LE were located

next to each other on the surface of the embryo (Fig. 1A). LE cells then started to crawl over pAS with extensive F-actin accumulation in their dorsal margin (Figs. 1C, D). pAS cells simultaneously constricted their apical surface (Fig. 1A) and extended numerous filopodia in their basal side to the ventral orientation (Fig. 1C, arrowhead). We further noted that pAS formed a sharp line of F-actin accumulation in the region that precisely faced the leading edge of DC (Fig. 1C, arrow). We consider this F-actin accumulation as contributing to the purse string of F-actin that had previously been attributed exclusively to LE cells. After 90 min, we found that pAS had constricted its apical cell surface and was nearly completely covered by LE cells; the purse string of F-actin in LE and pAS had reached the dorsal side of pAS; and ventral filopodial activity in pAS had decreased. It was at this point that DC commenced (time = 0).

We noted an unusual distribution of E-cadherin-GFP in pAS cells. Although E-cadherin-GFP was detectable at the majority of apical epithelial cell junctions (Fig. 1A, asterisk), its intensity in the pAS-DE interface was significantly weaker than in other cell–cell interfaces (Fig. 1A, arrow) at the beginning of DC, and further decayed as DC progressed.

DC proceeded over the next 150 min, with pAS tightly associated with a part of the basolateral surface of the DE and found to steadily progress toward the dorsal midline (Figs. 1E, F, asterisks). The number of ventral filopodia in pAS remained small (Fig. 1E) and under low magnification we observed that the outer surface of the pAS appeared smooth (Fig. 6A). Sporadic expression of GFP-moesin in internal AS cells revealed that they continuously reduced the surface area and internalized (Figs. 1A, F). After 120 min, DE from each side met at the midline and by 150 min, a firm association of DEs was established. pAS maintained its tight association with DE until all of the internal AS cells left from the surface, with the area of contact between DE and pAS gradually decreasing (Fig. 1F) before pAS cells were finally completely dissociated from the DE and underwent apparent apoptosis (Fig. 6A). These observations suggest that the cell adhesion and cell motility of pAS are unique among epithelial cells.

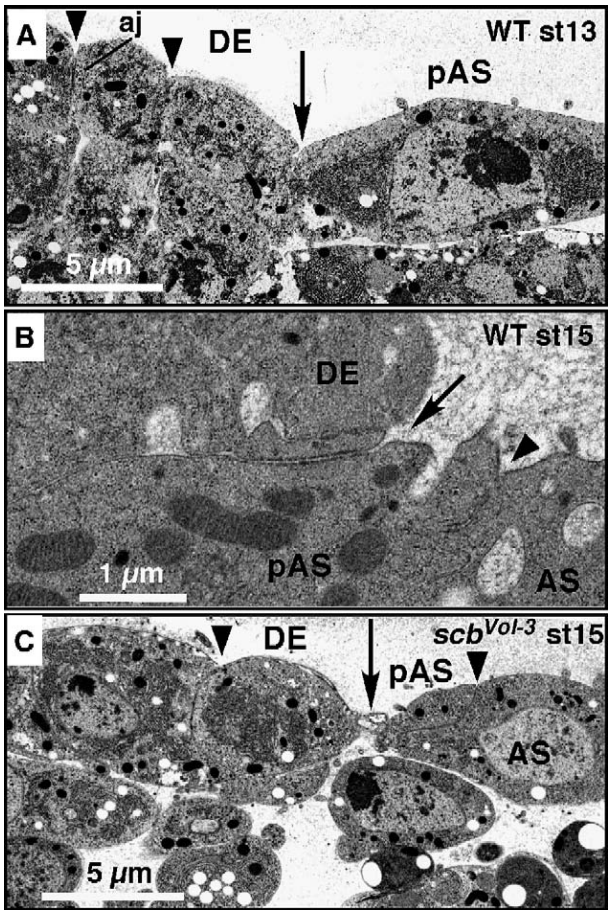

Fig. 2. Transmission electron microscope images of the border between the peripheral amnioserosa and dorsal epidermis. (A, B) Control (stage 13 and 15) and (C) $scb^{Vol\text{-}3}$ (stage 15) embryos. (A) Arrowheads indicate the border between two epidermal cells with adherens junction (aj) labeled. Arrows indicate the border between the DE and pAS, where no obvious aj-like structure was found. (B) When DE covered the surface of pAS, the two cells formed a series of electron-dense structures beneath the apposed plasma membrane near the outer surface. (C) In a $scb^{Vol\text{-}3}$ embryo, a gap appeared between the DE and pAS and the cells were barely connected with filopodia (arrow). The border between two epidermal cells appeared unchanged (arrowhead).

Structural properties of amnioserosa-epidermis interface

Taking advantage of transmission electron microscopy, we elucidated the structural basis for the adhesion of the pAS with DE. In stage 13 wild-type embryos (Fig. 2A), the columnar epithelial cells of the DE were tightly associated with each other by electron-dense adherens junctions (AJ) at their interface (Fig. 2A, arrowheads), with the AS cells taking on a flat, squamous shape and coming into close contact with the DE (arrow). At the beginning of DC by stage 15, when DE has covered pAS (corresponding to time 0 in Figs. 1A, C), the two cells formed a large attachment area with their lateral membranes, which was occasionally associated with electron dense structures at both sides of the interface—corresponding approximately to the position of purse-string like F-actin cables (Fig. 2B; compare to Fig. 1C).

In an attempt to elucidate the molecular components of this cell adhesion interface, we monitored the expression pattern of αPS3 and compared this to the distribution of βPS (Narasimha and Brown, 2004). We used an antibody against the unique C-terminal sequence of αPS3 (Figs. S1A, B) to double label embryos with anti-βPS. In stage 15 embryos, αPS3 was strongly expressed in the closing dorsal midline, AS, midgut, salivary gland, and antenno-maxillary complex (Fig. 3A). We were also able to detect weaker signals in the hindgut, musculature, and central nervous system. In transverse sections of late stage 14 embryos (Fig. 3B), the pAS appeared flat, and bridged both the DE and the yolk membrane. αPS3 was detected at the interface of the pAS and the DE (arrowhead), and between the pAS and the yolk membrane (arrow), where βPS was also expressed. At late stage 15, when the AS was internalized (Fig. 3C, corresponding to 120′ in Fig. 1E), a strong αPS3 signal was detected in the dorsal midline where the leading edges of the DE made contact (Fig. 3C′, arrowhead). The distribution of αPS3 corresponded to a subset of the tissues

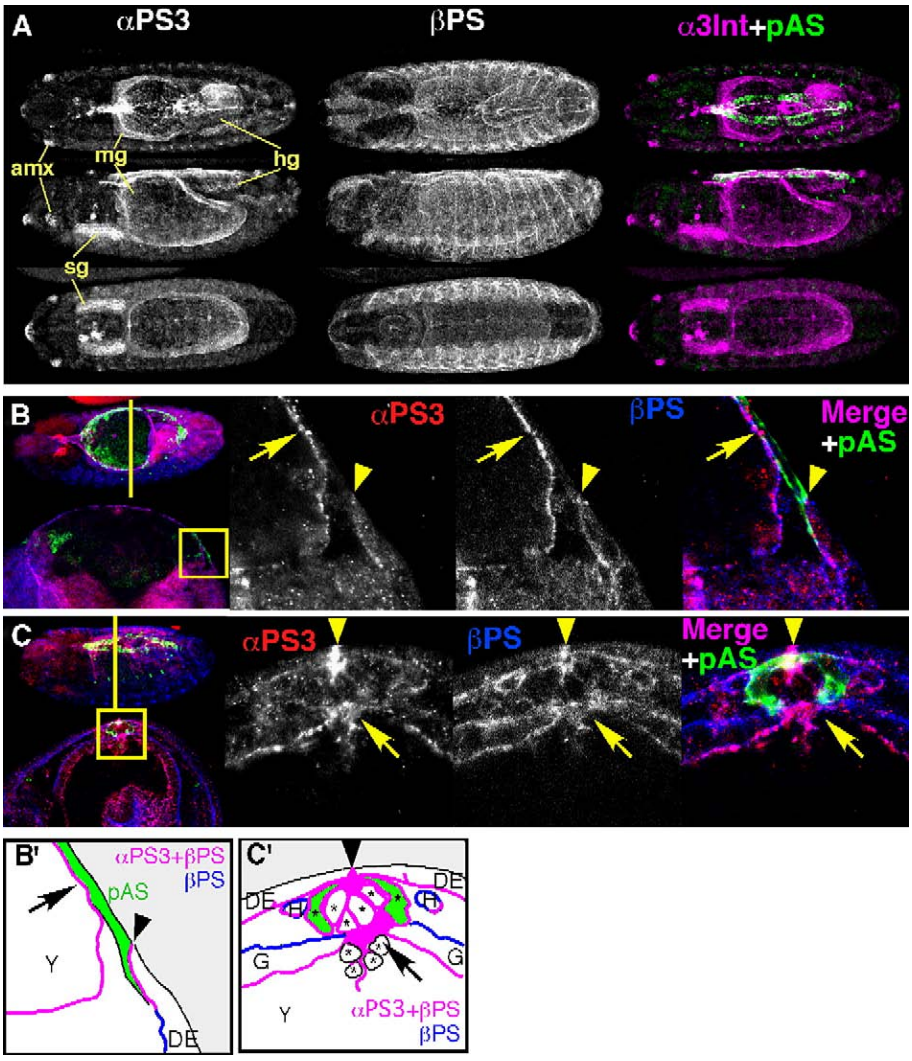

Fig. 3. Expression of αPS3 and βPS during dorsal closure. (A–C) Embryos expressing GFP-moesin under control of *pAS-Gal4* stained with antibodies against αPS3 and βPS. (A) Dorsal, lateral, and ventral views of a stage14 embryo. amx, antennomaxillary complex; mg, midgut; hg, hindgut; sg, salivary glands. (B and C) Cross-sections of stage 14 (B) and 15 (C) embryos. (Top left) Embryos before dissection. Lines indicate positions where the sections were cut. (Bottom left) Low-magnification views of sections. Rectangles indicate the area for magnified images. (B) Arrowheads indicate the border between the AS and DE. Arrows indicate the border between the AS and the yolk membrane. (C) Arrowheads and arrows indicate the fusion points of the DE and visceral mesoderm, respectively. (B′ and C′) Schematic diagrams of the area enclosed in yellow boxes in panels B and C, respectively. DE, dorsal epidermis; pAS, peripheral amnioserosa; Y, yolk; H, heart precursor; G, gut.

expressing βPS (Narasimha and Brown, 2004), and the colocalization of αPS3 and βPS in pAS suggested that αPS3 expression in pAS participates in the integrin-dependent adhesion between AS and DE (Narasimha and Brown, 2004).

Adhesion between dorsal epidermis and amnioserosa fails in αPS3 mutants

Although a previous study by Stark et al. (1997) had reported that αPS3 mRNA was expressed in the tracheal system and that αPS3 mutants (*scb*^{2}) showed severely disrupted tracheal tubules, in this study we were unable to detect the significant expression of the αPS3 protein in the tracheal system (Fig. 3A). In the hope of clarifying this apparent discrepancy, we analyzed a molecularly characterized null mutation *scb*$^{Vol\text{-}3}$, which carries a deletion that removes the region including the three divalent cation-binding domains essential for integrin function (Rohrbough et al., 2000). We were only able to find a mild phenotype in the trachea showing a stalled dorsal branch extension (Fig. S1C). This observation prompted us to reanalyze the role of αPS3 in DC by specifically focusing on the adhesion between pAS and DE.

Time-lapse analysis of *scb*$^{Vol\text{-}3}$ embryos expressing GFP-moesin under the control of the *ush* promoter demonstrated variable degrees of defects in DC, ranging from partial scarring in DE to a complete dorsal open phenotype (Fig. 4, Supplemental movies 4, 5). Even in the event of successful cases, *scb*$^{Vol\text{-}3}$ embryos showed slack DE leading edges, and we found F-actin accumulation in the leading edge to be discontinuous (Fig. 4B, arrowheads) with mutant embryos taking twice as long to complete DC as control embryos. In more severe cases (Fig. 4C), the edges of the DE suddenly separated from the pAS as they approached the dorsal midline (Fig. 4C, 120′) and started to show

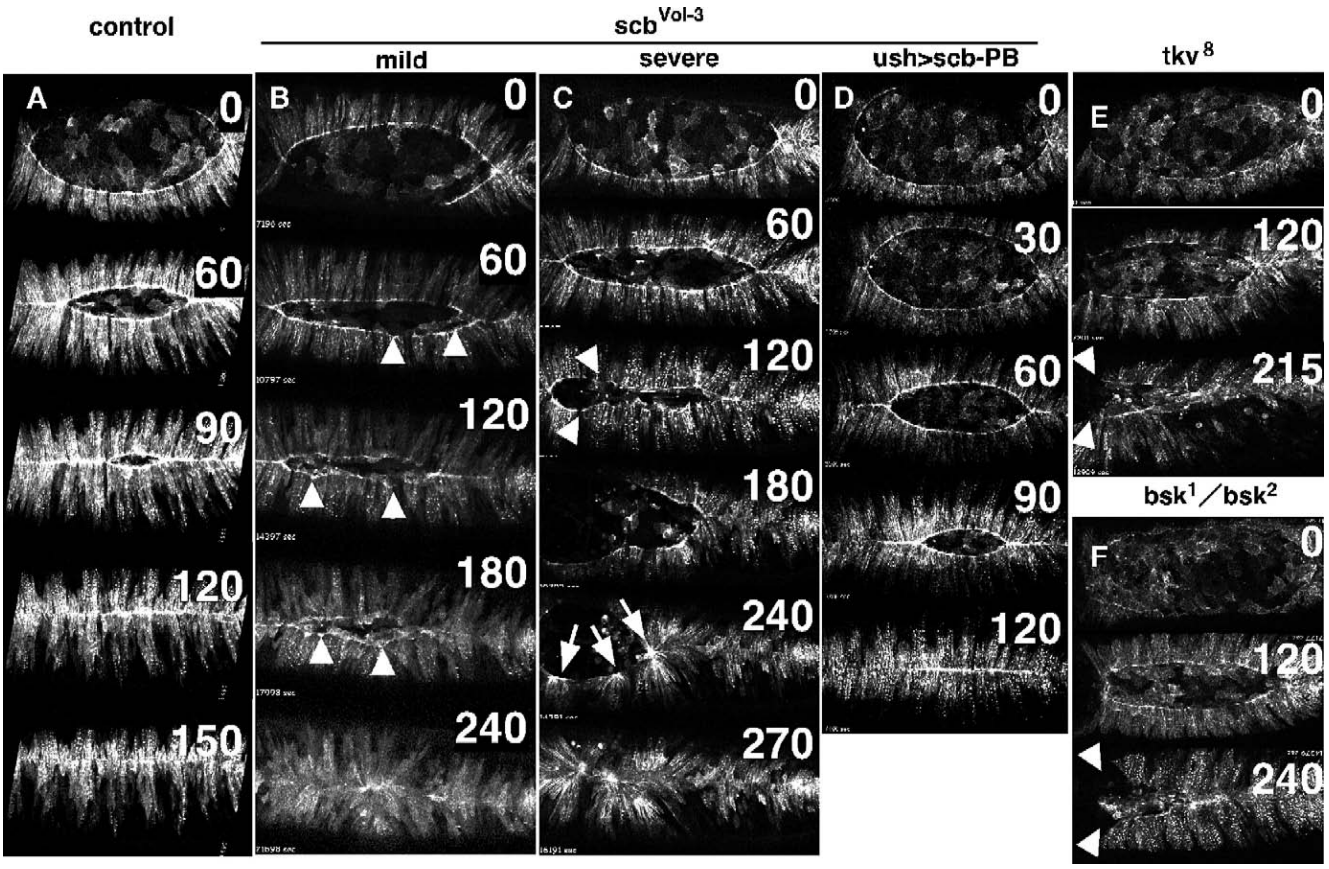

Fig. 4. Adhesion between peripheral amnioserosa and dorsal epidermis requires αPS3. (A–C) Control and two examples of the $scb^{Vol\text{-}3}$ phenotype visualized by GFP-moesin driven by *ush-Gal4*. (A) Control. (B) Modest phenotype. Arrowheads indicate where F-actin accumulation becomes discontinuous. (C) Severe phenotype. Arrowheads in the 180-min image indicate the place where detachment of pAS and DE occurred. Arrows in the 240-min image indicate elevated F-actin concentration. (D, E) Rescue experiments. (D) A $scb^{Vol\text{-}3}$ embryo expressing the long isoform of α3 integrin cDNA (scb-PB) in the amnioserosa and dorsal epidermis. DC followed the normal time course. Rescue experiment with the short isoform (scb-PA) was also successful. (E) A tkv^{8} embryo showing a delay in DC and rapture of the DE-pAS interface. (F) bsk^{1}/bsk^{2} embryo showing defective DC. Arrowhead indicates a scar left by rapture between pAS and DE.

abnormal F-actin accumulation (240′, arrows). Despite the separation of the DE and pAS in $scb^{Vol\text{-}3}$ embryos, the DE from the opposing sides continued to seal the gap with a delayed time course (Fig. 4C), and closure was accomplished in most cases that we observed. From this, we speculate that the dorsal holes created by the loss of the AS invoked a wound-healing reaction to repair the hole (Wood et al., 2002).

Labeling with nuclear localized GFP allowed us to distinguish the large nuclei of amnioserosa from the smaller nuclei found in DE (Fig. 5). Consistent with our observations with GFP-moesin (Fig. 1), the nuclei of pAS and the leading edge of DE overlapped during DC. In $scb^{Vol\text{-}3}$ embryos, however, we were able to observe that the nuclei of pAS and DE were occasionally separated at the start of DC (Figs. 5B, D). These were the locations where the rupture of the pAS-DE interface took place. Expression of αPS3 cDNAs (scb-PA and scb-PB) in both AS and DE completely rescued the DC phenotype of $scb^{Vol\text{-}3}$ (Fig. 4D, data not shown). On the other hand, our attempt to rescue $scb^{Vol\text{-}3}$ with αPS5 – the closest relative of αPS3 – was marginal, at best (data not shown), suggesting the two αPS integrins are functionally distinct.

Transmission electron microscopy of $scb^{Vol\text{-}3}$ embryos at stage 15 revealed that the interface between pAS and DE appeared loose, and was barely connected by the filopodia extending from both types of the cells (Fig. 2C). In contrast to this, however, the interface between dorsal epidermal cells remained tight (Fig. 2C, arrowheads).

Our observations confirmed previous reports that αPS3 is required for DC (Homsy et al., 2006; Stark et al., 1997). Furthermore, the sudden backward retraction of the DE after detachment from the pAS (Supplemental movie 5) suggested to us that the contractile forces provided by AS and DE (Kiehart et al., 2000) are present in $scb^{Vol\text{-}3}$ mutants. The active filopodial activities in LE cells that successfully sealed the dorsal holes further suggested that the wound closure activities in the DE remained intact (Fig. 4C). Viewed as a whole then, these observations suggest that the failure to maintain stable adhesion between pAS and DE is the earliest defect to occur in $scb^{Vol\text{-}3}$ mutants undergoing DC.

Amnioserosa responds to, and requires Dpp signaling during DC

In an attempt to further elucidate the cellular mechanisms controlling the tight adhesion between pAS and DE, we next studied the role of Dpp signaling. *tkv* mutants labeled with

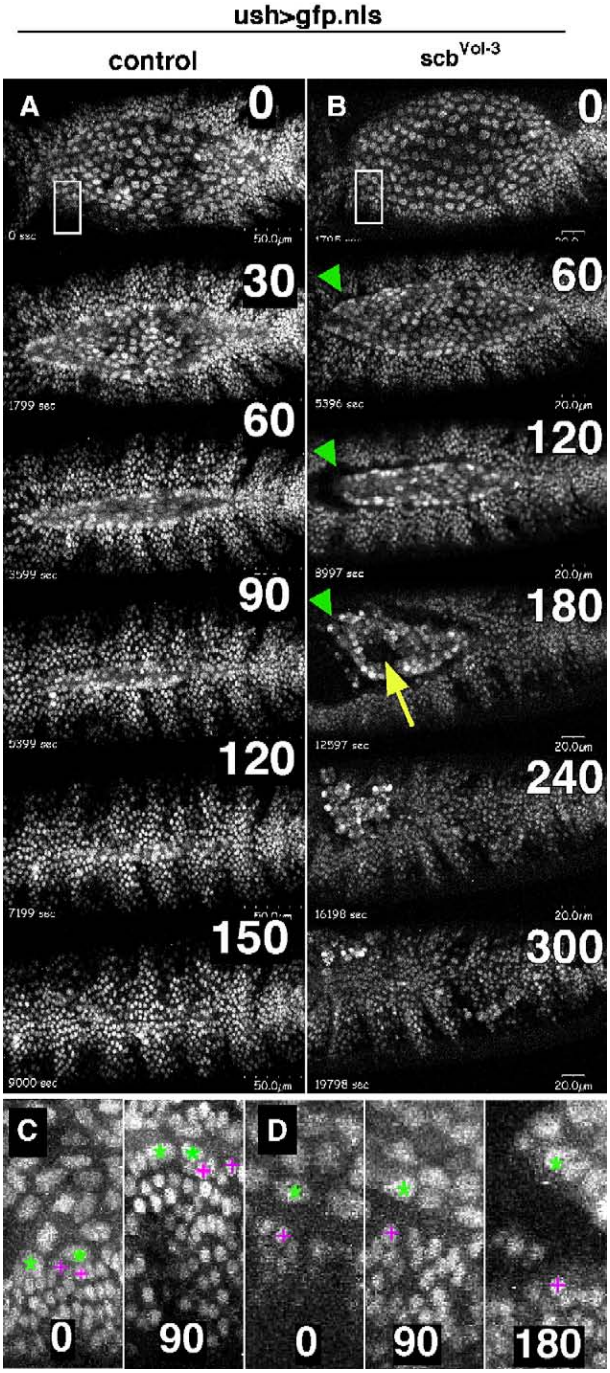

Fig. 5. Coordinated movement of amnioserosa and dorsal epidermis required αPS3 integrin. Time-lapse images of dorsal closure visualized by GFP.nls, driven by *ush-Gal4*. Numbers indicate the minutes after the onset of DC at stage 13. Anterior is to the left. (A, B) Dorsal surface views of a control (A) and $scb^{Vol\text{-}3}$ (B) embryos. Arrowheads indicate the breakpoint between the AS and DE showing where the AS and DE detached. Arrow indicates the collapsed amnioserosa cells. (C, D) Close-up view of the border between the pAS and DE taken from boxed regions in panels A and B. * and + indicate pAS and DE nuclei that stayed together in control embryos (C) but were separated in $scb^{Vol\text{-}3}$ embryo (D).

ush>gfp-moe marker mutants showed a delay in DC (Affolter et al., 1994) and discontinuity in F-actin in the LE (Fig. 4E). The mutants also exhibited rupture of the AS–DE interface (Fig. 4E, arrowhead), and similar defects were observed in *bsk* mutants (Fig. 4F, see also Homsy et al., 2006).

As *scb*, *tkv* and *bsk* mutants all failed to maintain pAS-DE adhesion, we next decided to study the behavior of pAS in these mutants. In *bsk* mutants, we found that F-actin cable formation in pAS was less prominent and the outer (ventral) edge of pAS was able to maintain a higher activity of filopodia (Fig. 6B′). The effect of the *bsk* mutation in pAS is likely to be indirect, however, as JNK signaling activity is inactive in the AS during the early to mid stage of DC (Martin-Blanco et al., 1998; Reed et al., 2004). We found that *dad*, a marker for the readout of Dpp signaling (Tsuneizumi et al., 1997), was broadly expressed in the AS, with an elevated level in the pAS (Figs. 6G–I), suggesting that pAS is responding to the Dpp secreted from the DE. In *tkv* mutants, we found that the level of pAS-Gal4 activity was reduced and the F-actin cable was absent in pAS (Fig. 6C), suggesting that pAS specification was incomplete. Our subsequent time-lapse recording of the remaining pAS revealed that the progression and removal of pAS was delayed (Fig. 6C). In the hope of addressing the cell autonomy of Dpp signaling in pAS, we overexpressed Dad to attenuate Dpp signaling and found that progression of pAS was arrested (Fig. 6D). In both *tkv* and *pAS>dad* embryos, the pAS became highly disorganized and its purse string F-actin was reduced and showed rapidly moving filopodia along the entire outer side (Figs. 6C′, D′). In control embryos, however, we were only able to observe such an active filopodial movement at the stage in which DE crawled up over the pAS (Fig. 1C).

This loss-of-function analysis of Dpp signaling suggests that Dpp is required to stabilize filopodia activity in pAS. This prompted us to then raise the level of Dpp signaling by expressing a constitutively active form of *tkv* (Fig. 6E). We found that DC progressed normally with a smooth outer edge of pAS and with internal AS cells being removed over the normal time course. Following completion of DC, however, pAS remained associated with DE, which subsequently delayed entry into apoptosis. Seen in this light, then we think that sustained Dpp signaling slows down the detachment of pAS and its subsequent apoptosis (Fig. 6E). As the pAS in these embryos maintained a smooth outer edge (Fig. 6E′), we inferred that the association of pAS with its neighboring cells was hyperstabilized. Finally, our observations revealed that pAS of $scb^{Vol\text{-}3}$ mutants exhibited a similar delay in apoptosis and sustained hyperactivity, with a significant reduction of F-actin purse strings (Fig. 6F). These results suggested that the precise control of Dpp signaling is essential for motility and cell adhesiveness in pAS.

Discussion

The amnioserosa of the *Drosophila* embryo plays a number of key roles in the dorsal closure process by generating a contractile force for DC. The tight connection between the pAS and DE is essential for transmitting the force to the entire embryonic epidermis. In this study, we have shown that in addition to E-cadherin-GFP being specifically down-regulated at the interface between pAS and DE during DC (Fig. 1A),

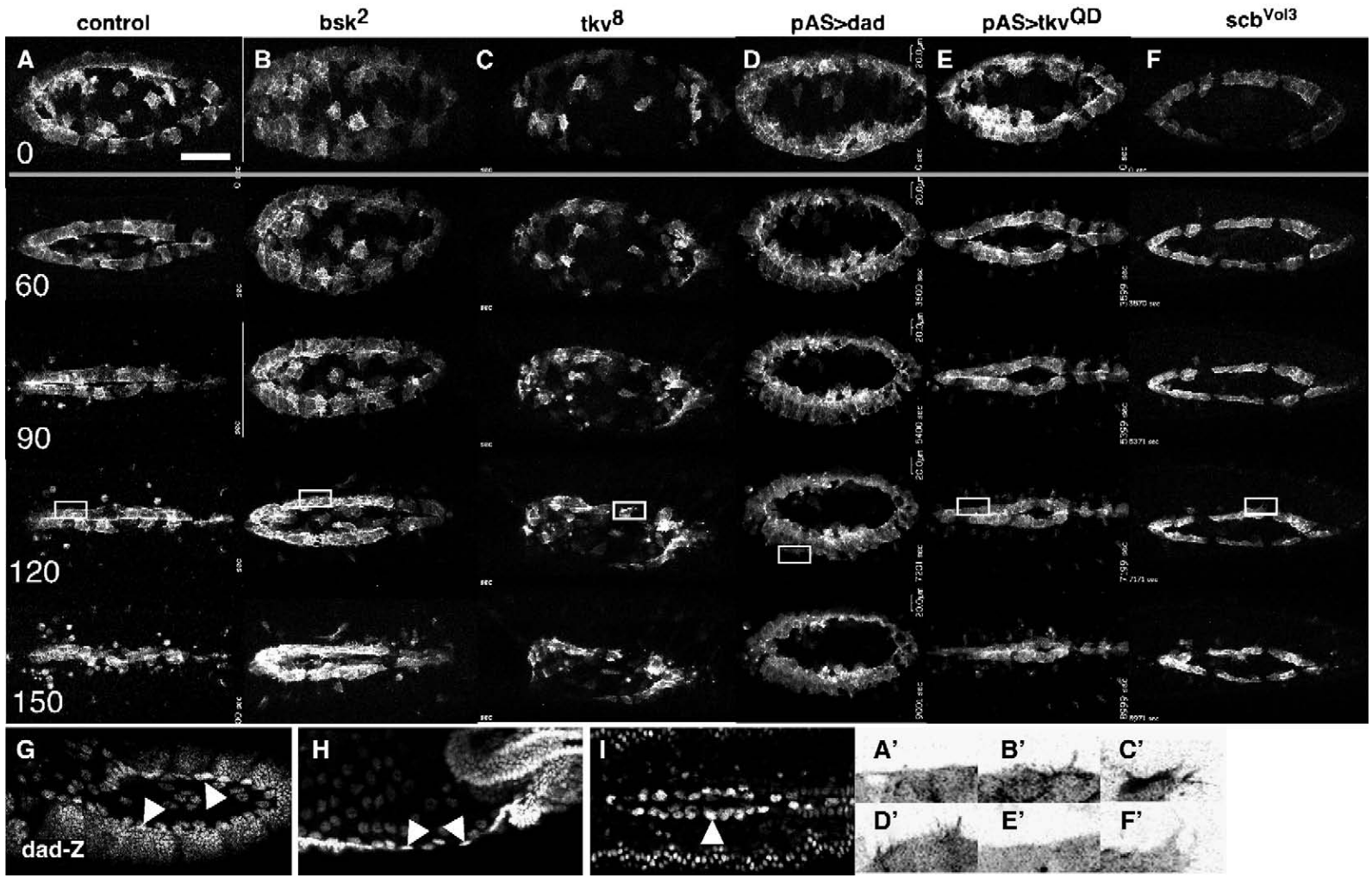

Fig. 6. The roles of Dpp, JNK, and integrin on the adhesion between peripheral amnioserosa and dorsal epidermis. (A–F) Time-lapse confocal images of dorsal closure in embryos visualized by the GFP-moesin reporter expressed by *pAS-Gal4*. Control and mutants deficient in JNK or Dpp-signaling, or αPS3 integrin activity were examined (genotype indicated at the top). Dorsal view. Anterior is to the left. Original movie files are provided as Supplemental movies 9–14. The onset of DC (stage 13) was set as 0 min and elapsed time was indicated in panel A. Boxed areas in panels A–F are enlarged in panels A′–F′. Note delayed time course of DC (B–D) and sustained activity of filopodia in bsk^2, tkv^8, and *pAS>dad* embryos (B′–D′). Sustained activation of Dpp signaling by Tkv^{Q253D} delayed the detachment of pAS from DE (E). In scb^{Vol-3} embryos, DC was delayed and the outer edge of pAS was rough (F, F′). (G–I) Expression of *dad-lacZ* in stage 9 (G), late stage 12 (H), and stage 15 (I) embryos. *dad-lacZ* was expressed in the DE and AS, and was especially high in the pAS (arrowheads). Scale bar, 50 μm (A).

adherens junctions were not obvious in our transmission electron microscope analysis (Fig. 2), suggesting that E-cadherin-mediated cell–cell adhesion does not mature into a solid junctional structure in this region. Instead, we observed that the pAS and DE attach with a large basolateral contact area. Here we show that αPS3 is required to maintain the adhesion of this interface. Since an integrin ligand, α1-laminin, accumulates in this region (Narasimha and Brown, 2004), we take this to infer that the laminin-containing ECM holds the amnioserosa and dorsal epidermis together via integrin-mediated cell adhesion.

We were also able to show that intense F-actin cables were formed in pAS in the place precisely apposing the dense F-actin cables in the leading edge of DE, suggesting that pAS may contribute to the contraction of purse string-like F-actin cables in the leading edge. Roughly corresponding to the positions of F-actin cables, we found electron-dense structures beneath the plasma membranes using an electron microscope. As the formation of supracellular F-actin cables in pAS and DE depends on αPS3 function, the integrin complex seems to serve as a platform for organizing an F-actin network in both pAS and DE, thereby promoting cell–cell adhesion and contraction of the leading edge.

αPS3 is expressed in a subset of βPS-expressing cells. A previous study has indicated that the only other β subunit, β*ν*, is apparently not required for DC (Devenport and Brown, 2004), suggesting that it is likely that an αPS3βPS heterodimer, together with αPS1βPS (Homsy et al., 2006) are the major integrins supporting the adhesion of pAS to DE. We suggest that the special morphological properties of the interface between AS and DE, as a transition point between squamous and columnar epithelium, make it vulnerable to the physical tension applied by the AS when integrin activity is reduced.

We further found that pAS responds to Dpp emanating from the DE, and performs a specialized function in maintaining the integrity of the AS and DE and to drive DC. This study also showed that F-actin cable formation and down-regulation of filopodial activity in pAS were dependent on the cell autonomous activity of Dpp signaling, and JNK, an upstream regulator of Dpp expression in the DE. Dpp may act as a downstream, non-cell autonomous mediator of JNK signaling in regulating cytoskeletal dynamics in the pAS. Furthermore, Homsy et al. (2006) recently reported that αPS1 and αPS3 expression can be activated by JNK signaling; this JNK activity would help to enforce the adhesion between pAS and DE. Although further details remain obscure, these observations suggest a model in which Dpp from the DE induces reactions in the AS that support contraction and re-enforce its adhesion to the DE, with the result that the contractile force is transmitted back to the DE to promote DC. This signaling circuit should be able to persist until the leading edges of the DE establish direct contact with each other, when Dpp expression in DE is terminated and the AS has been completely removed from the DE.

In lower insects the extraembryonic epithelia can be separated into two regions, the serosa and amnion; the latter making direct contact with the embryo proper (Zeh et al., 1989). van der Zee et al. (2005) recently reported that, in the embryo of the beetle Tribolium, the serosa can be removed completely by RNAi-mediated knockdown of *zen1*. Such embryos nevertheless complete dorsal closure, suggesting that the serosa is dispensable for DC. This observation strongly supports the idea that the amnion plays a major role in driving DC. In this study, we have been able to show that the pAS of the *Drosophila* embryo connects the embryonic epidermis to the rest of the extraembryonic membranes and plays a major role in driving DC. Such similarities suggest that the pAS is the *Drosophila* equivalent of the amnion.

An analogous population of cells linking the embryonic body wall to the extraembryonic tissue can also be found in vertebrates, such as the yolk syncytial layer in zebrafish, the yolk stalk of the chick, and the mammalian umbilical cord. We believe that further investigation into the mechanism of insect dorsal closure will hopefully shed new light on the evolutionarily conserved roles of the linker cells that connect the embryonic body wall and the extraembryonic tissues.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.09.020.

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