Armadillo/β-catenin-dependent Wnt signalling is required for the polarisation of epidermal cells during dorsal closure in *Drosophila*

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Summary

At the end of germband retraction, the dorsal epidermis of the *Drosophila* embryo exhibits a discontinuity that is covered by the amnioserosa. The process of dorsal closure (DC) involves a coordinated set of cell-shape changes within the epidermis and the amnioserosa that result in epidermal continuity. Polarisation of the dorsal-most epidermal (DME) cells in the plane of the epithelium is an important aspect of DC. The DME cells of embryos mutant for *wingless* or *dishevelled* exhibit polarisation defects and fail to close properly. We have investigated the role of the Wingless signalling pathway in the polarisation of the DME cells and DC. We find that the β-catenin-dependent Wingless signalling pathway is required for polarisation of the DME cells. We further show that although the DME cells are polarised in the plane of the epithelium and present polarised localisation of proteins associated with the process of planar cell polarity (PCP) in the wing, e.g. Flamingo, PCP Wingless signalling is not involved in DC.

Key words: Dorsal closure, Armadillo, Wingless, Planar cell polarity (PCP), *Drosophila*

Introduction

Half way through embryogenesis, the dorsal epidermis of the *Drosophila* embryo exhibits a discontinuity that is covered by an epithelium of large and flat polyploid cells, the amnioserosa. During the next 4 hours, the cells of the epidermis and the amnioserosa undergo coordinated and spatially orientated cell shape changes that will result in epidermal continuity. This process is known as dorsal closure (DC) and represents a good model for the study of epithelial movements that can be compared with processes such as wound healing or the seaming of the eyelids in vertebrates (Jacinto et al., 2001).

At the end of germband retraction, the cells of the amnioserosa undergo cell shape changes through a reduction of their apical surface (Harden et al., 2002). The net result of these changes is a pulling force exerted by the amnioserosa on the epidermis, which appears to be the main force driving DC during these early stages (Harden et al., 2002; Hutson et al., 2003; Kiehart et al., 2000). Simultaneously, the epidermal cells elongate in the dorsoventral direction, first the dorsal-most epidermal (DME) cells and then the cells positioned more ventrally. There is no cell division during DC and these cell shape changes account for the increase of the epidermal surface, which eventually enables the epidermis to enclose the embryo (Foe, 1989; Young et al., 1993). In a second step, the two edges of the epidermis meet at the ends of the embryo and initiate a zippering process powered by filopodia and lamellipodia that protrude from the actino-myosin cable (Jacinto et al., 2000; Kaltschmidt et al., 2002). These filopodia are thought to contribute to the interactions between the two edges of the epidermis both by facilitating adherens junction establishment and contributing to a correct segment-matching of the two sides of the epidermis (Jacinto et al., 2000). The interactions between the two sides of the epidermis proceed from both the anterior and the posterior edges in a double zipper-like fashion until the continuity of the epidermis is achieved. At the same time, the amnioserosa ingresses, dislodges itself from the epidermis and undergoes apoptosis (Hartenstein, 1993).

Genetic screens, which are mainly based on surveying defects in the patterning of the dorsal epidermis, have identified a large number of genes required for DC. These can be grouped into two main categories: genes involved in signalling pathways, e.g. JNK, Wnt and Dpp pathways, and genes coding for components of cellular architecture or its regulation, e.g. cytoskeletal components like Myosin or proteins associated with the adherens junctions like Canoe/L-afadin or Abl (Harden, 2002). Although early studies focused on large defects in the patterning process, more recent ones have targeted cellular activities (Harden et al., 2002). These studies have revealed a number of specialisations of the DME cells and in particular a progressive polarisation of their membrane components in the plane of the epithelium (Kaltschmidt et al., 2002). At the end of germband retraction, the DME cells and the more ventrally located epidermal cells exhibit an isotropic shape. At the onset of DC, the microtubules of the DME cells bundle in the dorsoventral (DV) direction, which defines an axis along which these cells begin to elongate (Fig. 1A′). Simultaneously, Flamingo (Fmi), an atypical cadherin (Chae et al., 1999; Usui et al., 1999), localises to the membrane of the DME cells, except at their leading edge (LE, Fig. 1A). A similar localisation pattern is also adopted by other membrane associated proteins such as Discs-large (Dlg) and Fasciclin 3 (Fas3; also known as Fas III). As the process of polarisation progresses, actin begins to accumulate at the LE and

concentrates in actin nucleating centres (ANCs) (Kaltschmidt et al., 2002), while Myosin accumulates at the LE forming a 'beads on a string' pattern (Young et al., 1993) (V.M., unpublished; Fig. 1A″). By the end of that first phase, the DME cells are thus elongated in the DV direction and characterised by a polarised localisation of both cell membrane associated proteins and cytoskeletal components. At present, the function of this polarisation is unclear. It might contribute to the correct actin dynamics because in *wingless* and *dishevelled* mutants, in which the polarisation is affected, actin dynamics are defective.

Wingless (Wg) and Dishevelled (Dsh) are essential elements of Wnt signalling that can act through either of two different but related signal transduction pathways. One pathway, called 'canonical' signalling, is dependent on β-catenin (Armadillo in *Drosophila*) and targets the nucleus. A second pathway, tightly linked to the process of planar cell polarity (PCP), targets the cytoskeleton (for a review, see Veeman et al., 2003). The PCP pathway provides directional organisation to epithelial cells in the plane of the epithelium and has been well characterised in the wing and in the eye (Eaton, 1997; Mlodzik, 2002). In vertebrates, it seems to play a role in convergent extension and gastrulation (Heisenberg et al., 2000; Lawrence and Morel, 2003; Tada and Smith, 2000).

PCP manifests itself in the asymmetric organisation in the plane of the epithelium of membrane associated proteins, including Fmi, and cytoskeletal components, such as microtubules. These act as a scaffold for the directional bundling of actin at the distal vertex of each cell and the formation of the hairs. At first sight, the polarisation of the DME cells during dorsal closure can be seen as a similar process with polarised actin dynamics instead of actin bundling. A similarity between the polarisation of the wing hairs and the DME cells of the embryo is also suggested by the requirement for *dishevelled* in both processes.

In *Drosophila* both PCP and 'canonical' Wingless signalling require Dishevelled, but there is no solid evidence for an involvement of Wingless in other than the 'canonical' pathway. The observation that during DC the polarity of the DME cells is abnormal in *wingless* and *dishevelled* mutants has raised the possibility that Wingless is involved in establishing PCP in these cells. Here, we test the possibility of a role for the PCP pathway and Wingless in the polarisation of the DME cells. Our results reveal that Armadillo-mediated Wingless signalling is necessary for the correct polarisation of the DME cells during embryogenesis, while the PCP pathways seems dispensable.

Materials and methods

Drosophila strains

We used Canton S flies as the wild-type stock. The *wg* mutant embryos are derived from the stock wg^{CX4}/CyoftzlacZ;daGal4. The overexpression experiments in *wg* mutant background were made using wg^{CX4}/CyoftzlacZ;daGal4 females for ubiquitous overexpression and wg^{CX4},332.3Gal4/CyoftzlacZ females for overexpression in the amnioserosa. These females were crossed with males from the stocks wg^{CX4},UAS Wg^{E1}/CyoftzlacZ, wg^{CX4},UAS Arm^{S10c}/CyoftzlacZ, wg^{CX4}/CyoftzlacZ;;UAS Dsh, wg^{CX4},UAS Dsh ΔA^{4-2}/CyoftzlacZ, wg^{CX4},UAS Dsh ΔC^{15-21}/CyoftzlacZ, and wg^{CX4}/CyoftzlacZ;UAS Tkv^{Q253D}/TM6b for overexpression of Wg, Arm^{act}, Dsh, DshΔDIX, DshΔDEP and Tkv^{QD}, respectively. *dsh* and *dsh,sgg* germline clones were generated using the Flp-FRT system as described by Chou and Perrimon (Chou and Perrimon, 1992) using, respectively, dsh^{V26} FRT101/ FM6 and dsh^{V26},sgg^{M11} FRT101/ FM7c females (dsh^{v26} is dsh^{3} in FlyBase).

Immunostaining

Embryos were fixed and stained as described previously (Kaltschmidt et al., 2002). We used the following primary antibodies (and concentration): rabbit antisera against Discs-large (1/200; a gift from P. J. Bryant), mouse monoclonal against Flamingo (1/10; from T. Uemura) (Usui et al., 1999), mouse antibodies against Fas3 [1/50; Developmental Studies Hybridoma Bank (DSHB), University of Iowa; developed by C. Goodman], mouse antibodies against βTubulin (1/100; DSHB; developed by M. Klymkowsky), rabbit antibodies against Myosin (1/500; gift from R. Karess), mouse antibodies against Wingless (1/250; DHSB; developed by S. M. Cohen), rabbit antibodies against β-galactosidase (1/10,000; from Cappel), mouse antibodies against Engrailed (1/100; DSHB; developed by C. Goodman). The following conjugated secondary antibodies were used at 1/200: Cy5-conjugated antibodies from Jackson ImmunoResearch; Alexa488- and Alexa568-conjugated antibodies from Molecular Probes and biotin-conjugated antibodies from Vector Laboratories. With biotin-conjugated secondary antibodies we used the Elite ABC kit (Vector) before staining with diaminobenzidene.

Fluorescently labelled embryos were mounted in Vectashield (Vector) and examined under confocal microscope (BioRad). Diaminobenzidene stained embryos were mounted in DPX (Fischer) and examined using an Axioplan2 microscope (Zeiss) and a KY-F55B camera (JVC).

RNA in situ hybridisation

We fixed embryos using standard protocols in 4% formaldehyde. In situ hybridisation was carried out using a digoxygenin-labelled DNA *dpp* probe using standard methods (Lecourtois and Schweisguth, 1995). After staining, the embryos were washed several times in PBTw (PBS, 0.1% Tween 20), dehydrated in 70% ethanol, rehydrated in PBT-BSA (PBS, 0.1% Triton, 1% BSA) and blocked for 1 hour at room temperature. They were incubated overnight at 4°C with rabbit anti-β-galactosidase antibody and anti-rabbit Alexa-488-conjugated secondary antibody was added as described above. Embryos were hand sorted under a MZFLIII GFP-scope (Leica) and mounted in Spurr embedding medium (Fullam).

Cuticle preparation

Two-hundred and fifty embryos were collected from an overday egg collection and aged 48 hours at 25°C. The unfertilised embryos were removed and deducted from the count and the remaining embryos were mounted in acetic acid/Hoyers 50/50. The slide was cooked overnight and the different phenotypes counted.

Results

wingless and *dishevelled* mutant embryos show defects in polarisation of the DME cells

Embryos mutant for *wingless* (wg^{-}) and *dishevelled* (dsh^{-}) show defects in the polarisation of the DME cells. In both mutants, cells elongate and bundle microtubules in the anteroposterior (AP) direction instead of the DV direction (McEwen et al., 2000; Kaltschmidt et al., 2002). In addition, Myosin fails to accumulate at the LE and Fmi displays a dotty cytoplasmic staining instead of the wild-type localisation at the membrane (Kaltschmidt et al., 2002) (Fig. 1B-B′ and not shown). Later on, the zippering process is initiated only at the posterior region of the embryo, which results in an asymmetric closure. At this late stage, the main axis of many DME cells switches to the DV

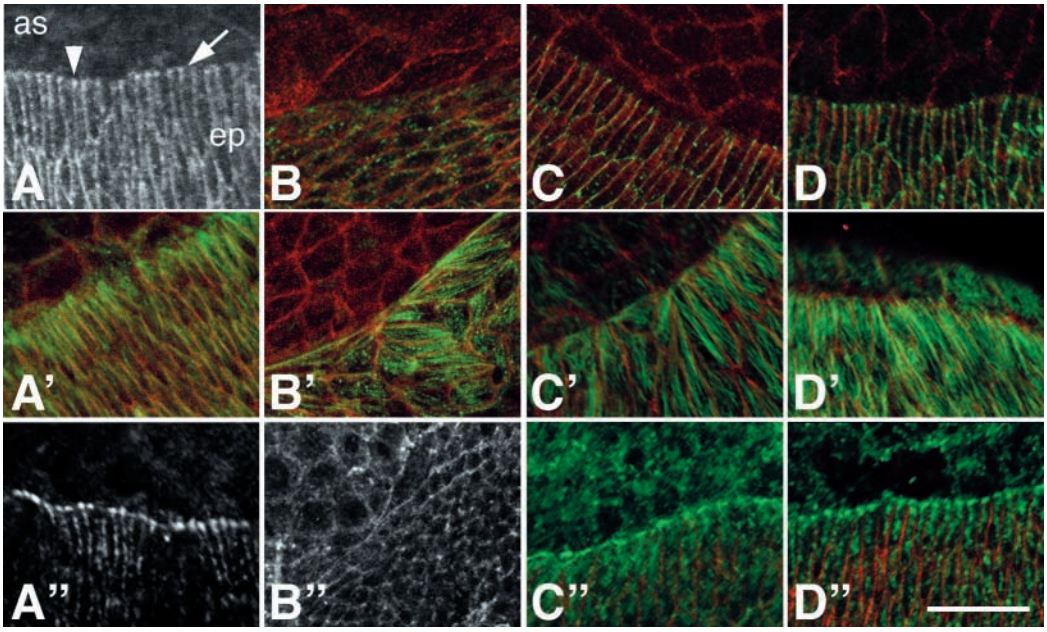

Fig. 1. Ubiquitous overexpression of Wg or activated Arm rescues the polarity of DME cells in *wingless* mutant embryos. Confocal images of stage 13 embryos showing the subcellular localisation of Fmi and Dlg (A-D, green and red, respectively), βTubulin and Fas3 (A′-D′, green and red, respectively), and Myosin (A″-D″; green, C″,D″, red is Fas3). (A-A″) In wild-type embryos, the DME cells (outlined by Fas3 in A′) are elongated in the DV direction. Fmi (A) localises at the membrane, but is excluded from the LE (arrow), and accumulates at the level of the ANCs (arrowhead). The microtubules bundle and orient in the DV direction (green in A′) and Myosin shows a 'beads on a string' pattern at the LE (A″). (B-B″) In *wg*⁻ embryos, the cells are stretched in the AP direction and Fmi, which weakly labels the membrane, fails to accumulate at the ANCs (B, green). The microtubule bundles are stretched in the main direction of the cells (B′) and the weak Myosin staining is characterised by the lack of accumulation at the LE (B″). In embryos ubiquitously overexpressing Wg (C-C″) or Arm^{act} (D-D″), although few cells show a triangular shape, the elongation of the DME and the more ventrally located epidermal cells (C,D) is rescued. The localisation of Fmi at the membrane and its concentration in ANCs (green C,D), the orientation of the microtubule bundles (green C′,D′) and the 'beads on a string' pattern of Myosin (green, C″,D″, red is Fas3) are comparable with wild type. In all the figures, anterior is leftwards and dorsal upwards. as, amnioserosa, ep, epidermis. (A-A″,B′,B″) Modified, with permission, from Kaltschmidt et al. (Kaltschmidt et al., 2002). Scale bar: 20 μm.

direction but only some of these cells undergo an elongation of any kind. Fmi is then localised at the membrane and excluded from the LE as is observed at earlier stages of DC in wild-type embryos. Neither during the initial or the late phases of DC do the more ventrally localised epidermal cells display any elongation (not shown). There is, however, one difference between *wg* and *dsh* mutant embryos. In *dsh*⁻ embryos the amnioserosa detaches from the epidermis, which results in a dorsal hole. By contrast, *wg*⁻ embryos close and show most of the time only a very small dorsal hole, next to the anterior hole, which can be correlated with the defect of zippering initiation anteriorly (this work) (McEwen et al., 2000).

The 'canonical' Wingless pathway is required for dorsal closure

The involvement of both *wingless* and *dishevelled* in the polarisation of the DME cells and the similarity of this process in the planar polarisation of epithelial cells (Eaton, 2003) led us to enquire which of the two Wingless signalling pathways, the 'canonical' or the PCP pathway, is involved in this process. We tested this by attempting to rescue *wg* mutant embryos with effector elements of Wg signalling pathways. In these experiments we made use of the Gal4 targeted expression system (Brand and Perrimon, 1993) to express different signalling molecules in the *wg* mutant background.

We first examined the ability of different levels of Wingless signalling to rescue *wg*⁻ embryos. To do this, we used an ubiquitous Gal4 driver, daughterless-Gal4 (daGal4) to express either Wingless (*wg*>da>Wg) or an activated form of Armadillo (Arm^{act}, *wg*>da>Arm^{act}). We assumed that if the 'canonical' Wingless pathway is involved in DC, overexpression of both Wg and Arm^{act} should lead to some rescue of DC. However, if the 'canonical' pathway is not involved but the function of Wg during DC involves the PCP pathway, one should observe some rescue only with the overexpression of Wg. As an internal control of the experiment, we monitored the rescue of two features of the 'canonical' pathway. One with a low requirement for Wingless signalling, the presence of naked cuticle on the ventral side of the embryo, and one with a higher requirement for Wingless, the expression of Engrailed (En) in stripes in the ectoderm. Both overexpression of Wg and Arm^{act} allowed rescue of those two features (Fig. 2), validating our experimental conditions.

Ubiquitous expression of Wingless rescues the DC defects of *wg*⁻ embryos (Kaltschmidt et al., 2002), including the double zipper (not shown). Detailed analysis of these embryos reveals that they have normal elongation of the DME cells in the DV direction and correct subcellular localisation of Flamingo (Fig. 1C). In addition the bundling of the microtubules (Fig. 1C′) and the localisation of Myosin at the LE of the DME cells (compare Fig. 1A″,B″,C″) are normal. A similar rescue was observed when Arm^{act} was used instead of Wingless (Fig. 1D-D″).

Therefore both Wg and Arm^{act} are able to rescue the DC defects of a *wg*⁻ embryo to a similar extent. These experiments indicate that the 'canonical' Wingless pathway is required for the correct behaviour of the DME cells during DC.

Spatial requirement for the Wingless 'canonical' signalling pathway

During germband elongation and until the beginning of

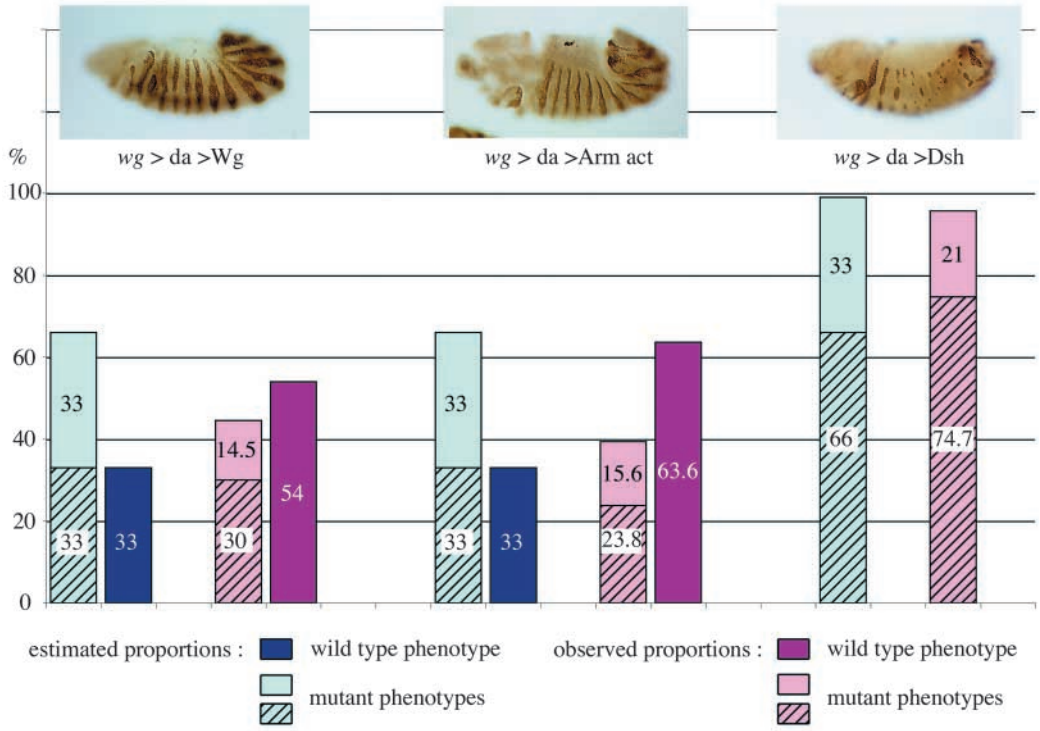

Fig. 2. Rescue of 'canonical' Wg pathway features following overexpression of Wg, Arm^{act} or Dsh in *wg*$^{-}$ embryos. Analysis of the different cuticle phenotypes from the following crosses: females *wg*^{CX4}/CyoftzZ;daGal4 × males *wg*^{CX4},UASWg/CyoftzZ (*wg*>da>Wg), *wg*^{CX4},UASArm^{act}/CyoftzZ (*wg*>da>Arm^{act}) and *wg*^{CX4}/CyoftzZ;;UASDsh (*wg*>da>Dsh). The embryos shown above are stained for Engrailed. *wg*>da>Wg: 44.5% of the embryos overexpress Wg and present a fully naked cuticle, and are organised in two categories of different strength (30%, 14.5%). 21% of the embryos overexpress Wg and have a wild-type phenotype (out of the 54% showing a wild-type phenotype, 33% do not overexpress Wg). Wg overexpression in *wg*$^{-}$ embryos is thus able to rescue the naked cuticle identity and to some extent suppress the denticules specification. En expression is rescued to normal. *wg*>da> Arm^{act}: 39.4% of the embryos overexpress Arm^{act} and show a fully naked cuticle, and 30.6% of the embryos overexpress Arm^{act} and show a wild-type phenotype. Arm^{act} overexpression is thus able to rescue the naked cuticle identity. En expression, as shown by the above embryo, is normal. *wg*>da>Dsh: all the embryos from this cross overexpress Dsh. A mild rescue of the naked cuticle identity (see Fig. 5C′) is observed in 21% of the embryos (33% are *wg*$^{-}$). A partial rescue of En expression is observed.

retraction, *wg* is expressed in the epidermis of the embryo in a two-cell-wide stripe in each segment. During stage 11, expression in the lateral epidermis is lost and a new pattern emerges with a ventral stripe and a dorsal patch, just below the DME cells, per segment. There is never *wg* expression in the amnioserosa cells. Wingless protein expression pattern reflects the dynamics of the RNA expression (Gonzalez et al., 1991) (L. E. Owen, PhD Thesis, University of Cambridge, 1994).

The experiments described above support the suggestion that a localised source of Wingless may not be required for DC. However, there might be a differential requirement for Wg signalling between the amnioserosa and the epidermis for DC to proceed. We previously showed that when activated in both tissues Wg signalling rescues DC and epidermis defects. We have now tested the requirement for Wg signalling in the amnioserosa by overexpressing Wingless or Arm^{act} in the amnioserosa of *wg*- embryos and assessing its effects on DC and the epidermis.

When Wingless is expressed using the amnioserosa specific driver, 332.3-Gal4 (ASGal4) (Wodarz et al., 1995), Wingless can be seen to be expressed and secreted by the amnioserosa cells from the stage 11 onwards. In these embryos, Wingless can be detected in the cytoplasm of the amnioserosa cells but not in the epidermis. At stage 12 [mid-way through retraction of the germband, stages as in Hartenstein (Hartenstein, 1993)], some Wingless can be observed in the head epidermis up to 5-6 cells away from the amnioserosa. At the onset of DC, late stage 12 and stage 13, some Wingless can be detected in the form of dots over the DME cells and the lateral epidermis up to five cells away from the amnioserosa (Fig. 3D). This pattern persists until the end of closure. Although at stages late 12 and 13 the dots are on the apical side of the epidermis, later on they are located on the basal side of the epidermis (data not shown).

We assessed the ability of Wingless secreted from the AS to rescue *wg*$^{-}$ embryos (*wg*>AS>Wg) by following several markers of cell shape and polarity. In these embryos the DME cells become oriented in the DV direction as early as stage 13 but they do not elongate significantly (Fig. 3A). The epidermal cells located ventrally do not even show a preferential direction in the DV axis but show an isotropic shape (Fig. 3A, asterisk). Even though the DME cells do not stretch, they show signs of polarisation, e.g. Fmi is correctly localised at the ANCs (Fig. 3A) and the microtubules bundle in the DV axis (not shown). However the organisation of other elements of the cytoskeleton is not fully rescued. Myosin localises only weakly to specific locations in the leading edge and does so at late stage 14 rather than the wild-type stage 13 (Fig. 3B,C). The overexpression of Wg in the amnioserosa is thus not able to fully rescue the Myosin localisation or the cell elongation at the early stage of DC, although it is sufficient to rescue both Fmi localisation and microtubule bundles orientation. Later on however, DME cells elongate in the DV direction, although to a lesser extent than in wild-type embryos. It is interesting that this elongation does not propagate to the more ventral epidermal cells (data not shown).

Although we observe Wingless protein over the epidermis, the rescue of the *wg*$^{-}$ phenotype is restricted to the DME cells. This raises the possibility that the rescue is due to an interaction between the epidermis and the amnioserosa rather than to a

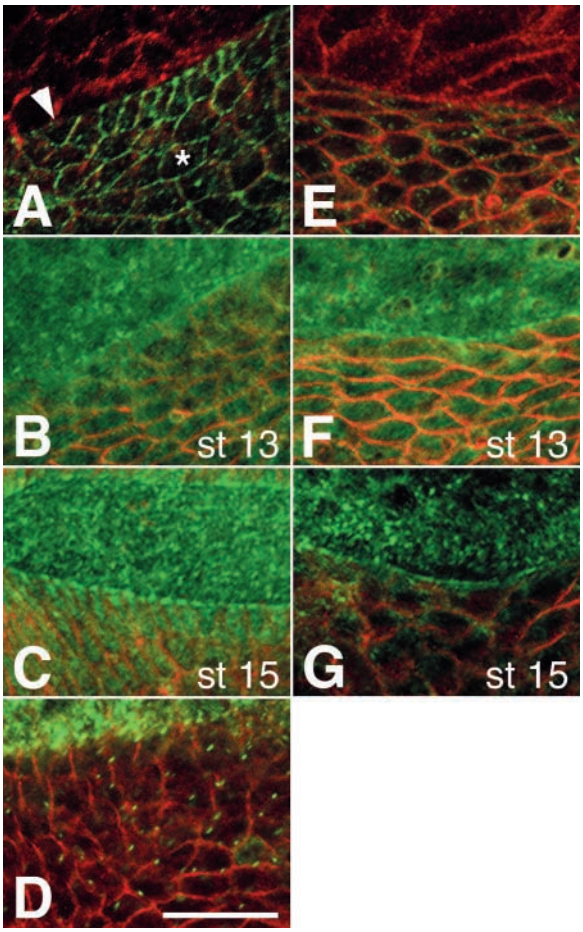

Fig. 3. Activation of the 'canonical' Wg pathway is required in the epidermal cells but not in the amnioserosa. Subcellular localisation of Fmi, Dlg, Myosin, Fas3 and Wg in *wg*$^{-}$ embryos overexpressing either Wg or Arm^{act} in the amnioserosa. (A,E) Stage 13 embryos stained for Fmi (green) and Dlg (red). Although the DME cells are oriented in the DV direction in the *wg*>AS>Wg embryos (A), they do not elongate and even sometimes show a triangular shape (arrowhead). The ventrally located epithelial cells present an isotropic shape (asterisk). In these embryos, however, Fmi localises at the membrane and concentrates at the level of the ANCs as in wild type (compare with Fig. 1A). By contrast, in *wg*>AS>Arm^{act} embryos (E), DME cells are not oriented in the DV direction but rather stretched in the AP direction as in *wg*$^{-}$ embryos. In these embryos, Fmi shows a 'dotty' staining similar to that observed in *wg*$^{-}$ embryos (compare with Fig. 1B). (B,C,F,G) Subcellular localisation of Myosin in stage 13 (B,F) and stage 15 (C,G) embryos. At a stage when Myosin already shows accumulation at the LE in wild type (compare with Fig. 1C), no accumulation is observed in *wg*>AS>Wg embryos (B). However, when the zippering process has been initiated at both ends, accumulation is observed at the LE (C). By contrast, neither at stage 13 (F) nor at stage 15 (G) does Myosin accumulate at the LE of *wg*>AS>Arm^{act} embryos. (D) Subcellular localisation of Wg (green) in a stage 13 *wg*>AS>Wg embryo. After overexpression, a strong Wg expression is observed in the amnioserosa and Wg-containing dots are seen on the apical side of the epithelium up to six cells away from the LE (the cells are outline in red by Dlg). All panels are single confocal sections, dorsal is upwards and anterior leftwards. Scale bar: 20 μm.

direct effect of Wingless on the epidermal cells. To circumvent this, we overexpressed Arm^{act} in the amnioserosa (*wg*>AS>Arm^{act}). As Arm is an intracellular protein, any observed effect is likely to be cell autonomous and thus associated with activation of the Wingless pathway within the amnioserosa. Under these conditions, neither the DME cell shape, nor the localisation of Fmi, the organisation of the microtubule bundles nor the localisation of the Myosin were significantly rescued when compared with similar staining in *wg*$^{-}$ embryos (compare Fig. 1B, Fig. 3E and Fig. 1B″, Fig. 3F). Activation of the 'canonical' Wingless pathway within the amnioserosa cells is thus not sufficient to rescue the cellular aspects of DC.

These results show that the polarisation and cytoskeletal reorganisations of the DME cells require the activity of the 'canonical' Wingless signalling pathway within the epidermis. In addition, they indicate that different cellular events respond to different thresholds of Wingless signalling and that activity, and elongation, of the DME cells does not trigger elongation in more ventrally located cells.

Ubiquitous activation of the Dpp pathway does not rescue the polarity defects of *wingless* mutant embryos

The activity of the DME cells during dorsal closure requires the activation of the JNK pathway and the expression of two target genes, *puckered* (Glise et al., 1995; Riesgo-Escovar et al., 1996) and *decapentaplegic* (*dpp*) (Glise and Noselli, 1997; Harden, 2002; Hou et al., 1997; Riesgo-Escovar and Hafen, 1997; Zeitlinger et al., 1997). McEwen et al. (McEwen et al., 2000) reported that *wg* is required for *dpp* expression in the DME cells. As the 'canonical' Wingless pathway leads to the activation of gene expression, we investigated its contribution to *dpp* expression. Reciprocally, we investigated if all the effects of the 'canonical' Wingless pathway are mediated by Dpp.

In wild-type embryos, *dpp* shows a complex and dynamic pattern of expression. During retraction of the germband, *dpp* expression is restricted to a single row of cells corresponding to the DME cells. As DC begins, the level of *dpp* expression in the DME cells decreases (Fig. 4A) and during the zippering phase *dpp* transcripts cannot be detected anymore. In *wg*$^{-}$ embryos, *dpp* is expressed in the DME cells at the onset of DC but its levels, as detected by in situ hybridisation, are decreased compare with wild type (Fig. 4B). Nevertheless, the dynamic of *dpp* expression seems to be conserved, and, as in wild-type embryos, *dpp* expression disappears during the zippering process. In *wg* mutant embryos expressing high levels of either Wg or Arm^{act} with daGal4, the levels of *dpp* are restored to wild type levels (Fig. 4C,D). Interestingly, regardless of the ubiquitous overexpression of either Wg or Arm^{act} throughout the epidermis, the pattern of *dpp* expression during DC is preserved, suggesting that there is only a subpopulation of cells that are competent to express *dpp*. However, amnioserosa-specific expression of Wingless, but not of Arm^{act}, in *wg* mutants results in a rescue of *dpp* expression in the DME cells (Fig. 4E,F). This confirms that the effects that we observe are due to Wingless signalling directly to the DME cells and not to secondary signalling across cell types.

The correlation between *dpp* expression in the DME cells and the rescue of many of their cellular elements and activities by Wingless signalling raises the possibility that some of the

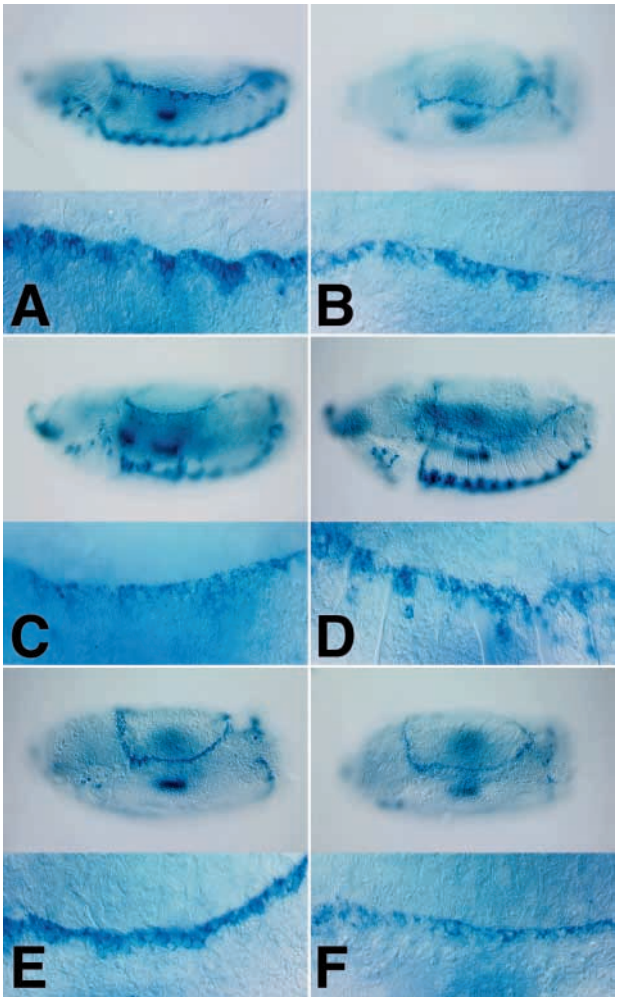

Fig. 4. Rescue of *dpp* expression levels following overexpression of Wg or Arm^{act}. Lateral view of stage 13 embryos stained by in situ hybridisation against *decapentaplegic*. *dpp* is expressed in the DME cells of wild-type embryos (A), and in one or two additional cells per segment ventrally localised, compare with the LE. In *wg*$^{-}$ (B), expression levels are lower and *dpp* expression is often lost in the posterior DME cells. After ubiquitous overexpression of Wg (C) or Arm^{act} (D), *dpp* expression levels are restored to normal. Interestingly, although the overexpression is ubiquitous, the expression pattern in stage 13 embryos is not altered. (E) In *wg*>AS>Wg embryo, *dpp* expression levels are rescued, in contrast to *wg*>AS> Arm^{act} embryos (F), which show a weak expression of *dpp* similar to the levels observed in *wg*$^{-}$ embryos.

effects of Wingless are mediated indirectly by Dpp. To test this, we increased the levels of Dpp signalling by overexpressing an activated form of the Dpp receptor Thick-veins (Tkv^{QD}) (Nellen et al., 1996) in *wg*$^{-}$ embryos. These levels of signalling are sufficient to cause a dorsalisation of the epidermis (Nellen et al., 1996) but only lead to a mild rescue of DME cell polarisation. Fmi localises to the membrane and is excluded from the LE in *wg*>da>Tkv^{QD} embryos. This localisation is observed with some delays compared with wild type and does not correlate with any elongation of the DME cells in the DV direction (data not shown). Similarly, activating the *dpp* pathway in the lateral epidermal cells does not result in their elongation. This suggests that the effect of the 'canonical' Wg signalling pathway on DME cell polarity is not simply mediated by activation of the Dpp pathway.

Altogether, these results challenge the proposal that in wild-type embryos the elongation of the ventral epidermal cells is induced by Dpp secreted from the DME cells and suggest that if Dpp contributes to the elongation of the epidermal cells, it requires an additional input from Wingless signalling.

Overexpression of Dishevelled in *wg* mutant embryos is not sufficient to rescue polarity defects

To analyse the possibility of a contribution of the PCP pathway in DC, we tested the ability of Dsh to rescue the *wg* mutant phenotype. In these experiments, we overexpressed Dsh with the ubiquitous driver daGal4 (*wg*>da>Dsh). We first assessed the ability of Dsh to rescue the naked cuticle and the expression of En in *wg*$^{-}$ embryos. Although overexpression of either Wg or Arm^{act} restored naked cuticle and exhibited a dominant effect, the overexpression of Dsh exhibits a partial rescue and only restores some naked cuticle (compare Fig. 5B′,C′, Fig. 2). A similar effect is observed with regard to the expression of En, which is fully rescued by ubiquitous expression of Wg or Arm^{act}, but only weakly rescued by the ubiquitous overexpression of Dsh (compare Fig. 5B″,C″, Fig. 2). Thus, overexpression of Dsh in a *wg*$^{-}$ embryo partly rescues the 'canonical' pathway, as assessed by the naked cuticle and En expression.

We then turned our attention to the process of dorsal closure. The cuticle of the *wg*>da>Dsh embryos is longer and its pattern much improved relative to that of *wg*$^{-}$ embryos (compare Fig. 6A,B with Fig. 6A′,B′). However, these embryos also show some 'warts' on the dorsal side, which are not observed following the overexpression of Wg or Arm^{act}, and which probably result from cell sorting defects associated with the defects of En expression (Fig. 5C″). Analysis of these embryos at the cellular level reveals that most of the DME cells show a weak elongation in the DV direction, which is badly coordinated along the LE (Fig. 6B″). Their polarity, as assessed by the subcellular localisation of Fmi, is rescued (Fig. 6B″), and the microtubule bundles are correctly oriented (not shown). However, the Myosin concentration at the LE is very delayed and is observed only very late while the zippering process is well advanced (data not shown). Dsh is thus not potent to rescue all the polarisation features of the DME cells in these experimental conditions.

We wondered if this might reflect low expression levels of Dsh in our experimental conditions. However, these experimental conditions provide *dsh* activity to rescue *dsh* mutant embryos (Fig. 7A,B), and cuticles of *wg*>da>Dsh embryos made at 29°C, a temperature at which the Gal4 is more active, did not show an improvement of the naked cuticle phenotype compared with the experiments carried out at 25°C (not shown).

These results indicate that there are some important differences between the activities of Wg, Arm^{act} and Dsh in these rescue experiments. Some of these effects could be due to interactions between the 'canonical' and PCP signalling pathways.

DIX and the DEP domains of Dishevelled have different contributions to DC

Dsh contains three highly conserved domains, the DIX, PDZ

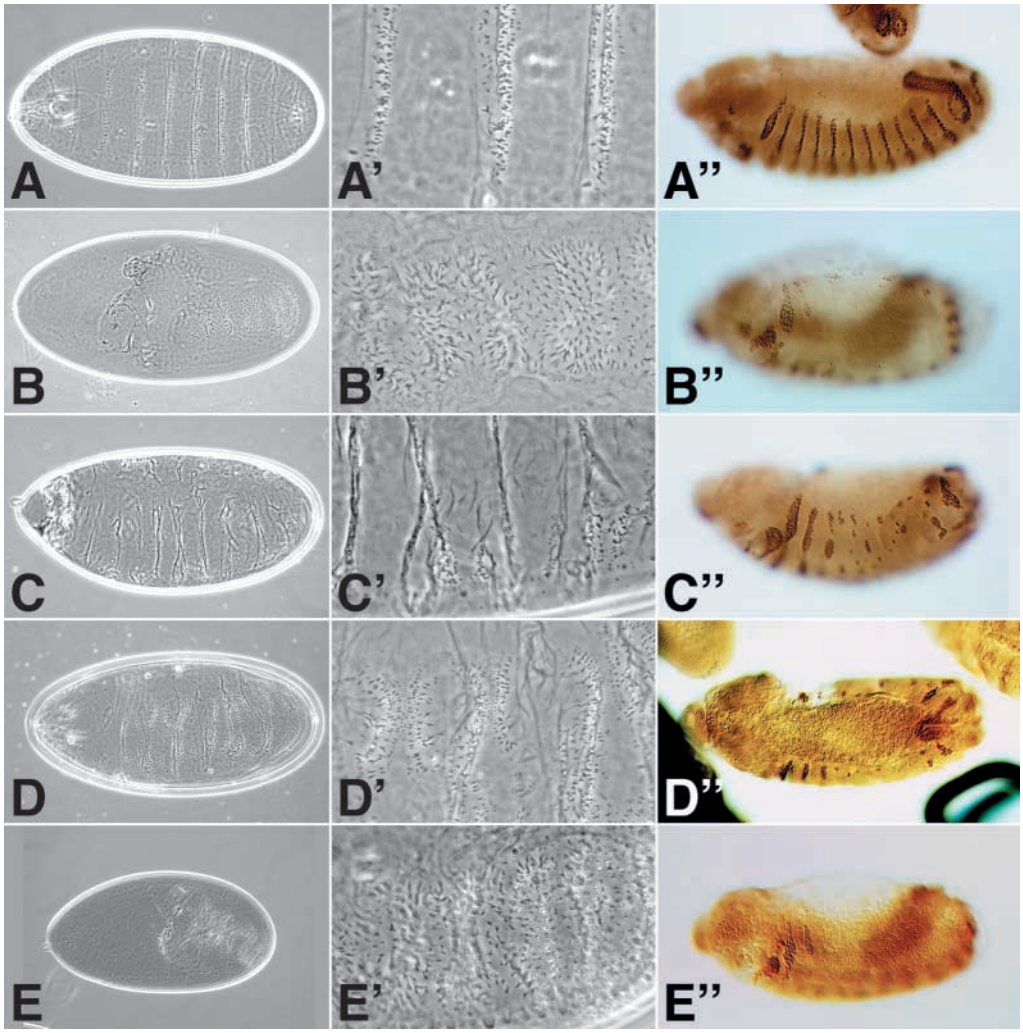

Fig. 5. Contribution of the Dsh domains to the rescue of 'canonical' features in *wg*⁻ embryos. (A′-E′) Magnification of the ventral side of the cuticle shown in A-E. (A″,B″,C″,E″) Lateral views; (D″) dorsal view. Cuticle (A-A′) and En expression pattern (A″) of wild-type embryos. (B-B″) *wg*⁻ embryos show a short cuticle (B) and a lawn of denticules on the ventral side (B′). The expression in stripe of En in the lateral and dorsal ectoderm is lost, except for very few cells (B″). Upon ubiquitous overexpression of Dsh (C,C′) or DshΔDEP (D,D′), the length of the cuticle and the presence of naked cuticle on the ventral side are rescued. A mild rescue of En expression is observed in *wg*>da>Dsh embryos (C″) and to a lesser extent in *wg*>da>DshΔDEP (D″). No rescue of either the length of the cuticle (E), the presence of naked cuticle (E′) or the expression of En (E″) is observed after ubiquitous overexpression of DshΔDIX. Moreover, the cuticle of *wg*>da>DshΔDIX embryos is shorter than the cuticle of *wg*⁻ embryos (compare E with B).

and DEP domains (for a review, see Wharton, 2003). The DEP domain mediates interaction of Dsh with the cell cortex and is required for PCP but not 'canonical' Wg signalling (Axelrod, 2001; Axelrod et al., 1998; Rothbacher et al., 2000), while the DIX domain is required for the 'canonical' Wg signalling but seems dispensable for PCP (Axelrod, 2001; Penton et al., 2002). To investigate further an involvement of the PCP pathway in the activities of the DME cells during DC, we repeated the rescue experiments of *wg*⁻ embryos using truncated forms of Dsh deleted for either the DEP (DshΔDEP) or the DIX (DshΔDIX) domain. Although overexpression of DshΔDEP leads to the partial rescue of naked cuticle (compare Fig. 5B′,D′) and of En expression (compare Fig. 5B″,D″), no naked cuticle nor rescue of En expression are observed in *wg*>da>DshΔDIX embryos (Fig. 5E′,E″). We thus confirm that DshΔDEP is able to signal within the 'canonical' Wg pathway but not DshΔDIX.

We then tested the ability of either protein to rescue DC in *wg*⁻ embryos. *wg*>da>DshΔDEP embryos are longer than *wg* mutants and their dorsal cuticle is improved as no hole is observed and only occasional warts can be seen (Fig. 6C′). The DME cells are oriented in the DV direction and most of them show a slight elongation in the DV direction when the zippering process has started. Simultaneously, Fmi is observed at the membrane and accumulates at the level of the ANCs (Fig. 6C″). Although no clear elongation of DME or ventral epidermal cells is observed, DC process is improved as two zippers, at the anterior and posterior ends of the embryo, are initiated, whereas only the posterior one is observed in *wg*⁻ embryos (not shown). By contrast, *wg*>da>DshΔDIX embryos have a shorter cuticle than *wg* mutants and show a more severe puckering and hole on the dorsal side (compare Fig. 6A,D with Fig. 6A′,D′). Furthermore, neither the shape nor the polarisation of DME cells is improved in these embryos (Fig. 6D″).

Thus, although DshΔDEP can rescue partially the DC defects of *wg* mutants, ubiquitous overexpression of DshΔDIX does not rescue any of the observed features confirming the requirement for the Wg 'canonical' pathway during DC.

The activity of Dsh is not required for dorsal closure when the Wg 'canonical' pathway is constitutively activated

Although overexpression of DshΔDEP in *wg* mutant embryos leads to a rescue comparable with the one observed with overexpression of Dsh, one cannot exclude the possibility that the PCP pathway plays a role in this process. Dsh function is not affected in *wg*⁻ embryos and the endogenous Dsh protein

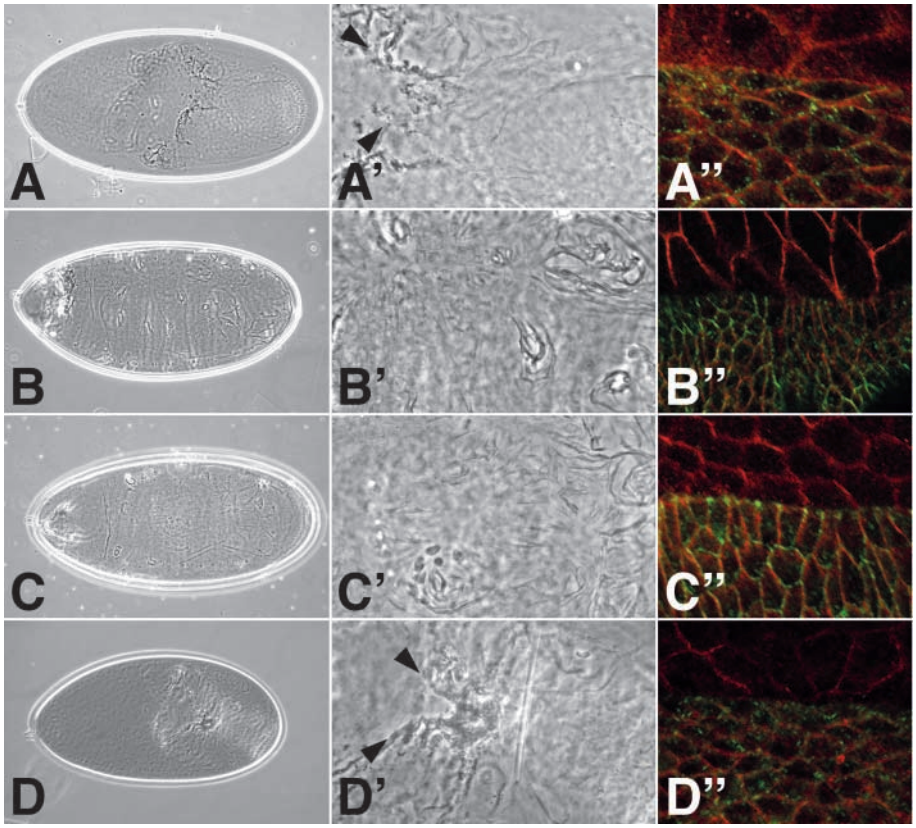

Fig. 6. Contribution of the Dsh domains to the rescue of dorsal closure features in *wg*⁻ embryos. (A-D′) Dorsal view of the cuticles shown in Fig. 5. Confocal high magnification pictures of late stage 13 embryos (A″,B″,D″) and early stage 14 (C″), oriented dorsal upwards and anterior leftwards. (A-A′) Cuticle of a *wg*⁻ embryo showing an anterior and a anterodorsal hole (arrowheads), as well as a short and folded cuticle on the dorsal side. After ubiquitous overexpression of Dsh (B) or DshΔDEP (C) in *wg*⁻ embryos, the length of the dorsal side of the cuticle is rescued and the anterodorsal hole suppressed. The dorsal cuticle of *wg*>da>Dsh embryos (B′) shows some warts in addition to a puckering on the midline, while the dorsal cuticle of *wg*>da>DshΔDEP embryos (C′) is disorganised with occasional warts. By contrast, the dorsal cuticle of *wg*>da>DshΔDIX embryos is very short and a strong dorsal hole is observed (D-D′, arrowheads). The cell organisation, as revealed by Fmi (green) and Dlg (red) staining (A″-D″), is partially rescued after overexpression of Dsh (B″) or DshΔDEP (C″), with DME cells showing a mild elongation in the DV direction and accumulation of Fmi at the membrane and weakly at ANCS. No rescue of cell shape or Fmi localisation is observed following overexpression of DshΔDIX (compare D″,A″).

is potent to mediate PCP. Thus, it is possible that in many of our experiments some of the observed rescue is due to an activity of the endogenous Dsh induced by interactions of the overexpressed forms with other regulatory proteins. To rule out this possibility, we generated *dsh* mutant embryos in which the 'canonical' Wg signalling pathway is constitutively activated through a loss of function of *sgg* (*sgg,dsh*^{GLC}).

In agreement with a role for the 'canonical' Wg pathway in DC, the cuticle of the *sgg,dsh*^{GLC} embryos is improved compared with that of *dsh*⁻ embryos. The embryos exhibit a severe defect in germband retraction but no dorsal hole is observed and the dorsal cuticle appears severely puckered (compare Fig. 7A,C with Fig. 7A′,C′). Furthermore, although in *dsh* mutants the DME cells do not elongate in the DV direction and show a 'dotty' cytoplasmic localisation of Fmi (Fig. 7A″), in embryos derived from *sgg,dsh*^{GLC} females DME cells are elongated in the DV direction and Fmi localises to the cell membrane as it does in the wild type (Fig. 7C″). These observations show that the function of Dsh is dispensable for the organisation of the DME cells when the 'canonical' Wg pathway is activated. They also indicate that the rescue of *wg* mutants by Armadillo is due to an activation of the 'canonical' Wg signalling pathway without a major contribution of the PCP pathway.

Discussion

The initiation of DC in *Drosophila* embryos correlates with the elongation and polarisation of the DME cells in the DV axis of the embryo. In parallel with this polarisation, a cable of F-actin assembles on the dorsal-most surface of these cells and promotes the formation of filopodia and lamellae during the final phases of the process. A functional link between the polarisation and the assembly of the cable of actin is supported by the observations that in mutants in which the DME cells do not elongate, there is no actin cable and no dynamic protrusions (Kaltschmidt et al., 2002). As a consequence, these embryos display defects and delays in the closure process. Embryos mutant for *wg* and *dsh* are good examples of this class.

Transcriptional control of the polarisation of the DME cells and DC by the β-catenin-dependent Wg pathway

The polarisation of the DME cells occurs in the plane of the epithelium and can be seen as a manifestation of the phenomenon of planar cell polarity (PCP). As a specific branch of Wg signalling has been implicated in PCP and there is evidence for an interaction between Dishevelled and JNK signalling during dorsal closure (Boutros et al., 1998), we tested whether there is a role for this mode of Wg signalling in the process of DC. Our results clearly show that the 'canonical' Wg signalling pathway that leads to activation of Armadillo and of the transcription of target genes is necessary and sufficient to restore the polarity of the DME cells and to promote a normal process of dorsal closure in a *wg* mutant embryo. Surprisingly, we find that the PCP pathway does not appear to play a major role in DC or the polarisation of the DME cells as activation of the 'canonical' pathway in the absence of *dsh* activity rescues the polarity and function of the DME cells. This conclusion is supported by the observation that although a moiety of Dishevelled that promotes Armadillo signalling is capable of rescuing the defects of *wg* mutants, a moiety that promotes JNK

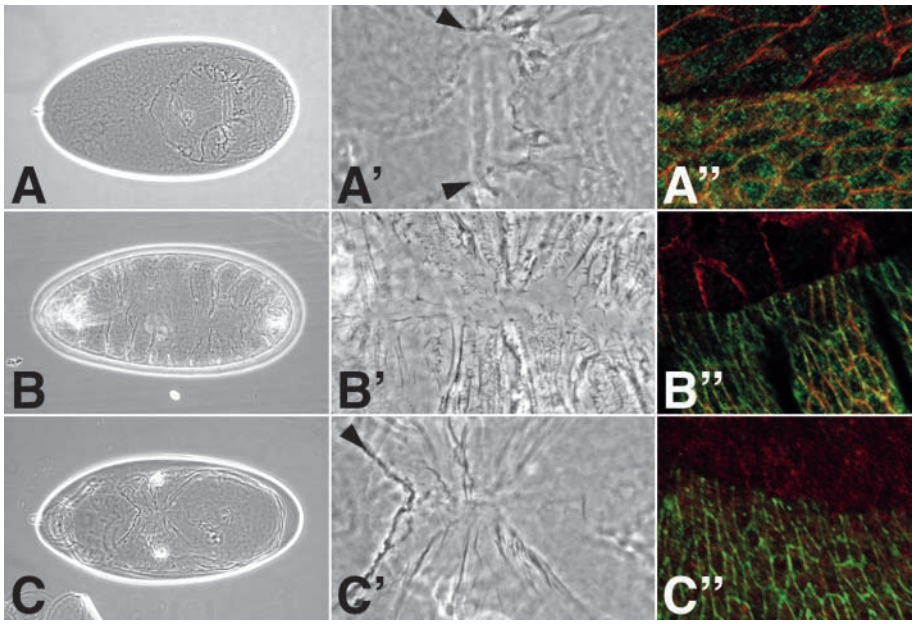

Fig. 7. Dsh is not required for dorsal closure to proceed when the 'canonical' Wg pathway is activated. Cuticles (A-C′), Fmi (green) and Dlg (red) antibody staining (A″-C″) of *dsh*^{GLC} (A-A″), *dsh*^{GLC}>da>Dsh (B-B″) and *sgg,dsh*^{GLC} (C-C″) embryos. The cuticle of *dsh*^{GLC} embryos presents an anterodorsal hole (A′, arrowheads) and is very short with most of the dorsal epidermis missing (A). In *dsh*^{GLC}>da>Dsh embryos, the length and the hole of the cuticle are rescued (B) and a strong puckering is now observed dorsally (B′). The cell morphology and polarity in *dsh*^{GLC} embryos is very similar to that observed in *wg*$^{-}$ embryos (compare A″ with Fig. 1B): the DME cells are stretched in the AP direction while the other dorsal epidermal cells present a rather isotropic shape. Fmi does not localise at the membrane but shows a 'dotty' localisation in the epidermis. Upon ubiquitous overexpression of Dsh in *dsh*^{GLC}, the DME cells elongation in the DV direction is rescued (B″). Fmi does not localise at the membrane in stage 13 embryos (not shown), but shows membrane localisation and ANCs accumulation in late stage 13-early stage 14 embryos (B″). The cuticle of *sgg,dsh*^{GLC} embryos (C), which lacks both *dsh* and *sgg* function, presents a strong retraction defect and a ventral naked cuticle characteristic of the constitutive activation of the 'canonical' Wg pathway associated with the loss of *sgg* function. They present an anterior hole (arrowhead) and a puckering of the dorsal midline (C′). Although the retraction defect makes the observations difficult, the cuticle is longer than in *dsh*^{GLC} embryos (compare with A). Fmi localises at the membrane and concentrates at ANCs in *sgg,dsh*^{GLC} embryos (C″) and the main direction of DME cells is restored to the DV axis.

signalling and PCP does not. Altogether, these results indicate that the polarisation and activity of the DME cells during dorsal closure requires Armadillo/β-catenin-dependent Wg signalling. Furthermore, this requirement is restricted to the epidermis as activation of Wg signalling in the amnioserosa has no effect on the epidermis.

The polarisation of the DME cells and subsequent dynamics of actin at the LE can be construed as the development of the leading edge of a motile cell and to a certain extent is akin to an epidermal/mesenchymal transition (EMT), as one of the features of this process is the reorganisation of the actin cytoskeleton and the acquisition of motility by the cells. In this regard, it is interesting to note that β-catenin-dependent Wnt signalling has been implicated in EMT both in normal and cancerous cells (Martinez Arias, 2001; Muller et al., 2002) and that therefore there are precedents for the involvement of the β-catenin-mediated transcriptional regulation in the development of actin dynamics. However, the targets of the Wnt pathway mediating this process are not known.

Dpp is not the central target of the β-catenin-dependent Wingless signalling

It has been suggested that the *Drosophila* BMP homologue Dpp is a central effector of dorsal closure (Affolter et al., 1994; Glise and Noselli, 1997; Hou et al., 1997; Zeitlinger et al., 1997). Embryos mutant for *dpp* signalling exhibit defects in dorsal closure. *dpp* is expressed in the DME cells and has been proposed to act as a long range signal for the elongation of the more ventral cells (Glise and Noselli, 1997; Hou et al., 1997; Riesgo-Escovar and Hafen, 1997). Here, we show that Wingless is required for the correct maintenance of *dpp* expression in the DME cells, although in our experiments the input is less significant than has been reported before (McEwen et al., 2000). Altogether, these observations suggest that some of the activity of Wingless during DC is mediated by Dpp. Indeed, when we ubiquitously activated the Dpp pathway by the means of an activated form of its receptor Tkv, we observe some rescue of the polarity of the DME cells. However, although in this case the DME cells orient themselves in the DV direction and Fmi localises as it does in wild type, neither the DME nor the ventral epidermal cells elongate, and the DC process is not substantially improved. This contrasts with the full rescue of both the polarisation of DME cells and the DC process following ubiquitous activation of the β-catenin-dependent Wg pathway. Thus, if Dpp contributes to DC, it is not as the only target of Wg signalling.

Expression of Wingless from the amnioserosa in *wg* mutants induces high and continuous levels of *dpp* in the DME cells together with some rescue of the polarity of the DME cells but without any effect on the elongation of these or the more ventral cells. This rescue is very similar to the one observed with ubiquitous expression of the activated Tkv. These results indicate that Dpp does not act as a long-range signal for the elongation of the more ventral epidermal cells as rescue of Dpp expression in the DME cells or activation of Dpp signalling throughout the epidermis in *wg* mutants does not lead to the elongation of the more ventral cells. A similar conclusion had been suggested from the observation that epidermal cells initially elongate in the absence of Dpp signalling but resume their polygonal shape soon after (Ricos et al., 1999; Zeitlinger et al., 1997). However, an alternative explanation for our observations is that the elongation of the ventral epidermal cells requires inputs from both Dpp and Wingless signalling.

Altogether, these observations indicate that Dpp is not the only effector of Wingless during DC and indicates that Wingless signalling via Armadillo controls genes that act either in parallel or together with those regulated by JNK and Dpp.

β-catenin dependent Wg signalling plays a permissive role for the polarisation of the DME cells and dorsal closure

We have shown that Wingless is required in the epidermal cells but does not act as a polarising signal, as ubiquitous activation of the pathway rescues the defects of *wg* mutants. An important observation of our experiments is that the DME cells of *wg* mutant embryos display a polarity and an elongation at the very final stages of DC, suggesting that the polarisation signal is received correctly by the DME cells but that in the absence of Wingless signalling there is a delay either in its interpretation or in its materialisation. This, together with the lack of importance of a fixed source of Wingless for the polarisation of the DME cells (Kaltschmidt et al., 2002), suggests that Wingless makes the DME cells competent to interpret a pre-existing polarisation signal. Such a permissive role of Wingless signalling had been suggested by McEwen et al. (McEwen et al., 2000). It has, furthermore, been emphasized in other transcriptional events (Martinez Arias, 2003).

In the case of DC, the permissive function of Wingless signalling translates itself in the correct coordination of the different events, i.e. the cells have to elongate at the right time and the activity of their cytoskeleton has to be properly linked to other events some of which are transcriptional. Failure to do this will result in defects in dorsal closure. These observations raise the question of the temporal requirements for Wg signalling during DC.

ASGal4 drives expression of Wingless from the elongation of the germband to the end of DC. However, when driven by ASGal4, Wg can only be detected over the epidermal cells during the first phase of DC. This is probably due to the inability of Wingless to cross the deep fold existing between the AS and the epidermis during germband retraction and the zippering process. The provision of Wg from the amnioserosa rescues the defects of the DME cells of *wingless* mutants but not those of the more ventral cells. Although the DME cells, in contact with the AS, might have received Wg signal at the very onset of the overexpression (around stage 9-10), the more ventral epidermal cells seem to see the signal too late to elongate, suggesting that Wg signalling is required before the beginning of DC for the cell shape and polarity changes. A hint at the timing of Wnt requirement for DC is provided by experiments using a temperature sensitive allele of *wingless* (Bejsovec and Martinez Arias, 1991). Removal of *wingless* function between 4 and 4.5 hours after egg laying, i.e. at stages 9-10, affects the shape of the dorsal cuticle in a way similar to DC defects. This suggests that the polarising signal must occur very early, during germband elongation.

Establishment versus propagation. What is the function of the PCP pathway?

The notion of PCP has emerged from studies of the mechanism that determines the orientation of the hairs in the cells of the wing of *Drosophila* (Eaton, 2003). A number of studies have revealed the existence of protein complexes that mediate this orientation by becoming asymmetrically distributed between the proximal and distal membranes of the epidermal cells. Thus, while Flamingo becomes localised equally between the proximal and the distal sides of the cell (Usui et al., 1999), the distal side of the cell accumulates a complex composed of Frizzled and Dishevelled (Axelrod, 2001; Shimada et al., 2001; Strutt, 2001) and the proximal side accumulates a complex formed by Strabismus and Prickled (Bastock et al., 2003; Tree et al., 2002). Genetic analysis of these complexes has led to the formulation of a model which describes the propagation of the polarity from one cell to its neighbours (reviewed by Eaton, 2003), but which says nothing about the origin of the polarity that is being propagated. In this model, Dsh, like Strabismus, Prickled or Frizzled, is an essential component of the mechanism that propagates the polarity.

The observation of polarised distributions of Fmi, Dsh and Fz in the DME cells during dorsal closure has led us to suggest a link between the polarisation of these cells and the process of PCP. However, we have not found a requirement for elements of this pathway in dorsal closure. In particular, the PCP function of Dsh is not required for the polarisation of the DME cells and the polarised localisation of Fmi, which was quite unexpected considering the interdependence of Dsh and Fmi for their asymmetric localisation in the wing cells (Shimada et al., 2001). This asymmetric distribution of Fmi is likely to play a role in the polarised actin dynamics in response to the polarity signal. Although this may appear surprising, it also invites a consideration of the notion of PCP.

The PCP pathway has been defined in a context of propagation of a polarity but not of its initial definition. In fact none of the experiments performed in the wing of *Drosophila* address the origin of the polarity that is being propagated. In DC, however, the process that we observe in the asymmetric distribution of proteins in the DME cells reflects the establishment of a polarity and not its propagation. From this perspective, the lack of a requirement for the PCP branch of Wnt signalling might not be that surprising as PCP Wnt signalling might be related to propagation or coordination of a polarity signal that has been generated in a different manner. However, the requirement for the β-catenin-dependent Wg pathway might be significant and indicate the requirement for a transcriptional event in the establishment of PCP. This observation might also apply to the wing.

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