Thorax closure in Drosophila: involvement of Fos and the JNK pathway

Julia Zeitlinger and Dirk Bohmann*

European Molecular Biology Laboratory (EMBL), Meyerhofstrasse 1, 69117 Heidelberg, Germany

*Author for correspondence (e-mail: bohmann@embl-heidelberg.de)

Accepted 5 June; published on WWW 5 August 1999

SUMMARY

Dorsal closure, a morphogenetic movement during Drosophila embryogenesis, is controlled by the Drosophila JNK pathway, D-Fos and the phosphatase Puckered (Puc). To identify principles of epithelial closure processes, we studied another cell sheet movement that we term thorax closure, the joining of the parts of the wing imaginal discs which give rise to the adult thorax during metamorphosis. In thorax closure a special row of margin cells express puc and accumulate prominent actin fibres during midline attachment. Genetic data indicate a requirement of D-Fos and the JNK pathway for thorax closure, and a negative regulatory role of Puc. Furthermore, puc expression co-localises with elevated levels of D-Fos, is reduced in a JNK or D-Fos loss-of-function background and is ectopically induced after JNK activation. This suggests that Puc acts downstream of the JNK pathway and D-Fos to mediate a negative feed-back loop. Therefore, the molecular circuitry required for thorax closure is very similar to the one directing dorsal closure in the embryo, even though the tissues are not related. This finding supports the hypothesis that the mechanism controlling dorsal closure has been co-opted for thorax closure in the evolution of insect metamorphosis and may represent a more widely used functional module for tissue closure in other species as well.

Key words: Drosophila, Fos, Dpp, JNK, Dorsal closure, Metamorphosis

INTRODUCTION

The molecular control of morphogenesis is still poorly understood, even in simple and genetically readily accessible organisms. An advance has been the genetic dissection of dorsal closure during mid-embryogenesis of Drosophila. Before this morphogenetic movement is initiated, the dorsal side of the embryo is covered by amnioserosa, an extraembryonic tissue that disintegrates at later stages. During dorsal closure, a concerted dorsalward stretching of the lateral embryonic epidermis encloses the amnioserosa from both sides. Eventually, the two epidermal edges meet at the dorsal midline and attach. In this way, the entire embryo becomes surrounded with epidermis.

Genetic studies have revealed a requirement for cytoskeletal components and a number of signal transduction molecules for dorsal closure (reviewed by Knust, 1996; Martin-Blanco, 1997; Noselli, 1998). The latter include the Drosophila AP-1 transcription factors, D-Jun (Hou et al., 1997; Kockel et al., 1997; Riesgo-Escovar and Hafen, 1997b) and D-Fos known as Kayak (Kay) (Riesgo-Escovar and Hafen, 1997a; Zeitlinger et al., 1997) and an upstream kinase cascade homologous to the Jun-NH2-terminal kinase (JNK) pathway in mammals. The Drosophila homologue of JNK, Basket, has been shown to phosphorylate D-Jun in vitro (Riesgo-Escovar et al., 1996; Sluss et al., 1996). Basket itself is phosphorylated and activated by Hemipterous (Hep), the homologue of JNK-kinase (JNKK) (Glise et al., 1995). The upstream components of the Drosophila JNK pathway are not yet completely defined but include the kinase Misshapen, of which the closest human homologue is NCK-interacting kinase (NIK) (Su et al., 1998; Treisman et al., 1997). All components of the pathway are required for the expression of decapentaplegic (dpp) and puckered (puc) (Glise and Noselli, 1997; Hou et al., 1997; Riesgo-Escovar and Hafen, 1997a,b; Sluss and Davis, 1997; Zeitlinger et al., 1997). Puc is a dual-specificity phosphatase that selectively inactivates Basket and, thus, is thought to act in a negative feed-back loop to limit the strength and/or duration of JNK activity and dpp expression (Martin-Blanco et al., 1998). Dpp, a member of the BMP family, is also essential for dorsal closure since some loss-of-function mutations affecting dpp signalling through its cognate receptors thick veins (tkv) and punt (put) show defects in dorsal closure (Affolter et al., 1994; Letsou et al., 1995, Hudson et al., 1998).

We noticed that semilethal hypomorphic alleles of some of the genes that are required for dorsal closure give rise to an interesting abnormal adult phenotype, suggesting that there is an additional requirement for these genes during later development: homozygous animals of mutant alleles of D-fos (Zeitlinger et al., 1997), hep (Glise et al., 1995; Zeitlinger et al., 1997), pannier (pnr) (Heitzler et al., 1995) and components of the Dpp pathway (Chen et al., 1998; Hudson et al., 1998; Morimura et al., 1996; Simin et al., 1998; Spencer et al., 1982) show a cleft at the dorsal midline of the thorax and neighbouring bristles are abnormally parted to both sides.

How can we explain this phenotype? Since Drosophila is a holometabolous insect, the adult form (imago) is assembled de novo during metamorphosis. The epidermis develops from...
imaginal discs, epithelial sacs that derive from internal invaginations of the embryonic body wall and proliferate during larval stages. Such an epithelial sac consists of a columnar epithelium on one side, the imaginal disc proper, a squamous epithelium termed peripodial membrane on the other side, and a peripodial stalk through which the imaginal disc is attached to the larval epidermis. During and after puparium formation (pupariation), the imaginal discs evert (the epithelial sac is turned inside out) and then assemble to form the continuous epidermal structure of the future adult (Fig. 1). At the same time, the larval epidermis is histolyzed. The first 12 hours after pupariation (AP) in which these extensive morphogenetic movements occur are referred to as the prepupal stages (Fristrom and Fristrom, 1993). The dorsal thorax, termed notum, develops from the dorsal parts of the two wing imaginal discs (Fig. 1). They approach each other from both sides and fuse at the midline between 6 and 8 hours after pupariation to close the thorax (Fristrom and Fristrom, 1993). Thus, this process represents a form of ‘dorsal closure’, as well. To distinguish between embryonic dorsal closure and this later dorsal closure, which forms the adult thorax, we will refer to the latter as ‘thorax closure’.

We wondered whether thorax closure could be similar to embryonic dorsal closure, both in terms of morphogenesis and molecular control. Dorsal closure during embryogenesis is known to require cell shape changes but not proliferation or cell rearrangements (reviewed by Knust, 1996). In prepupal morphogenesis too, proliferation or major cell rearrangements do not appear to be the driving force (reviewed in Fristrom and Fristrom, 1993). Although proliferation still persists during thorax closure at a low level, it does not seem to be required for morphogenesis. When late third instar larvae are irradiated to prevent cell division, a complete thorax is still established. Furthermore, imaginal discs can develop and acquire their pupal shape in vitro under certain conditions (Fristrom et al., 1973). In this system, treatment with cytchalasin B, which depolymerizes filamentous actin, reversibly inhibits imaginal disc morphogenesis, whereas drugs that inhibit DNA synthesis do not interfere with their development (Fristrom and Fristrom, 1993; Mandaron and Sengel, 1973). Thus, the cytoskeleton appears to play a role in these morphogenetic movements.

Here we have analysed the process of thorax closure and investigated the role of the JNK pathway, D-Fos and Puc genetically. We describe striking similarities between the two epithelial movements, both at the morphological and molecular level. These findings support the existence of an evolutionarily related molecular programme that regulates epithelial closure in different contexts.

**MATERIALS AND METHODS**

**Drosophila strains and genetics**

Fly strains are as described: *pnr-Gal4* (Calleja et al., 1996), *ap-Gal4* (Calleja et al., 1996), *dpp^{h3}-Gal4* (Morimura et al., 1996), UAS-EGFP and UAS-GFP (Yeh et al., 1995; Brand and Perrimon, 1993), UAS-Gei (Boutros et al., 1998), UAS-D-Fos^{394} (Zeitlinger et al., 1997), *kan^1* and *kan^2* (Zeitlinger et al., 1997), *puc^{394}* (Martin-Blanco et al., 1998; Ring and Martinez-Arias, 1993).

For the analysis of prepupae, crosses and stocks to analyse were kept in cages. Eggs were collected from apple juice plates every day.

**Fig. 1.** The development of the dorsal thorax from the wing imaginal discs. A dorsal view is shown with anterior up. The expression domain of *pnr* (green) marks the dorsalmost stripe of the future adult epidermis. The cells at the margin of the wing disc proper (blue) will attach to other imaginal discs to give rise to a continuous adult epidermis. (B-D) At 6-9 hours after pupariation (AP), the margin cells are marked by the expression of *puc* (see Figs 4-6). Additional markers are the three oblique muscles (red) which do not histolyze during pupariation (Fernandes et al., 1991). (A) In third instar larvae, the wing imaginal disc is attached to the larval body wall by the peripodial stalk (ps). Starting during pupariation, the disc epithelium everts through the widened peripodial stalk (upper arrow) and spreads inside the pupal case to replace the larval epidermis. At the same time, the wing blade develops through invagination of the disc epithelium (lower arrow). (B) At 6 hours AP, the dorsal parts approach each other (arrows) until they finally attach to each other at around 7 hours AP. The disc margin on the ventral side (dashed line) attaches to the leg discs to complete the ring-like structure of the thorax complex. (C) At around 8 hours AP, the anterior part of the future notum folds inside (arrow and dashed line), presumably to attach to the eye-antennal imaginal disc before it moves anteriorly. (D) At 9 hours AP, during head eversion (arrow), the wing imaginal disc is attached to all its neighbour imaginal discs: anteriorly to the eye-antennal disc (ea) and the dorsal prothoracic disc (dp), and posteriorly to the abdomen (a) and haltere disc (h). The border between them is marked in blue. Evidently, in order to obtain the final shape of the notum, further tissue movements will be required. The scutellum (s) will presumably form by a dorsoposterior protrusion.
Fig. 2. pnr expression (green) and actin localisation (red or white) in wild-type and kay\(^2\) prepupae. (A-F) Dorsal views with anterior up; (G-I) xz sections with dorsal up. (A,D,E,G) The left side of the prepupa is shown only. (A) At 6 hours AP, the future dorsal thorax, as marked by pnr expression (dashed line), typically lies on top of the three oblique musculature (seen in red) and close to the degenerating larval epidermis (dle), which the imaginal disc replaces. (B) At 8 hours AP, the two wing imaginal discs have attached at the dorsal midline and an anterior folding-in is seen. Also note the haltere disc (h). (C) At 9 hours AP, the head formed by the eye-antennal discs (ea) has everted and is attached to the future notum. Together with the dorsal prothoracic discs (dp), the haltere discs (h) and the abdomen (a), they now form a continuous epidermal structure. Note that, in addition to pnr expression, puc expression has been visualised by X-Gal staining of puc\(^{E69}\). (D) In a kay\(^2\) homozygous background, thorax closure defects are observed at 6 hours AP. The thoracic epithelium has not moved over the trachea (t) and the three oblique muscles (m) underneath. (E) Even more severe defects are observed at later stages of kay\(^2\) prepupae. The epithelium has retracted and fallen back into a folded state similar to that in third instar larvae. Note that filamentous actin is nevertheless present in the putative dorsal midline cells (arrows). (F) Filamentous actin is particularly dense at the dorsal thoracic midline of 8 hours AP prepupae. Also note the shape of the cells which are, unlike in dorsal closure, not elongated. (G) In an xz section of the dorsal thoracic epithelium at 7 hours AP, actin bundles become visible at the future dorsal midline (arrow). Note the striated oblique muscle (m) underneath. (H) At 8 hours, an xz section reveals that actin bundles are particularly dense at the site of attachment of the degenerating larval epidermis (dle) underneath characterised by large nuclei. (I) Another example of a prepupa at 8 hours AP, probably slightly later than the one shown in H. The actin bundles at the midline are predominantly localised basally in the epithelium. The genotypes are (A,B,F-I) pnr-Gal4\(^+/+\), UAS-EGFP\(^+/+\), (C) puc\(^{E69}\), pnr-Gal4\(^+/+\); UAS-EGFP\(^+/+\), (D,E) pnr-Gal4, kay\(^2\)/UAS-GFP, kay\(^2\). The power of magnification used in A,D,E are two times higher than in B,D and F-I are four times higher.

Dissection and staining

For morphological studies and X-Gal stainings, prepupae aged 6-9 hours AP were dissected in PBS in a way that leaves the thorax complex intact and protected inside the anterior part of the pupal case. Using two pairs of forceps, the pupal case was torn into two halves. While holding the anterior spiracles with one pair of forceps, intestines, salivary glands and, preferentially, brain and eye imaginal discs were removed from the anterior half of the pupal case. In this way, only the thoracic complex, consisting of the wing and leg imaginal discs, stays inside by being attached to the mouth hooks. For immunostainings, isolated wing imaginal discs were dissected out of prepupae aged 5-7 hours AP, or third instar larvae were dissected by turning them inside out.

All samples were fixed for 15-30 minutes in 4% formaldehyde in PBS and transferred to an Eppendorf tube containing 1.5 ml PBS with 0.1% Tween 20 (PBT). They were then washed twice with PBT.

For X-Gal stainings, samples were incubated with X-Gal staining solution for 75-90 minutes at 37°C while shaking, followed by two washes with PBT. Immunostainings were performed using standard techniques. The antiserum diluted in the following way: rabbit α D-Fos 1:500 (Zeitlinger et al., 1997), rabbit α D-Jun 1:1500 (Bohmann et al., 1994), mouse α β-Gal (Promega) 1:1000. Phalloidin staining was performed by TRITC-coupled phalloidin (Sigma) in 4%
formaldehyde-PBT for 30 minutes (after immunostaining), followed by two washes with PBT.

70% glycerol, containing 2.5% DABCO, was added as mounting medium in which the samples sank to the bottom overnight before dissection. The intact thorax complex samples were dissected out of the piece of pupal case using forceps (as if squeezing out a tube of toothpaste) and mounted on a slide supported by coverslips.

Pictures were taken with a Leica confocal microscope. In most pictures shown, sections were made from the layer of interest and composite projections are shown.

RESULTS
The morphogenetic movement of thorax closure
Because numerous Drosophila mutants show defects in both dorsal closure and thorax closure, we investigated whether there might be morphological similarities between the two processes. In order to mark and visualise the dorsal parts of the wing imaginal discs that fuse during thorax closure, we used the UAS-Gal4 system (Brand and Perrimon, 1993) to express EGFP in the expression domain of pnr, a gene encoding a GATA transcription factor whose expression is restricted to dorsal tissues throughout development (Calleja et al., 1996; Heitzler et al., 1996). The prepupae were then dissected in a way that leaves the entire thorax complex intact (see Materials and Methods) and different stages were inspected by confocal microscopy.

In addition, actin filaments were visualised by staining with phallodin to monitor the behaviour of the cytoskeleton during this process. Phallodin also stains three oblique muscles on each side, a useful marker during thorax closure (described in Fernandes et al., 1991).

Already in third instar wing imaginal discs, pnr expression marks the dorsal part, the future medial notum (Heitzler et al., 1996; Fig. 1A). At around 6 hours AP (Figs 1B, 2A), after eversion, the dorsal parts of the two wing imaginal discs spread towards the dorsal midline, while the larval epidermis degenerates (Fig. 2A). When they subsequently meet and attach to each other at around 7 hours AP, filamentous actin becomes visible at the medial edge of the epithelium (Fig. 2F-H). These actin bundles at the dorsal midline are most abundant at 8 hours AP (Fig. 2F-H) and are predominantly localised basally (Fig. 2I).

In summary, the process of thorax closure resembles embryonic dorsal closure at a tissue-morphological level: two epithelial sheets with a straight margin approach each other, meet at the dorsal midline, and attach. The actin organisation seen along the margin of the epithelium is reminiscent of the accumulation of actin along the leading edge of the closing embryo (Young et al., 1993). However, in contrast to the simple epithelial stretching of embryonic dorsal closure, the morphogenetic movements involved in thorax closure appear to be more complex: most cells are of polygonal shape and not obviously elongated along the dorsoventral axis (Fig. 2F and data not shown). Furthermore, the tissue movements also include unfolding (as part of the eversion) and an anterior folding-in during midline fusion (Figs 1C, 2B) with subsequent back folding during head eversion (Figs 1D, 2C).

Having established a system to monitor the progress of thorax closure, we analysed the tissue movements in a mutant background that gives rise to a cleft phenotype in adults. We used the hypomorphic mutation in D-fos, kay2 in this experiment (Fig. 2D,E). It revealed that the dorsomedialward spreading of the epithelium is already abnormal at 6 hours AP in most kay2 prepupae. While, in a wild-type background, the pnr expression domain of the wing imaginal disc is found on top of the three oblique muscles and close to the degenerating larval epidermis (Fig. 2A), the corresponding epithelium in kay2 prepupae of this stage has failed to reach this position and is still located more laterally (Fig. 2D). At 8 hours AP, the spreading epithelium often appears to have retracted and fallen back into its original folded position found at earlier stages, although filamentous actin typical of this stage is detectable (Fig. 2E). These findings strongly argue that the defects observed in kay2 adult animals result from defects in thorax closure during prepupal stages.

Genetic requirement for D-Fos and JNK during thorax closure and negative regulation by Puc
The thoracic cleft phenotype observed with hypomorphic mutations in D-fos (kay2) and hep (hep1) (Zeitlinger et al., 1997) suggests that D-Fos and the JNK pathway are involved in thorax morphogenesis. To confirm that the cleft phenotype is a result of a D-fos loss-of-function condition, we expressed a dominant negative form of D-fos (UAS-D-Fos[ DEL]) under the control of pnr-Gal4 (see Figs 1 and 2 for pnr expression). This resulted in the appearance of a marked cleft in the thorax (Fig. 3D). A similar phenotype was obtained by overexpressing Puc (UAS-Puc) in the pnr (Fig. 3E). In the embryo, overexpression of Puc phenocopies loss-of-function mutations in the JNK pathway, consistent with the proposed function of Puc as a phosphatase that negatively regulates the JNK pathway by dephosphorylation of Basket (Martin-Blanco et al., 1998). The fact that this is also true in thorax closure represents further evidence that the JNK pathway is involved in thorax closure.

Next, we tested whether D-Fos genetically interacts with components of the JNK pathway during thorax closure. In contrast to the D-fos hypomorphic mutant kay2, kay1 represents a D-fos null allele (a deficiency, Zeitlinger et al., 1997). The heterozygous allelic combination (kay1/kay2) is strictly lethal, but can be rescued by ubiquitous expression of D-Fos under a heterologous promoter (Riesgo-Escovar and Hafen, 1997a; Zeitlinger et al., 1997). Strikingly, the lethality of kay1/kay2 could also be rescued by eliminating one copy of the wild type puc gene (kay1/kay1 puc[E69]) in Fig. 3F]. We could recover more than 50% of the expected Mendelian frequency (see also Materials and Methods). Thus, puc[E69] has a dominant effect in a kay1 mutant background, even though heterozygosity for puc[E69] has no phenotypic effects in an otherwise wild-type fly. Furthermore, not only the lethality but also the thorax cleft phenotype of kay1 mutant flies could be dominantly rescued. The cleft phenotype of the rescued kay1/kay1 puc flies ranges from strong to very mild (Fig. 3F). Heterozygous puc[E69] in a kay2 homozygous background (kay2/kay2 puc[E69]) gave rise to a stable stock in which most flies show a very mild or no thorax cleft at all (Fig. 3G). Therefore, the puc mutation has a dominant effect on thorax closure and two conclusions can be drawn. First, Puc must be expressed during thorax closure. Second, as in dorsal closure, Puc negatively regulates the pathway in which D-Fos is acting during thorax closure.

To confirm that Puc acts specifically on the JNK pathway
during thorax closure, we generated a stock containing both the \textit{hep}^1 hypomorphic mutation and a mutant \textit{puc} allele (\textit{hep}^1/FM6; \textit{puc}^{E69}/TM3). As shown previously (Martin-Blanco et al., 1998), heterozygous mutant \textit{puc} rescues the lethality of embryos which are maternally and zygotically \textit{hep}^1 (\textit{hep}^1; \textit{puc}^{E69}/\+)

Surprisingly, however, the converse was also true: homozygous or hemizygous \textit{hep}^1 also rescued the lethality of homozygous \textit{puc}^{E69} mutants (\textit{hep}^1; \textit{puc}^{E69}). The rescued flies look remarkably normal, with the exception that, consistently, some females show macrochaetes with a kink (Fig. 2H, arrows). These data suggest that \textit{Puc} acts specifically and exclusively on the JNK pathway in vitro, including thorax closure.

A trivial explanation for the results described above would be that the thorax cleft phenotype represents a secondary consequence of a viable mild dorsal closure defect. To exclude this possibility, we expressed \textit{UAS-D-Fos}^{E69} or \textit{UAS-Puc} with \textit{apterous-Gal4} (\textit{ap-Gal4}), a driver that expresses in the dorsal portion of the wing imaginal disc epithelium, including the future notum, but not in the embryonic ectoderm (Calleja et al., 1996; Cohen et al., 1992). In both cases, thorax fusion defects are observed (Fig. 3LJ). Therefore, D-Fos and JNK activity are required in the wing imaginal disc, independently of their embryonic function. Taken together, these data indicate a strong genetic interaction between \textit{puc}, \textit{hep} and \textit{puc} during thorax closure and provide evidence that these proteins act in the same signalling cascade as in the embryo.

**Puc expression in margin cells and co-localisation with regions of high D-Fos expression**

Next, we investigated in which cells \textit{Puc} and D-Fos are expressed during thorax closure and whether their expression patterns are analogous to the ones observed in the embryo. During dorsal closure, AP-1 and the JNK signalling cascade are required in the leading-edge cells for the expression of \textit{puc}. This requirement for AP-1 in the leading edge correlates with a higher expression of both D-Jun and D-Fos (Kockel et al., 1997; Zeitlinger et al., 1997).

We stained wing imaginal discs of third instar larvae and prepupae with antibodies specific for D-Fos and monitored \textit{puc} expression with the enhancer trap line \textit{puc}^{E69} (Figs 4, 5). In the wing imaginal disc of third instar larvae, \textit{puc} is first expressed in the peripodial stalk as it widens for the eversion of the disc. Subsequently, \textit{puc} expression spreads along the margin of the disc but remains especially abundant in the region close to the peripodial stalk that will give rise to the thorax. D-Fos (as well as D-Jun, data not shown), in contrast, is expressed throughout the disc but at different levels. The highest expression is found in the peripodial membrane, the peripodial stalk, a region close by the peripodial stalk and the margins of the disc proper (Fig. 4A). Remarkably, there is a correlation between \textit{puc} expression and high protein levels of D-Fos (and D-Jun, data not shown). With increasing distance from the region of \textit{puc} expression, D-Fos expression gradually fades out (Fig. 4E). This region of \textit{puc} expression also correlates with the accumulation of actin fibres (Fig. 4F), although filamentous actin is also found in other regions of the disc (Fig. 4C). Taken together, this co-localisation between elevated levels of D-Fos/D-Jun, \textit{puc} and actin is reminiscent of that observed at the onset of dorsal closure in the embryo.

This correlation also holds at a later stage, in the prepupae during thorax closure (Fig. 5). Cells that express \textit{puc} also show high levels of D-Fos/D-Jun expression. Moreover, there is another resemblance to dorsal closure, the morphological structure in which \textit{puc} is expressed. During the movement of the two epidermal sheets towards each other, \textit{puc} expression is observed in the cells along the margin that will form the future dorsal midline. It is particularly abundant during the epithelial fusion when actin filaments become prominent (Fig. 5A). It is possible to interpret this cell row with \textit{puc} expression as a structure analogous to the ‘leading edge’ in the embryo, although, it is not as well arranged. The number of \textit{puc}-expressing cells can vary slightly along the margin and some scattered \textit{puc}-expressing cells are found nearby. This correlates with the less orderly executed morphogenetic movement in thorax closure as compared to the very regular stretching of the epidermal sheet during dorsal closure.

In summary, D-Fos/D-Jun and \textit{Puc} are coexpressed during thorax closure in a manner that is comparable to dorsal closure, which is consistent with our genetic data. Moreover, the cells where D-Fos and Puc are coexpressed resemble the leading edge cells of dorsal closure, at least based on their position in the system and their tendency to accumulate filamentous actin.

In the embryo, the cells of the leading edge also express \textit{dpp} and genetic data confirm that Dpp is required for dorsal closure. Since relevant loss-of-function phenotypes indicate that Dpp is essential also for thorax closure (Chen et al., 1998; Hudson et al., 1998; Morimura et al. 1996; Simin et al., 1998; Spencer et al., 1982), we visualised \textit{dpp} expression in prepupae by expressing \textit{UAS-EGFP} under the control of \textit{dpp}^{	extit{bek},-Gal4} (Fig. 5). During larval stages, \textit{dpp} is expressed in a stripe along the anterior-posterior compartment boundary, which is oriented perpendicular to the future dorsal midline. We find that this expression pattern is essentially maintained during prepupal stages. A difference, however, is that in third instar larvae, the stripe of \textit{dpp} expression at the anterior-posterior boundary runs to the anteriorly located peripodial stalk in a smooth curve, whereas in the prepupa there is a sharp kink in the expression pattern. In this manner, the dorsal end of the \textit{dpp}-expressing stripe overlaps with the anterior part of the future dorsal midline, in which \textit{puc} is expressed (Fig. 5H). Therefore, \textit{dpp} expression during thorax closure only partially recapitulates the expression pattern in the embryo where all cells that express \textit{puc} also express \textit{dpp}.

**Puc is a target of the JNK pathway during thorax closure**

Although our genetic data show that \textit{Puc} negatively regulates D-Fos and the JNK pathway during thorax closure, they do not imply that \textit{Puc} is also a target of this cascade and thus a feedback regulator, as it is in dorsal closure. JNK activation and \textit{puc} expression could be established by independent mechanisms (parallel pathways) and their action would converge at the level of Basket by modulating its level of activity. To test whether \textit{puc} is a target of the JNK pathway during thorax closure, we analysed the expression of \textit{puc} in a JNK loss-of-function and a gain-of function background.

Compared to wild type (Fig. 6A,C), in homozygous \textit{hep}^1 prepupae (Fig. 6B,D), \textit{puc} expression was significantly reduced, indicating that the JNK cascade activated by Hep is required for full expression of \textit{puc} during thorax closure. Is Hep also sufficient for \textit{puc} induction or might additional
factors be required? To test this, we expressed Hep in the pnr expression domain to ectopically activate the JNK pathway. Overexpression of wild-type Hep had previously been shown to be sufficient for activation of the JNK pathway (Boutros et al., 1998). When we visualised puc expression in prepupae of the genotype pucE69, pnr-Gal4/UAS-Hep by X-Gal staining, a
accumulate at the dorsal midline, where (B) imaginal discs started to attach to each other. (A) Actin fibres (A-D) A prepupa is shown at 7 hours AP in which the two wing expression during thorax closure. Dorsal view with anterior up. (D) An overlay of the three channels shown separately in A-C. (E-H) The right wing imaginal disc of a prepupa at 6 hours AP is depicted to show (E) D-Fos (blue) is expressed at high levels. (F) puc expression, actin and (G) dpp expression as detected by UAS-EGFP driven by dpp(Gal4 (green). In the anterior compartment, but not in the posterior one, dpp is expressed at the medial margin such that it partially co-localises with (F) puc expression (red) and high levels of (G) D-Fos (blue). (H) An overlay of the three channels shown separately in E-G.

**Fig. 5.** Localisation of D-Fos, puc expression, actin and dpp expression during thorax closure. Dorsal view with anterior up. (A-D) A prepupa is shown at 7 hours AP in which the two wing imaginal discs started to attach to each other. (A) Actin fibres (visualised in green) accumulate at the dorsal midline, where (B) puc (red) is expressed and (C) D-Fos (blue) is expressed at high levels. (D) An overlay of the three channels shown separately in A-C. (E-H) The right wing imaginal disc of a prepupa at 6 hours AP is depicted to show (E) dpp expression as detected by UAS-EGFP driven by dpp(Gal4 (green). In the anterior compartment, but not in the posterior one, dpp is expressed at the medial margin such that it partially co-localises with (F) puc expression (red) and high levels of (G) D-Fos (blue). (H) An overlay of the three channels shown separately in E-G.

strong induction of puc expression exactly in the region of pnr expression was observed (Fig. 6F). pnr-Gal4/UAS-Hep also resulted in an abnormal adult phenotype (Fig. 6G). This phenotype was enhanced in the puc heterozygous mutant background (pucE69, pnr-Gal4/UAS-Hep, Fig. 6H) confirming that the phenotype was due to an excess of JNK activity (probably partially compensated by the induction of Puc expression). Thus, JNK activation is not only required but also sufficient to induce puc during thorax closure, suggesting that puc acts as a negative feed-back regulator that can confer stability to disturbances of JNK activity (see Discussion).

Finally, to test whether this negative feed-back loop is mediated by D-Fos, the dominant negative form of D-Fos (UAS-D-FosbZIP) was expressed with heterozygous pucE69, pnr-Gal4. X-Gal staining revealed a specific, albeit not fully penetrant, reduction of puc expression at the dorsal midline (Fig. 6E). Thus, it is likely that D-Fos acts downstream of the JNK signalling cascade to induce the expression of puc during thorax closure, as this is the case for dorsal closure.

**DISCUSSION**

**Similarities between dorsal closure and thorax closure**

We have characterised a morphogenetic movement, thorax closure, which occurs during *Drosophila* prepupal stages and shows morphological and genetic similarities to embryonic dorsal closure. In dorsal closure, two sheets of lateral epidermis approach each other, moving over and ultimately replacing the amnioserosa. In a similar mechanism, the wing imaginal discs move over and replace the larval epidermal cells. Eventually, the two epidermal sheets meet and firmly attach to each other, giving rise to the embryonic/larval epidermis, or the adult notum, respectively. As for dorsal closure, thorax closure is characterised by prominent actin organisation at the midline when the two wing imaginal discs meet to form the adult thorax. Finally, these cells at the medial margin are characterised by a distinct gene expression such as the expression of puc detected using the pucE69 enhancer trap line.

In addition to the morphological similarities between dorsal closure and thorax closure, we present genetic evidence that a similar signal transduction cascade regulates both processes. Regulated JNK activity is required for dorsal closure, as well as for normal thorax morphogenesis: under JNK loss-of-function conditions, e.g. homozygosity of the hepl allele, or overexpression of Puc, the thoracic epithelia fail to reach the midline and/or to fuse with each other. These prepupal defects give rise to adults with a thoracic cleft phenotype. Overexpression of Hep, in contrast, results in another abnormal phenotype, which can be interpreted to be the result of a gain-of-function of thorax closure activity. The most medial parts of the thoracic epidermis seem to have moved inside at the dorsal midline such that the notum appears to be narrower (Fig. 6F,H). This phenotype is enhanced after removal of one copy of wild-type puc, confirming that the defect is due to excess JNK activity. In addition, a distinct bristle phenotype is associated with both the gain-of-function and loss-of-function phenotype, but it is unclear whether these defects are secondary to thorax closure defects or whether they result from an independent requirement of JNK activity, as for example in tissue polarity or other aspects of bristle development.

In the same manner as in dorsal closure, JNK activity is autoregulated during thorax closure by induction of the negative feed-back regulator Puc. Hep activity is both required and sufficient for puc expression, suggesting that this gene is entirely under the control of the JNK pathway and, conversely, that puc expression reflects JNK activity. This suggests two possible modes for the negative regulatory action of Puc in thorax closure (which are not mutually exclusive). On the one
hand, Puc could act as a buffer against high JNK activity; thus, Puc would help to stabilise JNK activity at a medium level. On the other hand, Puc could help to repress JNK activity when it is no longer required (in this case, JNK and puc activities would be slightly out of phase). Our genetic data indicate that Puc does not solely act to shut off JNK activity. Most strikingly, Puc appears to be dispensable for development and viability when Hep activity is reduced, as in a homozygous hep1 background. Albeit viable, the homozygous double mutant combination hep1; pucE69 has an occasional bristle phenotype and cannot be kept as a stable stock. This is consistent with the idea of puc being required for the fine-tuning and/or stabilisation of JNK activity, rather than for a simple shut-off mechanism.

Another component of the pathway is D-Fos. The D-Fos hypomorphic allele kay2 gives rise to a cleft thorax that can be efficiently rescued by expressing wild-type D-Fos or by eliminating one copy of the puc gene. Furthermore, puc expression shows a remarkable correlation with high D-Fos (and D-Jun, data not shown) levels during thorax closure, and a dominant negative form of D-Fos can decrease puc expression in the medial margin cells. It is therefore likely that also during thorax closure, D-Fos acts downstream of the JNK pathway and contributes to the expression of puc. Since the expression of D-Jun is identical to D-Fos expression during thorax closure, it is conceivable that D-Fos acts together with D-Jun in a classical AP-1 transcriptional complex, as in embryonic dorsal closure. However, genetic evidence for a requirement of D-Jun in thorax closure must be awaited.

There is good genetic evidence that Dpp signalling is required for thorax closure. Hypomorphic mutations in components of the Dpp pathway such as dpp2 (Spencer et al., 1982), certain mutant combinations of thick veins (Chen et al., 1998; Morimura et al., 1996), punt (Simin et al., 1998) and medea (Hudson et al., 1998) cause a strong split-thorax phenotype. In the embryo, the expression of dpp in the leading edge is under the control of AP-1 and the JNK pathway. To test whether this could also be true in the wing imaginal disc, we visualised dpp expression during thorax closure (as measured by EGFP expression from dppb3::Gal4) and found that there is an overlap between dpp expression and puc expression, but only in the anterior part of the future dorsal midline. Since puc expression marks cells with JNK activity, but not all puc-expressing cells show dpp expression, JNK activity cannot be sufficient for dpp expression, as is the case in dorsal closure (Glise and Noselli, 1997; Hou et al., 1997; Riesgo-Escovar and Hafen, 1997b). However, it is possible that, in the anterior part of the future dorsal midline, JNK signalling and AP-1 contribute to the transcription of dpp. Alternatively, AP-1 might synergise with Dpp signalling further downstream, e.g. by cooperative transcriptional activation together with Medea (and possibly Mad), as was shown for their mammalian counterparts (Zhang et al., 1998).

**Thorax closure in prepupal morphogenesis**

We have analysed the signalling requirements for thorax closure because it is a process for which defects can easily be scored in adult animals. It should be stressed, however, that thorax closure is only one part of the morphogenetic movements that occur during prepupal stages. Indeed, puc expression in wing imaginal discs, together with high levels of D-Fos, is not restricted to the future dorsal midline of the thorax. Instead it is characteristic of all margin cells, i.e. those cells that lie at the border to the peripodial membrane (see Fig. 6A). Similar patterns are found in other imaginal discs, such as the haltere and leg discs. These tissues attach to each other at the same time as the wing imaginal discs and together complete the adult thorax complex (see Figs 6A and 2A). Moreover, puc is expressed in four rings in each elongating leg (data not shown). Thus, it is possible that JNK signalling, in conjunction with AP-1 activity, is not only involved in thorax closure but more generally in the assembly and morphogenesis of imaginal discs. Consistent with this idea is the fact that kay2 or hep1 homozygous adults often show abnormal phenotypes in addition to a thorax cleft phenotype. kay2 homozygous adults regularly have abdominal midline defects, kinked humeral macrochaetae and, in severe cases, forked scutellar macrochaetae, a crumpled or blistered wing and a malformed or bent-leg phenotype. In a homozygous hep1 background, entire imaginal disc derivatives can be missing in the adult (Glise et al., 1995), although it is unclear whether this reflects a failure in the eversion of the imaginal disc or a defect in the anlageplan.

If the signalling cassette, consisting of the JNK signalling cascade, AP-1 and Puc, is more generally involved in prepupal morphogenesis, it might be artificial to view thorax closure as an isolated process. For example, it is unclear whether the expression of puc in third instar imaginal discs marks the beginning of thorax closure or whether this might be part of the eversion process. In favour of the latter hypothesis would be the fact that both puc expression and filamentous actin are sparse after eversion and only reappear during epithelial spreading and fusion, respectively. However, the expression of puc in the third instar larval wing disc is remarkably similar to that at later prepupal stages. Thus, it seems unlikely that they are completely independent.

**Developmental and evolutionary implications**

The fact that a signalling cascade is used reiteratively during embryonic dorsal closure, thorax closure and perhaps other morphogenetic movements in *Drosophila*, raises obvious questions. Is it possible, by comparing the similarities and differences between dorsal closure and thorax closure, to determine the common principles underlying epidermal closure processes, and could one derive information about the relevant targets of the JNK (and Dpp) signalling cascades in these situations? Dorsal closure in the embryo involves dorsoventral stretching of the epidermis. During thorax closure, however, most cells are of polygonal shape. Therefore, either the downstream effectors of the pathway are different in the embryonic and prepupal epidermis, or, the morphogenetic mechanisms regulated by JNK do not primarily control cell elongation but other parameters important for morphogenesis (see von Kalin et al., 1995 for a review). An important role has been attributed to actin-mediated contractions in both dorsal closure and imaginal disc morphogenesis. However, we did not find evidence that Fos directly controls actin fibre formation since accumulation of actin at the future dorsal midline still occurs in kay2 mutant prepupae. Therefore, conclusions on the cellular mechanisms of JNK-dependent morphogenesis will require further genetic analysis on both dorsal closure and thorax closure.
Dorsal closure and thorax closure occur at different times, are not related (apart from being epidermal), and do not seem to depend on each other. This raises the question of how the two processes are evolutionarily related. Evolution is thought to occur largely by subtle genomic alterations. Hence, an important mechanism to generate novel biological forms is to recruit previously useful functional units in a different context. Such a co-option could have occurred in the evolution of insect metamorphosis. This hypothesis implies that, in an ancestral insect, there was only one form of dorsal closure, which occurred during embryogenesis. During the evolution of holometabolous insects, thorax closure was then co-opted from the existing form of dorsal closure. However, it is possible that the present form of embryonic dorsal closure in Drosophila has also evolved since its ancestral appearance. Therefore, the embryonic and prepupal forms of dorsal closure could have been originally even more similar than they are now, for example, in terms of tissue movements at the cellular level.

Remarkably, similar types of epithelial closures are also found in vertebrates, and some also share features of Drosophila dorsal closure (see also review by Goberdhan and Wilson, 1999). Examples include the convergent extension movements during gastrulation, embryonic wound healing and secondary palate closure in mice. For example, the latter has been shown to require TGFβ3, which is expressed in the medial edge epithelium before and during palate fusion (Kaartinen et al., 1995; Proetzel et al., 1995). Thus, the process described here, as well as its regulation, might be conserved from insects to vertebrates and Drosophila dorsal closure and thorax closure may represent a valid experimentally accessible model for the study of tissue closure processes.

We are indebted to N. Paricio, M. Strigini, S. Cohen, E. Martin-Blanco and M. Mlodzik for gifts of Drosophila stocks. We thank S. Cohen, U. Gritzan, L. Kockel, C. Ovitt and M. Mlodzik for comments on the manuscript and N. H. Patel, as well as members of the Bohmann laboratory, for discussions. J. Z. was supported by a grant of the HFSPO to D. B.

REFERENCES
