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The leading edge during dorsal closure as a model for epithelial plasticity: Pak is required for recruitment of the Scribble complex and septate junction formation

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SUMMARY

Dorsal closure (DC) of the *Drosophila* embryo is a model for the study of wound healing and developmental epithelial fusions, and involves the sealing of a hole in the epidermis through the migration of the epidermal flanks over the tissue occupying the hole, the amnioserosa. During DC, the cells at the edge of the migrating epidermis extend Rac- and Cdc42-dependent actin-based lamellipodia and filopodia from their leading edge (LE), which exhibits a breakdown in apicobasal polarity as adhesions are severed with the neighbouring amnioserosa cells. Studies using mammalian cells have demonstrated that Scribble (Scrib), an important determinant of apicobasal polarity that functions in a protein complex, controls polarized cell migration through recruitment of Rac, Cdc42 and the serine/threonine kinase Pak, an effector for Rac and Cdc42, to the LE. We have used DC and the follicular epithelium to study the relationship between Pak and the Scrib complex at epithelial membranes undergoing changes in apicobasal polarity and adhesion during development. We propose that, during DC, the LE membrane undergoes an epithelial-to-mesenchymal-like transition to initiate epithelial sheet migration, followed by a mesenchymal-to-epithelial-like transition as the epithelial sheets meet up and restore cell-cell adhesion. This latter event requires integrin-localized Pak, which recruits the Scrib complex in septate junction formation. We conclude that there are bidirectional interactions between Pak and the Scrib complex modulating epithelial plasticity. Scrib can recruit Pak to the LE for polarized cell migration but, as migratory cells meet up, Pak can recruit the Scrib complex to restore apicobasal polarity and cell-cell adhesion.

KEY WORDS: *Drosophila*, Pak, Scribble, Apicobasal polarity, Dorsal closure, Epithelial morphogenesis

INTRODUCTION

Epithelial cells are typically polarized in the apicobasal axis, with this polarity including a particular organization of cell-cell junctions characterized by a belt of adherens junctions known as the zonula adherens (ZA), and, in vertebrates, tight junctions apical to the ZA and, in *Drosophila*, septate junctions basal to the ZA (Gibson and Perrimon, 2003). The Scribble (Scrib) complex, comprising Scrib, Discs large (Dlg) and Lethal giant larvae (Lgl), is involved in establishing this pattern of junctions, and, in *Drosophila*, becomes incorporated into the septate junction during epithelial development. The morphogenesis of epithelia involves 'epithelial plasticity', in which cell-cell adhesions are disassembled and cells become motile, for example, in epithelial-mesenchymal transition (EMT) (Thiery and Sleeman, 2006).

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Recent mammalian cell culture studies indicate that Scrib is involved in setting up the leading edge membrane (LE) during cell migration. Scrib localizes to the LE where it recruits Cdc42 and Rac, key regulators of the actin-based membrane extensions driving cell motility (Dow et al., 2007; Osmani et al., 2006). Cdc42 is activated by recruitment of the Cdc42/Rac guanine-nucleotide exchange factor (GEF) βPIX to the LE by Scrib, which binds βPIX directly (Osmani et al., 2006). Furthermore, Scrib and βPIX recruit Dlg1 and Pak, a βPIX-binding Cdc42/Rac effector kinase, to the LE (Nola et al., 2008; Osmani et al., 2006). The Scrib complex contributes to epithelial fusion events during both development and in wound healing (Humbert et al., 2006). Mice mutant for *scrib* have defects in embryonic epithelial fusions and wound healing, and Scrib is required for cell migration in an in vitro wound healing assay (Dow et al., 2007; Murdoch et al., 2003; Zarbalis et al., 2004). Dorsal closure (DC) of the *Drosophila* embryo is used in the study of developmental epithelial fusions and wound healing. During the closure of epithelia, the first row of cells at the edge of the advancing epithelial sheets acquires specific characteristics, and DC enables the genetic analysis of events in these cells, referred to as the dorsal-most epithelial cells (DME) in the case of DC (Harden, 2002; Kaltschmidt et al., 2002). In DC, a hole in the dorsal epidermis is sealed by the migration of the epidermal flanks up over the amnioserosa, the tissue occupying the hole. Of particular interest in DC is the behaviour of the LE, the side of the DME cells facing the amnioserosa. Whereas the other sides of the DME cells show no obvious changes during DC, the LE exhibits polymerization of actin in the form of lamellipodia, filopodia and a contractile purse-string. Rac, Cdc42 and Pak all participate in

DC, as do Scrib, Dlg and Lgl, and DC presents an opportunity for an in vivo analysis of interactions between the Scrib complex and Cdc42/Rac signaling complexes at the LE during cell migration (Arquier et al., 2001; Bilder et al., 2000; Harden et al., 1995; Harden et al., 1999; Jacinto et al., 2000; Manfruelli et al., 1996; Woods et al., 1996; Woolner et al., 2005; Zeitler et al., 2004).

We demonstrate here that in *Drosophila*, as in mammals, Pak associates with and is localized by the Scrib complex. More importantly, we show that the relationship between Pak and the Scrib complex can act in the opposite direction at the end of epithelial cell migration, i.e. Pak at the LE is used to recruit the Scrib complex. During DC, the Scrib complex is lost from the LE and is restored in a Pak-dependent manner as opposing DME cells meet up. We provide evidence that the absence of the Scrib complex and associated septate junctions at the LE during DC allows the accumulation of lateral integrins, which are used to anchor Pak at the LE where it can recruit the Scrib complex at the end of DC. Thus, the absence of septate junctions allows the recruitment of proteins needed for the assembly of septate junctions. We propose that the bidirectional relationship between Pak and the Scrib complex contributes to the epithelial membrane being able to toggle back and forth between migratory and adhered states, and might therefore contribute to EMT and mesenchymal-epithelial transitions (MET).

MATERIALS AND METHODS

Fly stocks

Unless otherwise stated, all crosses were raised at 25°C. w^{1118} was used as a wild-type control strain. *pak3* mutations were generated by imprecise excisions of the P element, *EP(3)1191*. *UAS-pak*, pak^{22} and *UAS-*pak^{myr} were from H. Hing (State University of New York Brockport, USA); pak^{14} from B. Dickson (Institute of Molecular Pathology, Austria); *UAS-lgl* from J. Knoblich (Institute of Molecular Biotechnology, Austria); *tj-Gal4* from G. Tanentzapf (University of British Columbia, Canada); $scrib^{4}$, $scrib^{673}$, *UAS-scrib-GFP* from D. Bilder (University of California-Berkley, USA); $cora^{k08713}$ from V. Auld (University of British Columbia, Canada); $dpix^{p1036}$ from P. Haghighi (McGill University, Canada); *UAS-dlg-GFP* from U. Thomas (Leibniz Institute for Neurobiology, Germany) and mys^{G1} and mys^{XG43} from the late D. Brower. *UAS-pak-GFP* was previously described (Rasse et al., 2005) and *UAS-pak3-GFP* was made by fusing GFP to the C-terminal end of the 64 kD Pak3 isoform encoded by cDNA *RE01659*. All other fly strains were obtained from the Bloomington *Drosophila* Stock Center. Mutants were distinguished by lack of GFP balancer chromosome. For the rescue experiment, a double-balanced stock was created bearing a second-chromosome *hs-Gal4* driver and the $pak^{14}pak3^{76A}$ chromosome. In the control cross, siblings from this stock were mated to each other, for the rescue cross males from this stock were mated to females bearing the $pak^{14}pak3^{76A}$ chromosome and homozygous for a second-chromosome *UAS-pak* transgene. Crosses were done at 29°C to allow moderate, ubiquitous expression of *hs-Gal4*, and embryonic cuticle preparations were used to quantify phenotypes in the progeny.

Cuticle preparations, immunohistochemistry and live imaging

Embryonic cuticle preparations and fixing and immunostaining of embryos and egg chambers have been described (Harden et al., 1996; Verheyen and Cooley, 1994). In experiments in which phenotypes were quantified by cuticle preparations, at least 400 embryos were examined. For immunostainings, the following reagents were used: rabbit anti-Pak (Harden et al., 1996) (1:2000), mouse anti-Pak3 (1:1000), rabbit anti-GFP (Abcam; 1:500), mouse anti-Crb (Tepass and Knust, 1990) (1:25), mouse anti-Fasciclin III (Patel et al., 1987) (1:100), mouse anti-Dlg (Parnas et al., 2001) (1:5), rat anti-DE cadherin (Oda et al., 1994) (1:50), guinea pig anti-Scribble (Zeitler et al., 2004) (1:500), rabbit anti-Lgl (Betschinger et al., 2003) (1:100), mouse anti-βPS-integrin (Brower et al., 1984) (1:2), rabbit anti-phosphotyrosine (Santa Cruz; 1:200), guinea pig anti-Coracle (Fehon et al., 1994) (1:2000) and FITC-phalloidin (Sigma). All secondary antibodies were from Vector Laboratories and used at a 1:200 dilution. Images were acquired with either a Zeiss LSM 410 or Zeiss LSM 510 laser-scanning confocal microscope and processed in Adobe Photoshop. Live imaging was done as described (Reed et al., 2004) using a Quorum spinning disk confocal microscope.

Clonal analysis in egg chambers

Homozygous mutant clones were generated with the FRT/FLP system using *Ubi-GFP FRT* chromosomes as markers for wild-type cells (Xu and Harrison, 1994). Clones were induced by a heat-shock promoter-driven FLP (*hsFLP*) (Xu and Harrison, 1994). Third instar larvae were heat-shocked for 2 hours at 37°C for 3 consecutive days. After eclosion, females were aged for 3-5 days on yeasted medium prior to dissection of the ovaries. Mutant clones were identified by lack of staining with an anti-GFP antibody.

Co-immunoprecipitations

UAS-pak-GFP, *UAS-scrib-GFP*, *UAS-pak3-GFP* or *UAS-lgl* flies were mated to *hs-Gal4* flies and progeny were grown at 29°C to induce moderate, ubiquitous transgene expression. For DE-cadherin immunoprecipitations, wild-type flies were used. Approximately 60 flies were homogenized in 800 μl of IP Buffer I [475 mM Tris HCl pH 8.0, 0.5% Triton X-100, 1 complete protease inhibitor tablet per 50 ml (Roche)]. The sample was then centrifuged for 10 minutes at 4°C. The supernatant was removed from the debris and 50 μl kept for the lysate lane on the gel. The rest of the lysate was split between the immunoprecipitation and the control (incubated with beads but no antibody). For immunoprecipitation, lysate was incubated with rotation at 4°C overnight with the antibody of choice and 25 μl of either a slurry of Protein A or Protein G beads (Santa Cruz). The beads were then centrifuged briefly, supernatants removed and beads washed 3 times with IP Buffer II (50% 1 M NaCl, 50% IP Buffer I). These beads were then resuspended in SDS-PAGE sample buffer, boiled for 10 minutes, run out on an SDS-PAGE gel together with lysate sample and western blotted with the antibody of interest. Western blot signals were detected by the BM chemiluminescence system (Roche). Antibodies used in co-immunoprecipitation experiments were as described above, and in addition, mouse anti-Armadillo (Peifer et al., 1994) was used.

Preparation of Pak3 antibody

Anti-Pak3 antibodies were raised in mice using a GST-Pak3 fusion protein as antigen. The GST-Pak3 fusion construct was generated by PCR amplification of *pak3* sequences encoding the N-terminal 300 amino acids and cloning into the pGEX vector.

RESULTS

Pak is a component of the Scrib complex in *Drosophila*

To determine if *Drosophila* Pak is a component of the Scrib complex in epithelia, we looked for co-localization of these proteins in the embryonic epidermis. Pak showed co-localization at the lateral membrane with the Scrib complex marker Dlg and the septate junction protein Fasciclin III (FasIII) (Fig. 1A-B″). To test for Pak association with the Scrib complex proteins, we ubiquitously expressed a *pak-GFP* transgene in flies and immunoprecipitated Pak using either anti-Pak or anti-GFP antibodies. We found that Scrib and Lgl, but not Dlg, co-immunoprecipitated with Pak-GFP (Fig. 1C,E; data not shown). Although Scrib exists as multiple isoforms (Bilder and Perrimon, 2000; Ganguly et al., 2003; Li et al., 2001), which ran as a smear in our lysates, both anti-Pak and anti-GFP antibodies co-immunoprecipitated a single anti-Scrib immunoreactive band of about 185 kD, suggesting that Pak specifically interacts with the predicted 184.7 kD isoform (Fig. 1C; data not shown). In a reciprocal experiment, we expressed biologically active Scrib-GFP (Zeitler et al., 2004) and immunoprecipitated it with anti-GFP, but

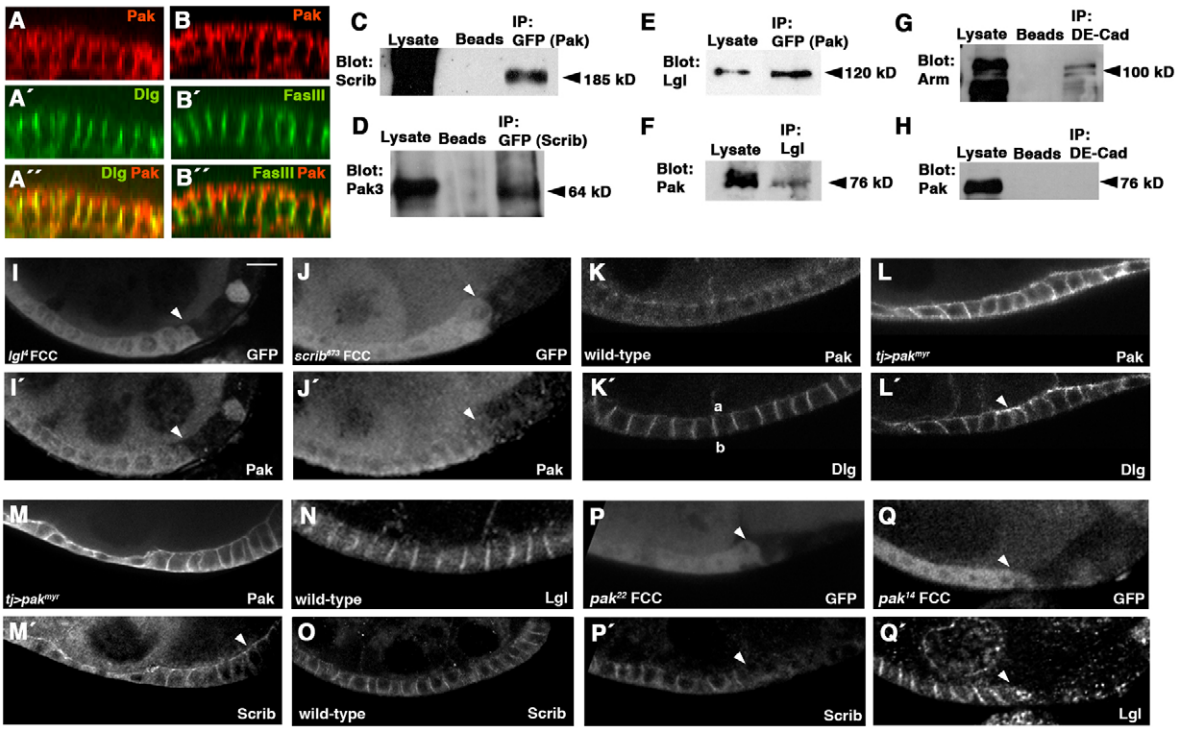

Fig. 1. Pak is a component of the Scrib complex. (**A-B″**) Views of the apicobasal axis of rows of DME cells, viewed ventral to the leading edge membrane (LE), double-stained with anti-Pak and anti-Dlg (A-A″) or anti-FasIII (B-B″). Pak shows both a cytoplasmic and cortical distribution, with cortical Pak colocalizing with Dlg/FasIII at the lateral membrane. Embryos were optically sectioned along the anteroposterior axis. (**C**) The 184.7 kD Scrib isoform co-immunoprecipitates with Pak-GFP immunoprecipitated from whole flies with anti-GFP antibodies. The smear in the lysate lane is probably due to detection of multiple isoforms of Scrib. (**D**) The 64 kD Pak3 isoform co-immunoprecipitates with Scrib-GFP immunoprecipitated from whole flies with anti-GFP. (**E**) Lgl co-immunoprecipitates with Pak-GFP immunoprecipitated from whole flies with anti-GFP. (**F**) Pak co-immunoprecipitates with Lgl immunoprecipitated from whole flies with anti-Lgl. (**G**,**H**) Pak does not co-immunoprecipate with DE-cadherin under conditions where Arm is pulled down. (G) DE-Cad immunoprecipitation stained with anti-Arm. (H) Same blot as in G stained with anti-Pak. (**I-Q′**) Views of follicular epithelium in contact with nurse cells in developing egg chambers. (I-J′) Egg chambers bearing *lgl* (I,I′) and *scrib* (J,J′) mutant follicle cell clones showing that Pak localization is dependent on these Scrib complex members. Clones are distinguished by lack of GFP staining (arrowheads in these and subsequent panels mark clone boundaries). (K,K′) Wild-type chamber showing Pak distribution (K) and localization of Dlg (K′) to lateral membranes of follicle cells. a, apical; b, basal. (L-M′) pak^{myr}-expressing egg chambers showing heavy accumulation of Pak^{myr} at follicle cell membranes (L,M) and ectopic Dlg (L′) and Scrib (M′) accumulation at the apical membrane (arrowheads). (N,O) Wild-type egg chambers showing localization of Lgl (N) and Scrib (O) to lateral membranes of follicle cells. (P-Q′) Egg chambers bearing *pak* mutant follicle cell clones showing that Scrib (P′) and Lgl (Q′) localization is dependent on Pak. Scale bars: 10 μm in I-Q′.

this did not co-immunoprecipitate Pak. This is not surprising, as the Scrib-GFP transgene expresses a 190.2 kD isoform of Scrib not detected in the Pak immunoprecipitates. However, we found that Pak3, a second Pak family member in *Drosophila* (see below), was pulled down with Scrib-GFP (Fig. 1D). Additionally, we found that Pak was pulled down with overexpressed Lgl (Fig. 1F).

We previously showed that Pak is required for apicobasal polarity in the follicular epithelium during oogenesis and we choose this tissue to address further the relationship between Pak and the Scrib complex (Conder et al., 2007). The Scrib complex proteins in *Drosophila* are codependent for their proper subcellular localization (Bilder et al., 2000). Consistent with Pak being a Scrib complex component, Pak localization was disrupted in *scrib* and *lgl* clones (Fig. 1I-J′). To determine if Pak could regulate membrane localization of the Scrib complex, we expressed a version of Pak constitutively localized to the membrane by myristoylation (Pak^{myr}) (Hing et al., 1999) in the follicular epithelium and looked at the distribution of Dlg and Scrib. Normally the Scrib complex proteins are restricted to the lateral membrane (Fig. 1K′,N,O), but they additionally accumulated along the apical membrane in Pak^{myr}-expressing cells (Fig. 1L′,M′). We previously demonstrated that membrane localization of Dlg was disrupted in *pak* clones in the follicular epithelium (Conder et al., 2007); this was also the case for Scrib and Lgl (Fig. 1P-Q′). Taken together, our results indicate that Pak is a component of the Scrib complex in epithelia and shows a mutual dependence with Scrib, Dlg and Lgl for membrane localization.

The LE membrane of the DME cells undergoes alterations similar to EMT and MET during DC

We are particularly interested in characterizing the relationship between Pak and the Scrib complex in migratory epithelial cells. The DME cells undergo changes during DC that are similar to EMT in that they become planar-polarized with regard to the actin cytoskeleton and exhibit actin-based protrusions at the LE used for cell motility (see Fig. S1A in the supplementary material) (Hay, 2005). Previous studies have shown that Dlg is lost from the LE during DC, while adherens junctions at the LE become restricted to nodes joining DME cells (Arquier et al., 2001; Fehon et al., 1994; Gorfinkiel and Arias, 2007; Grevengoed et al., 2001; Jacinto

et al., 2000; Kaltschmidt et al., 2002; Martinez-Arias, 1993; Wada et al., 2007). One study reported some retention of Dlg and Scrib at the LE (Gorfinkiel and Arias, 2007), but we found that Dlg, Scrib and Lgl were all absent from the LE by mid-DC, giving DME cells an open-ended appearance (see Fig. S1D-F in the supplementary material). Live imaging of Dlg-GFP (Koh et al., 1999) confirmed a lack of Dlg at the LE (see Fig. S1G and Movie 1 in the supplementary material). The Scrib complex proteins are present at the LE prior to commencement of closure (see Fig. S1C in the supplementary material) and the LE Dlg and Scrib reported in the earlier study could be the Scrib complex prior to LE downregulation and/or Scrib complex proteins on adjacent amnioserosa cell membranes. The Scrib complex becomes incorporated into the septate junction, which is also absent from the LE, as demonstrated by a lack of septate junction proteins such as FasIII, Coracle (Cora) and Neurexin IV (Fehon et al., 1994; Gorfinkiel and Arias, 2007; Manfruelli et al., 1996; Martinez-Arias, 1993). As an additional evaluation of the status of apicobasal polarity at the LE during DC, we looked at the distribution of Crumbs (Crb), a member of another complex regulating apicobasal polarity (Gibson and Perrimon, 2003), and found it lacking at the LE (see Fig. S1H in the supplementary material). Normally in EMT, epithelial cells lose apicobasal polarity and cell adhesion at all sides and exit the epithelium (Hay, 2005). In the DME cells, apicobasal polarity and adhesion are lost at only one side, the LE, and these cells remain part of the epidermis. The loss of apicobasal polarity at the LE that occurs during DC appears to be linked to planar polarization of the DME cells, as mutant embryos that fail to assemble the LE cytoskeleton have been reported to retain Dlg and/or FasIII at the LE during DC, indicating at least partial retention of apicobasal polarity (Arquier et al., 2001; Harden et al., 1995; Lin et al., 2007; Lu and Settleman, 1999; Magie et al., 1999; Manfruelli et al., 1996). The junctional changes at the LE are necessary, as at the end of DC the LEs from the migrating epidermal flanks must break any adhesion with the amnioserosa and adhere to each other. Contributing to breaking links between the LE and the amnioserosa is a lack of septate junction proteins in the amnioserosa, which never forms septate junctions (Narasimha et al., 2008; Tepass and Hartenstein, 1994). At the completion of DC, junctions are restored at the dorsal midline where the epidermal flanks meet up, an event with parallels to MET (Jacinto et al., 2000; Kaltschmidt et al., 2002; Martinez-Arias, 1993).

Pak is required for restoration of the Scrib complex and septate junction formation at the LE at the end of DC

Our previous studies with anti-Pak stainings of fixed embryos indicated that there are high levels of Pak at the LE and dorsal end of the DME cells, prior to restoration of the Scrib complex (Conder et al., 2004; Harden et al., 1996). We visualized the dynamic behaviour of LE Pak by live imaging embryos expressing Pak-GFP. Despite the high levels of cytoplasmic Pak-GFP, we were able to visualize the accumulation of Pak-GFP in puncta at the LE during DC, which persisted until shortly after the completion of DC (Fig. 2A-D; see also Movie 2 in the supplementary material). Given the requirement for Pak for membrane localization of the Scrib complex in the follicular epithelium, which is derived from mesenchymal cells (Conder et al., 2007; Tanentzapf et al., 2000), we wondered if Pak has a role in restoring the Scrib complex at the LE at the end of DC, i.e. essentially a reversal of the establishment of the LE seen in polarized migratory mammalian cells (Nola et al., 2008). Embryos maternally and zygotically mutant for *pak* fail to complete DC, precluding an evaluation of Pak function at the end of DC, whereas *pak* zygotic mutants survive embryogenesis (Conder et al., 2004; Hing et al., 1999). The ability of zygotic *pak* mutants to survive embryogenesis might be owing to redundancy with the other Group I Pak, Pak3 (Dan et al., 2001; Mentzel and Raabe, 2005; Morrison et al., 2000; Pirone et al., 2001). Two different *pak3* transcripts are produced through alternate splicing, encoding predicted 64 kD Pak3-A and 46 kD Pak3-B proteins, with Pak3-B missing sequences between the Cdc42/Rac-binding (CRIB) or auto-inhibitory domain (AID) and the kinase domain (see Fig. S2B,C in the supplementary material). Through excision of a P element, *EP(3)1191*, inserted 67 bp downstream of the start point

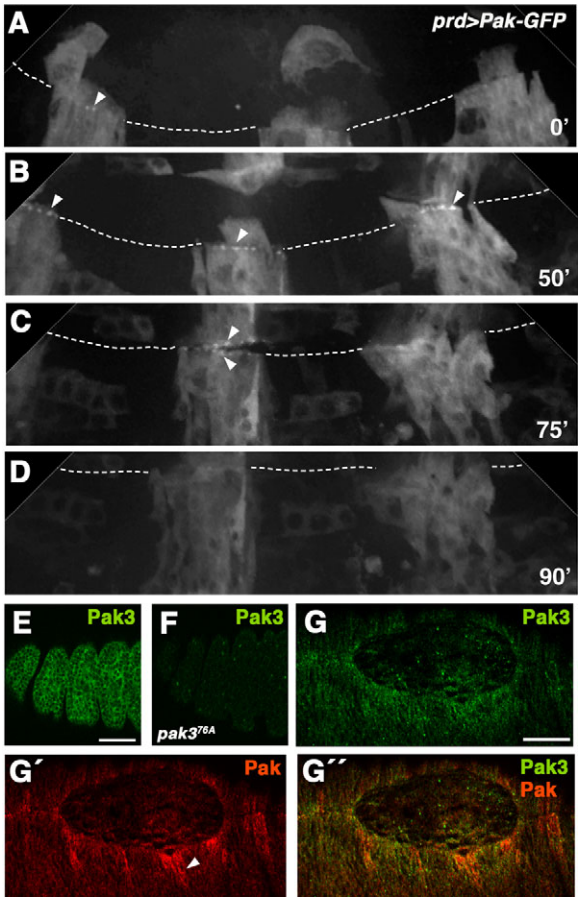

Fig. 2. Distribution of Pak proteins during embryogenesis. (**A-D**) Details from live imaging of Pak-GFP distribution in *prd* stripes at the LE. Cells expressing Pak-GFP ahead of the LE are due to *prd-Gal4* expression in the amnioserosa. As DC proceeds, Pak accumulates in puncta at the LE (arrowheads in A-C), which are lost after the completion of DC (D). In C, the epidermal flanks have just met at the dorsal midline. Dashed line denotes position of LE. See Movie 2 in the supplementary material. (**E**) Ventrolateral view of a stage-13 $pak3^{76A}$ heterozygous embryo showing Pak3 staining throughout the epidermis. (**F**) Ventrolateral view of a stage-13 $pak3^{76A}$ homozygous mutant embryo from the same collection and staining as that in E, showing lack of Pak3 staining. We conclude that $pak3^{76A}$ is probably null and that our anti-Pak3 antibody does not cross-react with Pak and is specific for Pak3. (**G-G″**). Dorsolateral view of a wild-type embryo midway through DC, double-stained for Pak3 (G) and Pak (G′), showing that both proteins are enriched in the dorsal epidermis around the dorsal hole but only Pak is elevated in segment border cells (arrowhead in G′). Scale bars: 50 μm in E,F; 25 μm in G-G″.

of transcription, we created two small deletions, *pak3*^{76A} and *pak3*^{27A}, removing the first 148 codons and 325 codons of Pak3-A, respectively (see Fig. S2C in the supplementary material). We raised a mouse polyclonal antibody against Pak3 that detected two bands on total protein lysates from embryos, with the larger, stronger band consistent with the size of Pak3-A (see Fig. S2A in the supplementary material). The faint, smaller band was possibly Pak3-B but was migrating below the predicted size. Pak3 showed an embryonic distribution somewhat distinct from Pak, although both were present in the dorsal epidermis (Fig. 2G-G″; data not shown).

We examined *pak3* mutant embryos and embryos mutant for both *pak3* and *pak* for defects in morphogenesis using embryonic cuticle preparations. Double mutants were made using the *pak*^{14} allele, which encodes a truncated Pak protein with no kinase domain (Newsome et al., 2000). The same phenotypes were seen in *pak3* mutant embryos, embryos homozygous mutant for one Group I *pak* gene and heterozygous for the other, or embryos homozygous for the *pak*^{14}*pak3*^{76A} and *pak*^{14}*pak3*^{27A} chromosomes, which included head holes and defects in the dorsal surface ranging from germband retraction failures to dorsal holes and puckers (Fig. 3B-D). The highest frequency of cuticle defects was seen in *pak*^{14}*pak3*^{76A} and *pak*^{14}*pak3*^{27A} embryos, indicating that the two Group I Paks cooperate during embryogenesis. In a count of 1061 embryos from the *pak*^{14}*pak3*^{76A} stock, 8.8% of embryos (an estimated 35.2% of *pak*^{14}*pak3*^{76A} homozygotes) exhibited both a germband retraction failure and a round dorsal hole, 7.4% of embryos (an estimated 29.6% of *pak*^{14}*pak3*^{76A} homozygotes) a dorsal pucker, and 1% of embryos (an estimated 4% of *pak*^{14}*pak3*^{76A} homozygotes) a large rectangular hole in the dorsal cuticle. Nearly all *pak*^{14}*pak3*^{76A} cuticles had head holes, suggesting a defect in head involution. Expression of a *UAS-pak* transgene at 29°C using an *hs-Gal4* driver efficiently suppressed defects in the dorsal surface of *pak*^{14}*pak3*^{76A} mutant embryos, but was less effective at rescuing head holes (data not shown).

To characterize DC defects further, we stained *pak*^{14}*pak3*^{76A} embryos with anti-Dlg antibody to evaluate the status of the Scrib complex. We chose not to focus on studying embryos with germband retraction failures and pronounced DC defects, but rather embryos that had a subtler defect probably corresponding to the puckered cuticle phenotype. In these embryos, the epidermal flanks had come together at the dorsal midline but, unlike wild-type embryos, they failed to restore Dlg at the LE (Fig. 3H) (Kaltschmidt et al., 2002). At first glance it appeared that the epidermal flanks had stopped just short of meeting up in *pak*^{14}*pak3*^{76A} embryos, leaving a dorsal cleft, but several results indicated that DC had proceeded to completion. Most notably, the dorsal vessel in such embryos had developed normally (data not shown). Rows of cardioblasts and pericardial cells migrate dorsally with the epidermis during DC and come together at the dorsal midline at the end of DC to create the dorsal vessel (Rugendorff et al., 1994). Secondly, if a cleft were left in these embryos, one would expect a dorsal hole in the cuticle. The vast majority of dorsal holes in cuticle preparations from the *pak*^{14}*pak3*^{76A} stock were accompanied by severe germband reaction defects that we didn't see in stained embryos failing to restore Dlg at the dorsal midline. The opposing LEs come together in a zigzag pattern at the end of DC, and this at least partly explains why the failure to restore LE Dlg gave a cleft-like appearance to the dorsal midline in *pak*^{14}*pak3*^{76A} embryos (Fig. 3S,T). Furthermore, we found more mildly affected embryos in which LE Dlg had been restored in some DME cells on one side of the midline but not the other; as discussed below, anti-DE-cadherin (DE-Cad) staining of such embryos demonstrated that the opposing LEs had indeed joined up (Fig. 3R-R″).

Occasionally, we found *pak*^{14}*pak3*^{76A} embryos in which it appeared that the epidermal flanks had come apart after successful DC, again these were lacking LE Dlg but had a normal dorsal vessel (Fig. 3O,O′). We suspect that these embryos correspond to the rare cuticle phenotype of a rectangular dorsal hole (Fig. 3D). We found that the entire Scrib complex was missing from the LE at the end of closure in *pak*^{14}*pak3*^{76A} embryos by staining for Lgl and Scrib. Similar to Dlg, these proteins failed to reappear at the LE after the epidermal flanks had closed up (Fig. 3I,J). As a complement to studies on fixed embryos, we followed the restoration of the Scrib complex at the LE in live embryos expressing Dlg-GFP. In wild-type embryos, Dlg-GFP appeared at the dorsal midline shortly after opposing DME cells made contact, whereas we identified *pak*^{14}*pak3*^{76A} embryos that failed to accumulate significant amounts of Dlg-GFP at the dorsal midline (Fig. 3K-N; see Movies 1 and 3 in the supplementary material). The lack of Scrib complex proteins at the LE indicated that the septate junction was not being restored in *pak*^{14}*pak3*^{76A} mutants, and this was confirmed with anti-FasIII antibody staining (data not shown).

Pak is required for restoration of the adherens junction at the LE at the end of DC

We simultaneously compared the effects of loss of Pak on LE adherens junctions with the effects on septate junctions and the Scrib complex by staining *pak*^{14}*pak3*^{76A} embryos with both anti-DE-Cad and anti-Dlg. Generally, DE-Cad and Dlg were mutually absent from the dorsal midline at the end of DC (Fig. 3Q; data not shown), although we found more mildly affected embryos with some restoration of Dlg and DE-Cad (Fig. 3R-R″). In such embryos, there were DME cells that had restored DE-Cad at the LE but not Dlg (Fig. 3R′). Given the requirement for Pak for adherens junction restoration and the presence of some Pak at the apical end of epithelial cells (Fig. 1A-B″), we tested to see if Pak existed in a complex with DE-Cad. Under conditions in which the adherens junction component Armadillo was successfully co-immunoprecipitated with DE-Cad, neither Pak nor Pak3 were co-immunoprecipitated (Fig. 1G,H; data not shown).

Pak localization to the lateral membrane is integrin-dependent and integrins are required for Scrib complex/septate junction recruitment in the follicular epithelium and at the LE

In cultured mammalian cells, Pak is a component of integrin-based focal complexes, and this mechanism of membrane localization appears to be conserved in *Drosophila* as Pak membrane localization in the follicular epithelium is integrin-dependent (Bokoch, 2003; Conder et al., 2007) (Fig. 4A′). Lateral Pak recruitment would require lateral integrins and βPS- and αPS-integrins are found at the lateral and apical membranes of follicle cells in addition to their expected basal localization (Dinkins et al., 2008; Fernandez-Minan et al., 2007; Goode et al., 1996) (Fig. 4B). In the embryo, there are modest levels of lateral βPS-integrin in the epidermis and high levels of lateral integrin in the amnioserosa and at the LE (Homsy et al., 2006; Narasimha and Brown, 2004; Wada et al., 2007) (Fig. 5A). To determine if Pak membrane localization in embryonic epithelia during DC was integrin-dependent, we looked at Pak distribution in myospheroid (*mys*) embryos mutant for βPS-integrin (Jannuzi et al., 2002). Localization of Pak to the LE was disrupted, as was cortical localization throughout the epidermis (Fig. 5C,E).

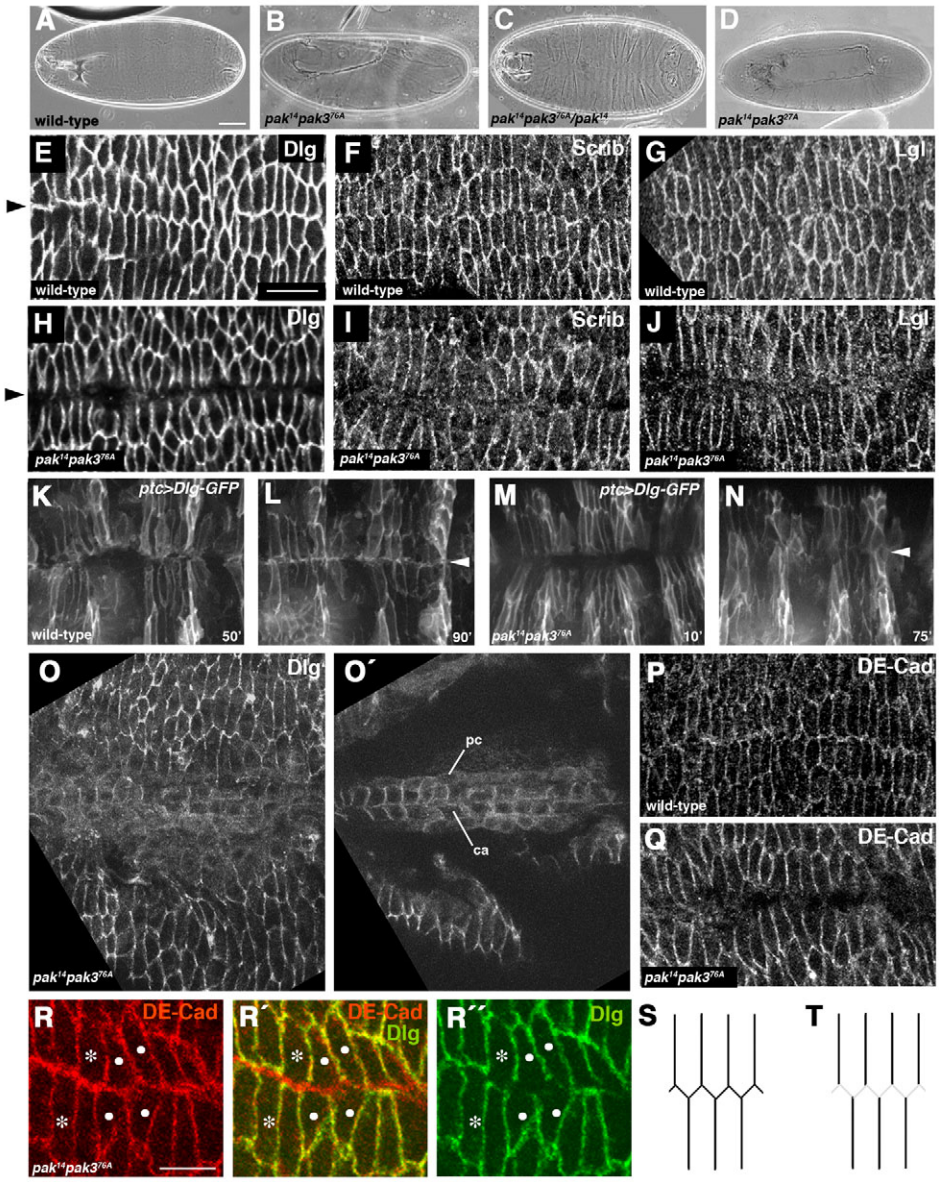

Fig. 3. The Scrib complex/septate junctions and adherens junctions are largely not restored when LEs meet at the dorsal midline following DC in embryos deficient in Pak. (**A-D**) Cuticle preparations of wild-type (A) and Pak-deficient embryos (B-D). (B) A *pak*^{14}*pak3*^{76A} embryo showing failure of germband retraction and a large dorsal hole extending into the head. (C) *pak*^{14}*pak3*^{76A}/*pak*^{14} embryo showing a head hole and puckering along the dorsal midline. (D) A *pak*^{14}*pak3*^{27A} embryo showing a rectangular dorsal hole. (**E-R″**) Views of the dorsal midline (marked with an arrowhead in E, H, L and N) of wild-type (E-G,K,L,P) and *pak*^{14}*pak3*^{76A} (H-J,M-O′,Q-R″) embryos stained with anti-Dlg (E,H,O,O′,R′-R″), anti-Scrib (F,I), anti-Lgl (G,J), or anti-DE-Cad (P-R′) or live-imaged with Dlg-GFP (K-N). Immunostainings show that the Scrib complex is restored at the dorsal midline in wild-type (E-G), but not *pak*^{14}*pak3*^{76A} (H-J), embryos. (K,L) Wild-type embryo viewed late in DC (K) and after DC (L), showing accumulation of Dlg-GFP at the dorsal midline in (L). See Movie 1 in the supplementary material. (M,N) *pak*^{14}*pak3*^{76A} embryo viewed late in DC (M) and after DC (N), showing lack of accumulation of Dlg-GFP at the dorsal midline in N. See Movie 3 in the supplementary material. (O) *pak*^{14}*pak3*^{76A} embryo in which epidermal flanks have come apart after DC. (O′) Same individual as in O, viewed deeper in the embryo to show the intact dorsal vessel. ca, cardioblasts; pc, pericardial cells. (P,Q) Adherens junctions are restored at the dorsal midline in wild-type (P), but not *pak*^{14}*pak3*^{76A} (Q), embryos. (R-R″) A *pak*^{14}*pak3*^{76A} embryo in which DE-cadherin has been restored at the LE in all cells along a stretch of the dorsal midline. Cells marked with an asterisk have failed to restore Dlg at the LE but their partners on the other side of the dorsal midline have. Cells marked with a dot are two pairs of dorsal midline partners that have failed to restore LE Dlg, giving a typical cleft-like appearance to the dorsal midline (similar to that seen in H). DE-cadherin staining shows that there is no actual cleft and that partners are adhering to each other. (**S**,**T**) Schematic diagram of Dlg staining in wild-type (S) and *pak*^{14}*pak3*^{76A} (T) embryos showing how the zigzag organization of the LEs would contribute to the cleft-like appearance of the dorsal midline in *pak*^{14}*pak3*^{76A} embryos. Scale bars: 50 μm in A-D; 10 μm in E-Q; 5 μm in R-R″.

Our results suggest that in situations where Pak participates in Scrib complex and/or septate junction recruitment, integrins should similarly be required. We looked at Scrib complex recruitment in clones of *mys* mutant follicle cells and found that, similar to *pak* follicle cell clones, lateral localization of Dlg was disrupted (Fig. 4C,C′). This result is in contrast to previous results in which follicle clones of the same *mys* allele showed retention of lateral Dlg (Fernandez-Minan et al., 2008; Fernandez-Minan et al., 2007). The

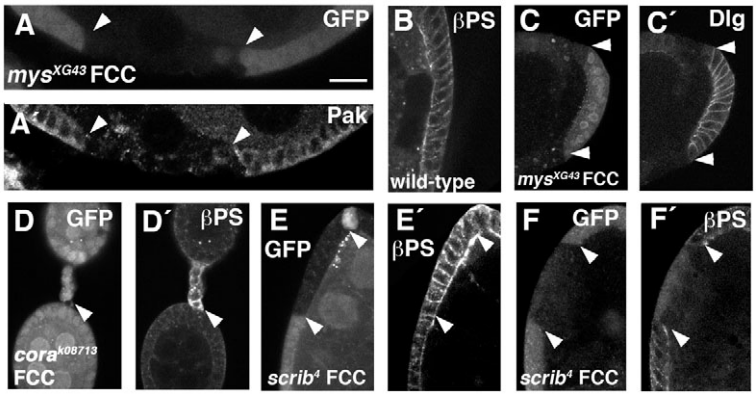

Fig. 4. Interdependence between Scrib complex or septate junction proteins and integrins for localization in follicular epithelium. (**A**,**A′**) Clone of *mys* mutant cells showing disruption of Pak localization in follicular epithelium in the absence of βPS-integrin. (**B**) Egg chamber showing βPS-integrin at the lateral and apical membranes in addition to basal membrane. (**C**,**C′**) Membrane localization of Dlg is disrupted in *mys* mutant cells. (**D**,**D′**) *cora* clones in stalk cells show elevated levels of βPS-integrin at the membrane. (**E**,**E′**) Egg chamber showing elevated integrin staining at the apical membrane in a *scrib* mutant clone. (**F**,**F′**) In egg chambers where a *scrib* follicle cell clone overlies a germline *scrib* clone (indicated by lack of GFP staining in the germline), integrin staining is lost in the follicle cell clone. Arrowheads indicate clone borders. Scale bar: 10 μm.

reason why our results differ from these previous findings might be the different approach we used to create *mys* clones. Whereas the earlier studies used *e22c-Gal4* to drive *UAS-FLP*, we used heat-shock and *hsFLP*. Given our findings in the follicular epithelium, one might expect Scrib complex recruitment and septate junction formation at the dorsal midline at the end of DC to be affected in *mys* mutant embryos. This is difficult to assess in *mys* embryos as they ultimately burst open (Hutson et al., 2003), but we found *mys* embryos in which the epidermal flanks were in contact at the dorsal midline but had failed to accumulate FasIII at the LE (Fig. 5G).

Strikingly, the distribution of lateral βPS-integrin during DC is complementary to the distribution of septate junction proteins. Whereas the septate junction protein Cora is absent from the lateral membranes of the amnioserosa and LE, but prevalent throughout the rest of the epidermis, integrin levels are high at the lateral membranes of the amnioserosa and the LE is the only epidermal lateral membrane with high integrin levels (Fig. 5A). An interesting possibility is that, in the developing follicular epithelium of the egg chamber and at the LE in the embryo, the absence of the septate junction diffusion barrier allows the accumulation of lateral integrin complexes, which then recruit Pak (see model in Fig. 6). We found that following the completion of DC, after the dorsal vessel had formed, integrin was downregulated at the dorsal midline (Fig. 5H). In $pak^{14}pak3^{76A}$ mutant embryos, elevated levels of integrin persisted at the dorsal midline, perhaps owing to a failure to restore septate junctions (Fig. 5I). We looked at integrin distribution in embryos mutant for the larval lethal $cora^{14}$ allele (Lamb et al., 1998) to see if there was any increase in integrin levels at lateral membranes. Embryos were indistinguishable from wild-type, with the exception that there were increased integrin levels in lateral membranes (including LE) of DME cells after DC (Fig. 5J,K). The restriction of this phenotype to these cells might be due to the initial high levels of integrin at the LE. In further support of the idea that septate junction proteins restrict integrin distribution, we found ectopic integrin accumulation in ovarioles in *cora* mutant stalk cells and *scrib* mutant follicle cells (Fig. 4D-E′). However, when *scrib* follicle cell clones occurred in contact with *scrib* germline clones, integrin staining was lost in the follicle cell clones, indicating a cell-non-autonomous effect of loss of Scrib in the germline, perhaps owing to disruption of germline-to-follicle cell signaling (Fig. 4F,F′).

DISCUSSION

Pak is required for restoration of apicobasal polarity as migratory epithelial cells complete DC

Some embryos lacking zygotic Group I Pak function successfully bring the epidermal flanks together at the dorsal midline but fail to restore septate junctions and adherens junctions at the LE in the DME cells. Thus, Pak at the LE membrane of the DME cells is regulating establishment of apicobasal polarity during a MET-like event. We suspect that Pak is acting through different routes in its regulation of adherens junction formation versus septate junction formation. Here, we have focused on Pak regulation of the Scrib complex in septate junction formation at the LE. Our data indicate that Pak is a component of the Scrib complex at the lateral membrane. Although Pak might be associating with the Scrib complex throughout epithelia, it might only be required for recruitment of the Scrib complex in epithelia derived from a mesenchymal-like intermediate such as the follicular epithelium (Conder et al., 2007) and the LE. With the exception of the LE, apicobasal polarity in the epidermis is determined much earlier in development with formation of the blastoderm by cellularization (Tepass et al., 2001). The epidermis is therefore a primary epithelium that does not arise from a mesenchymal intermediate, and Pak function does not appear to be required for apicobasal polarity in primary epithelia (this study) (Conder et al., 2004).

Relationship between integrins and septate junctions

Localization of Pak at the lateral membrane in both the follicular epithelium and in the epidermis is integrin-dependent. Studies using organ culture of embryonic kidney mesenchyme and MDCK cells demonstrate a requirement for integrins in apicobasal polarity of epithelia derived from MET (Matlin et al., 2003), and we have shown that βPS-integrin is required for Scrib complex and septate junction protein recruitment at the LE and in the follicular epithelium. Furthermore, previous studies in the follicular epithelium and another *Drosophila* epithelium derived from MET, the midgut, have demonstrated a requirement for integrins in the maintenance of apicobasal polarity (Conder et al., 2007; Devenport and Brown, 2004; Fernandez-Minan et al., 2008; Fernandez-Minan et al., 2007). We propose that, at the LE, the absence of the septate junction diffusion barrier allows the accumulation of integrin complexes along the lateral membrane. These lateral integrin complexes recruit Pak, around which the Scrib complex is assembled. Thus, the absence of septate junctions allows the recruitment of proteins needed for the assembly of septate junctions (Fig. 6). Our model suggests that there might be transient Pak-mediated links between integrin and the Scrib complex. Interestingly, Dlg and βPS-integrin have been shown to co-immunoprecipitate from fly head extracts, consistent with these proteins existing in a complex in the nervous system and/or in epithelia (Beumer et al., 2002).

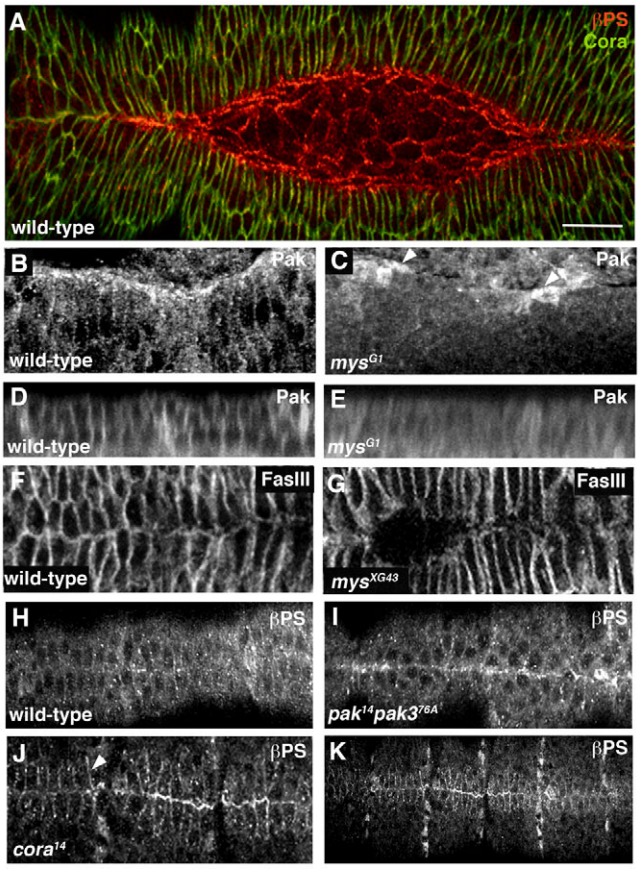

Fig. 5. Interdependence between Scrib complex or septate junction proteins and integrins for localization in embryonic epidermis. (**A**) βPS-integrin and Cora show complementary distributions during DC. (**B**) Wild-type embryo showing LE accumulation of Pak and some cortical Pak localization throughout the epidermis late in DC. (**C**) *mys* mutant embryo late in DC showing a lack of Pak accumulation at the LE and a lack of cortical Pak localization in the epidermis. Elevated Pak levels are maintained in DME segment border cells (arrowheads). (**D**,**E**) View of the apicobasal axis of a row of DME cells and underlying cardioblasts in wild-type (D) and *mys* (E) embryos, showing that localization of Pak to the lateral membrane is disrupted by loss of βPS-integrin. Embryos were optically sectioned along the anteroposterior axis. (**F**) View of the dorsal midline of a wild-type embryo at end of DC, showing FasIII accumulation at adhered LEs. (**G**) View of the dorsal midline of a *mys* mutant embryo at the end of DC, showing lack of FasIII accumulation at adhered LEs. The LEs have come apart over a short stretch. (**H**) View of the dorsal midline of a wild-type embryo after DC, showing downregulation of βPS-integrin at LE. (**I**) A $pak^{14}pak3^{76A}$ embryo of similar age to that in H showing persistent βPS-integrin at the dorsal midline. (**J**) A *cora* embryo after DC showing strong, sharp βPS-integrin staining at the dorsal midline and accumulation of βPS-integrin on lateral membranes between DME cells (arrowhead). (**K**) Low power view of the same embryo as in J, showing that ectopic βPS-integrin accumulation is apparent only in DME cells. Stripes of βPS-integrin staining are muscle attachment sites. Scale bar: 10 μm.

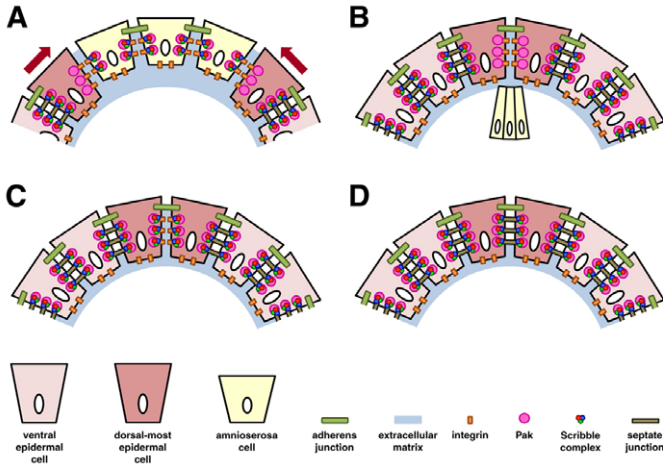

Fig. 6. Model for Pak-dependent septate junction formation in the LE membrane of DME cells. Embryo viewed in cross-section at various stages of DC. (**A**) During DC, Scrib complex and septate junction proteins are missing from the LE, the DME membrane in contact with the amniosersosa. Amnioserosa cells lack septate junctions and have high levels of lateral intergins, as does the LE, where integrins anchor Pak. (**B**) DME cells meet up at the dorsal midline as amnioserosa is internalized. (**C**) Pak recruits the Scrib complex to LE. (**D**) Following completion of DC, septate junctions at LE have matured and lateral integrins have been displaced.

Our data and recent studies on the amnioserosa support the idea that septate junctions restrict the accumulation of lateral integrins. The amnioserosa is devoid of septate junction proteins such as FasIII, and this might be owing to absence in this tissue of the transcription factor Grainy head, which promotes expression of septate junction proteins (Narasimha et al., 2008). The wild-type amnioserosa has high levels of lateral βPS-integrin, but ectopic expression of Grainy head in the amnioserosa leads to an accumulation of septate junction proteins and an accompanying disruption of βPS-integrin localization (Narasimha and Brown, 2004; Narasimha et al., 2008). Similarly, at the completion of DC, septate junctions appear at the LE and this is accompanied by downregulation of LE lateral integrins. In $pak^{14}pak3^{76A}$ and $cora^{14}$ embryos where LE septate junctions are deficient, lateral LE βPS-integrin persists.

Scrib complex proteins at the LE: a two-way street with regard to interactions with Cdc42/Rac signaling?

A recent study in mammalian cell culture indicates that Scrib recruits Pak to the LE (Nola et al., 2008), and we have shown that Pak localization in the follicular epithelium is Scrib-dependent. Our study of the LE at the end of DC demonstrates that the relationship between Cdc42/Rac signaling complexes and Scrib can act in the opposite direction: membrane-localized Pak recruits the Scrib complex. A bidirectional interaction between the Scrib complex and Cdc42/Rac signaling complexes, including Pak, might be a crucial regulator of events at the LE of closing epithelia during both wound healing and development in diverse systems. Scrib at the newly formed LE can lead to recruitment of the Cdc42/Rac signaling complex, allowing acquisition of mesenchymal characteristics and polarized cell migration. When the opposing epithelial flanks meet up, events can be reversed with Pak recruiting the Scrib complex to the lateral membrane, contributing to restoration of apicobasal polarity and cell adhesion at the LE during MET. We view the Scrib/Pak complex as a ‘toggle switch’, enabling the epithelial membrane to shift back and forth between a migratory state characterized by actin-based extensions and an apicobasal polarized state characterized by cell-cell adhesion.

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Competing interests statement
The authors declare no competing financial interests.

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