Hox-controlled reorganisation of intrasegmental patterning cues underlies *Drosophila* posterior spiracle organogenesis

Samir Merabet[1,2], James Castelli-Gair Hombria[3,4], Nan Hu[3], Jacques Pradel[1] and Yacine Graba[1,*]

[1]Laboratoire de Génétique et Physiologie du Développement, IBDM, CNRS, Université de la méditerranée, Parc Scientifique de Luminy, Case 907, 13288, Marseille Cedex 09, France
[2]Biozentrum der Universität Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland
[3]University of Cambridge, Department of Zoology, Downing Street, Cambridge CB2 3EJ, UK
[4]Centro Andaluz de Biología del Desarrollo, Universidad Pablo de Olavide, Carretera de Utrera, Km 1, Seville, 41013, Spain
*Author for correspondence (e-mail: graba@ibdm.univ-mrs.fr)

Accepted 4 May 2005

Development 132, 3093-3102
Published by The Company of Biologists 2005
doi:10.1242/dev.01889

Summary

Hox proteins provide axial positional information and control segment morphology in development and evolution. Yet how they specify morphological traits that confer segment identity and how axial positional information interferes with intrasegmental patterning cues during organogenesis remain poorly understood. We have investigated the control of *Drosophila* posterior spiracle morphogenesis, a segment-specific structure that forms under Abdominal-B (AbdB) Hox control in the eighth abdominal segment (A8). We show that the Hedgehog (Hh), Wingless (Wg) and Epidermal Growth Factor Receptor (Egfr) pathways provide specific inputs for posterior spiracle morphogenesis and act in a genetic network made of multiple and rapidly evolving Hox/signalling interplays. A major function of AbdB during posterior spiracle organogenesis is to reset A8 intrasegmental patterning cues, first by reshaping *wg* and *rhomboid* expression patterns, then by reallocating the Hh signal and later by initiating de novo expression of the posterior compartment gene *engrailed* in anterior compartment cells. These changes in expression patterns confer axial specificity to otherwise reiteratively used segmental patterning cues, linking intrasegmental polarity and acquisition of segment identity.

Key words: Hox, Signalling, Organogenesis, *Drosophila*

Introduction

Hox genes encode evolutionarily conserved homeodomain-containing transcription factors that play major roles in defining the diversity of animal body plans (Gellon and McGinnis, 1998; Graba et al., 1997; Krumlauf, 1994; McGinnis and Krumlauf, 1992). They deliver positional coordinates along the anteroposterior (A/P) axis that eventually instruct the development of distinct morphological structures between homologous groups of cells, termed segments in arthropods. In addition to Hox proteins, intrasegmental positional cues provide further information for segment morphogenesis. These cues are laid down by the segmentation gene cascade that culminates in the expression of segment polarity genes at distinct positions within each segment (Alexandre et al., 1999; Sanson, 2001). Three secreted signalling molecules provide key AP patterning information in controlling segmental denticle pattern: Wingless (*wg*), Hedgehog (Hh) and the ubiquitously expressed Egf ligand Spitz (Spi), which is locally cleaved by Rhomboid (Rho) to be turned into a secreted active ligand (Lee et al., 2001).

The definition and fate of metameric units constitute a paradigm to understand the function of Hox and intrasegmental signalling molecules (DiNardo et al., 1994; Lewis, 1978; Martinez-Arias and Lawrence, 1985). Functional interactions between Hox and signalling activities have been reported in a number of developmental processes (Chen et al., 2004; Dubrulle et al., 2001; Gieseler et al., 2001; Grienenberger et al., 2003; Immergluck et al., 1990; Joulia et al., 2005; Knosp et al., 2004; Marty et al., 2001; Panganiban et al., 1990; Ponzielli et al., 2002; Reuter et al., 1990; Zakany et al., 2001). Two studies have linked Hox and signalling molecules within the context of segment morphogenesis. The first concerns the regulation of *Serrate*, and ultimately that of *rho*, by the Hox proteins Ultrabithorax (Ubx) and Abdominal-A (AbdA) in the ventral ectoderm, to specify aspects of the abdominal denticle pattern (Wiellette and McGinnis, 1999). The second concerns the regulation of *rho* by AbdA in the lateral ectoderm of abdominal segments to allow oenocyte development (Brodu et al., 2002). Although these studies have provided important insights into how Hox proteins distinguish abdominal segments from more anterior ones, much remains to be learned about how Hox and signalling factors interact to specify segment-specific morphogenesis.

We have investigated how cells respond to axial and intrasegmental positional inputs during posterior spiracle morphogenesis and how Hox and signalling activities cooperate to control the formation of a segment-specific structure. The posterior spiracle develops in the eighth abdominal segment (A8) from an epithelial sheet of ectodermal cells that subdivides into two populations. The inner cells, that give rise to the spiracular chamber, invaginate and eventually

form an internal tube, the filzkörper, which constitutes the opening of the gas exchange system of first instar larvae (Hu and Castelli-Gair, 1999). The surrounding cells undergo rearrangements, in a manner similar to the process of convergent extension (Warga and Kimmel, 1990), to form the stigmatophore, the external part of the organ in which the filzkörper tube is located.

The Hox gene *Abdominal-B* (*AbdB*) initiates the developmental program of posterior spiracle formation (Hu and Castelli-Gair, 1999). This program is formed by two genetic modules that control morphogenesis of the spiracular chamber and stigmatophore, respectively. Each module comprises primary targets, the expression of which does not depend on the activity of the others: *cut*, *empty spiracles* (*ems*), *Klumpfuss* and *nubbin* for spiracular chamber cells; and *spalt* (*sal*) for stigmatophore cells. Enhancers that recapitulate expression in the posterior spiracle have been identified for *cut* (Jack and DeLotto, 1995) and *ems* (Jones and McGinnis, 1993), suggesting that these targets may be directly controlled by the Hox protein. These genes encode transcription factors that activate secondary targets, which also encode transcription factors. However, we lack an understanding of how AbdB or genes acting downstream cooperate with other developmental cues to promote posterior spiracle morphogenesis. We have found that posterior spiracle morphogenesis relies on a dynamic genetic network made of multiple Hox/signalling interplays, and that AbdB plays a fundamental role in reorganising intrasegmental positional cues during organogenesis.

Materials and methods

Fly stocks

The following fly strains were obtained from the Bloomington *Drosophila* stock centre: wg^{CX4} (BL-2980), hh^{1J35} (BL-5338), $Egfr^{f2}$ (BL-2768), $Egfr^{tsla}$ (BL-6501), hh^{ts2} (BL-1684), *UAS-rasv12* (BL-4847), *UAS-DN-Egfr* (BL-5364) and *UAS-DN-TCF* (BL-4784). The *ems-Gal4*, *sal-Gal4*, *arm-Gal4*, 69B-Gal4, *UAS-lacZ*, $UAS\text{-}Ci^{zn}$ (referred to as *UAS-Ci-DN*), UAS-AbdBm, en^{E}, lin^{G75} and $AbdB^{m5}$ strains are described elsewhere (Brand and Perrimon, 1993; Castelli-Gair, 1998; Castelli-Gair et al., 1994; Gustavson et al., 1996; Hepker et al., 1997; Kuhnlein and Schuh, 1996; Merabet et al., 2002; Sanchez-Herrero et al., 1985; Sanson et al., 1996). The *UAS-hh*, *UAS-en* and $UAS\text{-}spitz^{S}$ fly strains were obtained from S. Kerridge; the *UAS-wg*, *en-Gal4* and wg^{IL114} strains were from A. Martinez-Arias. The *UAS-GFP* (EGFP variants) lines are from C. Desplan.

Cuticule preparations, immunostaining and whole-mount in situ hybridisation

Embryo collection, cuticule preparations, in situ hybridisation and immunodetection of whole embryos were performed according to standard procedures. The anti-AbdB, anti-Cut and anti-En antibodies were obtained from the Developmental Study Hybridoma Bank (DSHB, Iowa University) and used at a 1:5 dilution. The rabbit anti-Spalt primary antibody was a gift from Reinhart Shuh (Kuhnlein et al., 1994) and used at a 1:50 dilution. The rabbit anti-Mirror antibody was provided by H. McNeill and used at a 1:1000 dilution. The anti-β-Galactosidase (Cappel) and anti-GFP (Promega) antibodies were used at a 1:500 dilution. Digoxigenin RNA-labelled probes were generated according to the manufacturer's protocol (Boehringer-Manheim) from *hh*, *wg*, *rho* and *ems* cDNAs cloned in Bluescript (Stratagene). Secondary antibodies were either coupled to alkaline phosphatase, biotin or peroxidase (Jackson ImmunoResearch Laboratories), or conjugated to Alexa-488 or Alexa-594 (Molecular Probes), and used at suppliers recommended dilutions. When needed, the signal was amplified with the aid of a Tyramide Signal Amplification kit (NEN Life Sciences). Embryos stained with fluorochromes were mounted in Vectashield (Vector Laboratories) for observation under a confocal microscope (Leica TCS SP2 or LSM 510 Zeiss). Images were processed with the Leica TSC NT 1.6, Zeiss LSM5 Image Browser and Adobe Photoshop 7.0 programmes. The Imaris software (Bitplane) was used for 3D reconstruction with the Shadow Projection function.

Thermosensitive experiments

The temporal requirement of Wg, Hh and Egfr signalling for posterior spiracle morphogenesis was assessed using temperature-sensitive alleles of *wg* (wg^{IL114}), *hh* (hh^{ts2}) and *Egfr* ($Egfr^{tsla}$). Embryos were collected over a 1 hour period at 18°C and left to develop at the same permissive temperature from 3 to 10 hours before shifting them to 29°C, a restrictive temperature for all alleles. Cuticles were prepared 36 hours after egg laying.

Results

Spatial distribution of Wg, Hh and Egf signalling sources in the dorsal A8 ectoderm

In the dorsal ectoderm of stage 10 embryos, *hh* and *wg* follow the same striped expression patterns in A8 as in other abdominal segments (Fig. 1C,I). *rho* expression, which marks cells secreting an active form of the Egf ligand (Lee et al., 2001), occurs in all primordia of tracheal pits, in A8 as in more anterior segments (Fig. 1O).

Specification of posterior spiracle primordia occurs at early stage 11 (Fig. 1A). The primordia can then be recognised by Cut expression in spiracular chamber cells and by Sal, the homogenous expression of which in A8 becomes restricted dorsally to stigmatophore cells that form a crescent surrounding Cut-positive cells (Fig. 1A′). From mid-stage 11, *wg* and *rho* adopt in the dorsal ectoderm expression patterns specific to A8, with *wg* transcribed in two cells only (Fig. 1J) and *rho* in a second cell cluster, dorsal and posterior to the tracheal placode (Fig. 1P). To localise *wg*- and *rho*-expressing cells with regards to stigmatophore and spiracular chamber cells, co-labelling experiments for *wg* or *rho* transcripts and for Cut or Sal proteins were performed: the two *wg* cells lie between Cut- and Sal-positive cells (Fig. 1K,L); the second cell cluster expressing *rho* in A8 also expresses Cut but not Sal (Fig. 1Q,R). This cluster is likely to produce the Egf ligand required for posterior spiracle development, as mutations that alleviate *rho* expression in the tracheal placodes (Boube et al., 2000; Isaac and Andrew, 1996; Llimargas and Casanova, 1997) do not abolish spiracles formation (Hu and Castelli-Gair, 1999). At mid-stage 11, the *hh* pattern in A8, along a stripe lying posterior and adjacent to the spiracular chamber (Fig. 1E) and overlapping stigmatophore presumptive cells (Fig. 1F), resembles expression in other abdominal segments (Fig. 1D). Analyses at later stages (Fig. 1B) indicate that the relationships between posterior spiracle cells (Fig. 1B′) and *hh* (Fig. 1G,H), *wg* (Fig. 1M,N) and *rho* (Fig. 1S,T) patterns are maintained.

Wg, Hh and Egfr signalling are required for posterior spiracle formation after primordia specification

Null mutations of *wg*, *hh* or *Egfr* result in the absence of posterior spiracles (Fig. 2C,E,G). The strong cuticular defects observed raise the possibility that the phenotypes result

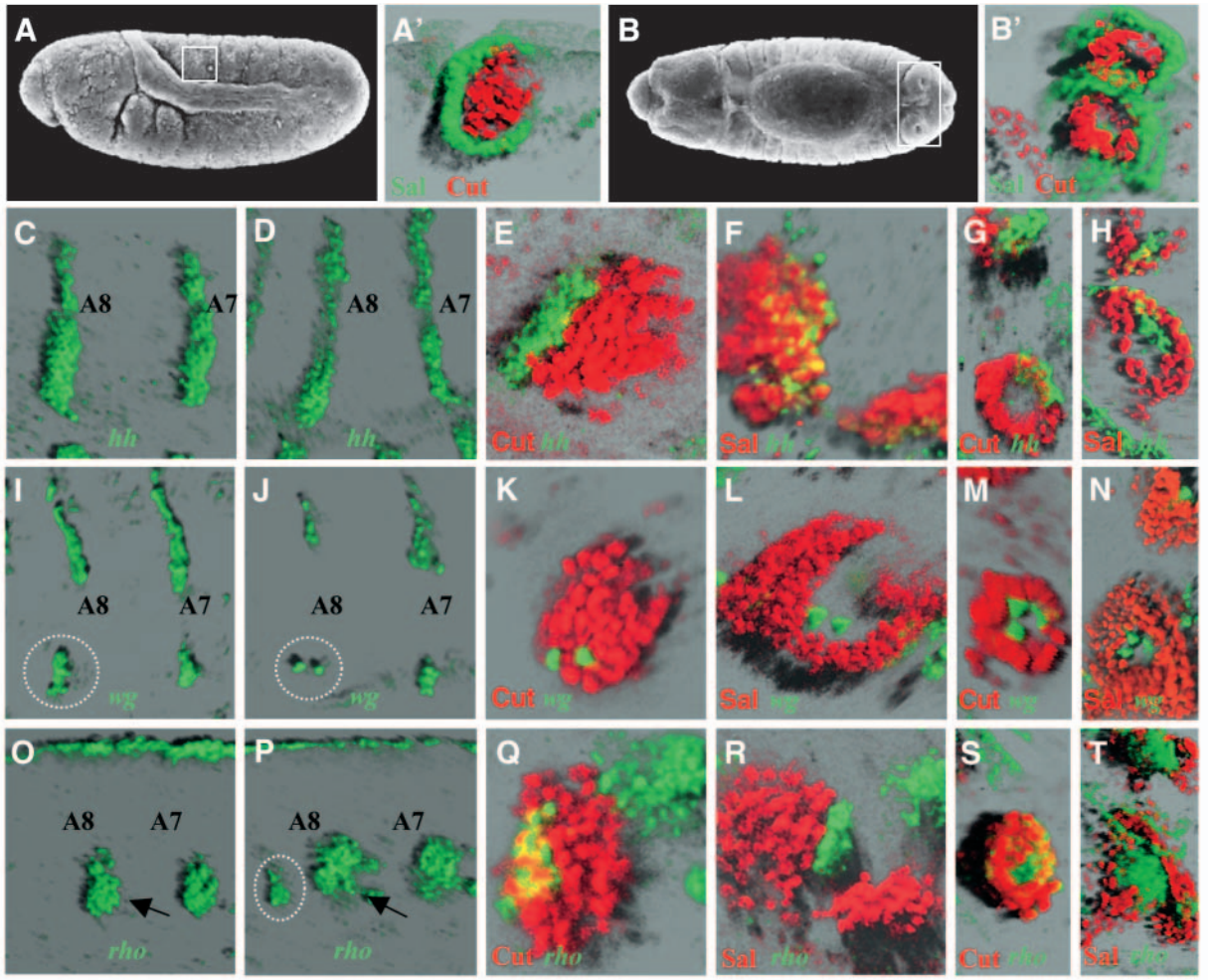

Fig. 1. Localisation of Wg, Hh and Egf signalling sources in the dorsal A8 ectoderm. (A) Electron micrograph of a stage 11 embryo [lateral view, from FlyBase (FlyBase Consortium, 2003)]. (A′) Magnified view of posterior spiracle primordia, showing prospective stigmatophore (stained in green by anti-Sal antibody) and spiracular chamber (stained in red by anti-Cut antibody) territories. (B) Electron micrograph of a stage 13 embryo (dorsal view, from Flybase). (B′) Magnified view of the posterior spiracle, stained in green for stigmatophore and in red for spiracular chamber cells. (C-H) In situ hybridisation to *hh* transcripts (green) in wild-type embryos at stage 10 (C), mid-stage 11 (D-F) and stage 13 (G,H). A7 and A8 mark the position of the seventh and eighth abdominal segments. (I-N) In situ hybridisation to *wg* transcripts (green) in wild-type embryos at stage 10 (I), mid-stage 11 (J-L) and stage 13 (M,N). At stage 13, three cells express *wg*, compared with two at stage 11. Circles in I and J indicate the expression of *wg* in the dorsal ectoderm of A8. (O-T) In situ hybridisation to *rho* transcripts (green) in wild-type embryos at stage 10 (O), mid-stage 11 (P-R) and stage 13 (S,T). Arrows in O and P indicate *rho* expression in the tenth tracheal placode. The circle indicates the appearance at stage 11 of a more dorsal and posterior cluster of *rho*-expressing cells in A8. Spiracular chamber cells are identified by Cut immunolabelling, in red in E,G,K,M,Q,S. Stigmatophore cells are identified by Sal immunolabelling, in red in F,H,L,N,R,T. In all magnified views, anterior is towards the right and posterior towards the left at stages 10 and 11; this orientation is reversed at stage 13 following germ-band retraction.

indirectly from early loss of segment polarity. Removing the Wg, Hh or Egfr signals from 5-8 hours of development using thermosensitive alleles causes strong segment polarity defects but allows filzkörpers (Fig. 2D), stigmatophores (Fig. 2F) or even complete posterior spiracles (Fig. 2H) to form. Thus, spiracular chamber and stigmatophore can develop in embryos that have pronounced segment polarity defects.

We next asked whether defects in primordia specification could account for posterior spiracle loss and examined Cut and Sal expression in the dorsal A8 ectoderm of *hh*, *wg* and *Egfr* mutant embryos. Expression of Cut (Fig. 3D,G,J) and Sal (Fig. 3F,I,L) is initiated at stage 11 in all of these mutants, although the somewhat disorganised patterns, especially from late stage 11 (data not shown), may reveal roles of these signalling in sizing or shaping the posterior spiracle primordia. Alternatively, these defects may result from altered morphology of mutant embryos. In any case, the induction of the early markers Sal and Cut in A8 dorsal ectoderm of mutant embryos indicates that posterior spiracle primordia specification does occur in the absence of signalling by Wg, Hh or Egfr. Transcription of *ems*, another AbdB target that is activated slightly later than Cut, although not affected in *hh* mutants (Fig. 3K), is lost in *wg* or *Egfr* mutants (Fig. 3E,H). Thus, proper regulation of AbdB downstream targets activated following primordia specification appears dependent on signalling activities.

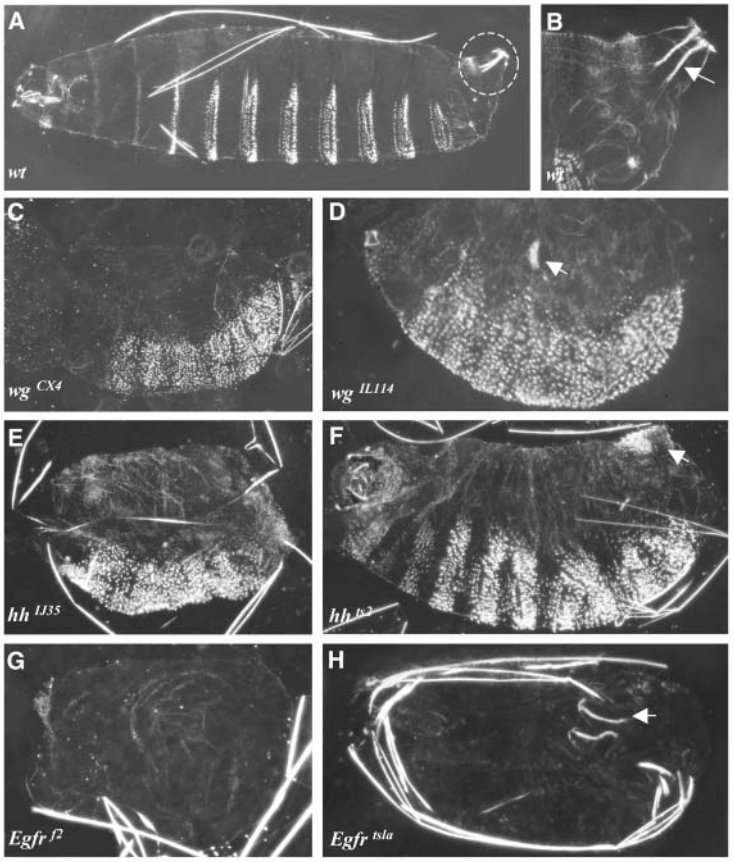

Fig. 2. Requirement of Wg, Hh and Egfr signalling for posterior spiracle formation. (A) Cuticle of a wild-type larva. (B) Magnified view of the posterior spiracle. The spiracular chamber cells are differentiated into filzkörpers (white arrow), which are located within a protruding dome-shaped structure, the stigmatophore. (C,E,G) No posterior spiracles are present in wg^{CX4} (C), hh^{IJ35} (E) and $Egfr^{f2}$ (G) mutants. (D,F,H) Cuticles of thermosensitive alleles of *wg*, *hh*, *Egfr* shifted to restrictive temperature from 5-8 hours of development. Differentiated filzkörpers are present in wg^{IL114} embryos (D), stigmatophores are present in hh^{ts2} embryos (F) and complete posterior spiracles are present in $Egfr^{tsla}$ embryos (H), despite severe segment polarity defects. In wg^{IL114} embryos, filzkörpers are often at abnormal positions, which reflects the absence of attachment to the disorganised mutant tracheal network.

Wg, Hh and Egfr signalling in spiracular chamber and stigmatophore cells is required for posterior spiracle organogenesis

We next investigated the role of Wg, Hh and Egfr signalling pathways in posterior spiracle organogenesis (i.e. after the specification of presumptive territories). Co-labelling experiments performed on embryos expressing GFP driven by *ems-Gal4* or by *sal-Gal4* indicate that whereas Cut and Sal are already expressed at early stage 11 (Fig. 4A,C), GFP is detected from late stage 11 only (Fig. 4B,D). These two drivers, which promote expression approximately 1 hour after primordia specification, were used to express DN molecules for each pathway, counteracting Wg (DN-TCF), Egfr (DN-Egfr) or Hh [DN-Cubitus interuptus (Ci)] signalling from that time on. Blocking either pathway in spiracular chamber cells does not perturb stigmatophore morphogenesis, but specifically leads to the loss of differentiated filzkörpers (Fig. 4E-G). Conversely, blockade in stigmatophore cells provokes in each case its flattening, while differentiated filzkörpers do form (Fig. 4H-J).

To ask how signalling inhibition interferes with the genetic modules initiated downstream of AbdB, we followed expression of Sal and Cut from stages 11 to 13. No major defects are seen until late stage 12 (shown for DN-TCF, Fig. 5B-E). Strong deviation from the wild-type patterns are, however, observed slightly later, from stage 13 onwards: Sal expression in basal cells of the stigmatophore are lost (Fig. 5F-I) and Cut expression remains in only a few scattered cells (Fig. 5J-M). The 2-hour delay seen between the onset of DN molecules expression and the detection of Sal and Cut (Fig. 5A) could reflect the time required for shutting down the pathways. Alternatively, Sal and Cut expression may not require signalling activities before stage 13. To discriminate between these possibilities, we forced the expression of the DN molecules earlier, using the *69B-Gal4*, known to promote protein accumulation by the onset of stage 11 (Castelli-Gair et al., 1994) (i.e. slightly before posterior spiracle primordia specification). Strong defects in Sal and Cut expression where again only seen in stage 13 embryos (data not shown), supporting that signalling activities are dispensable before the end of stage 12, but are required from stage 13 onwards to maintain Sal in basal stigmatophore cells and Cut in the spiracle chamber.

AbdB and Hh remodel *wg* and *rho* expression in A8 dorsal ectoderm at mid-stage 11

A8-specific modulation of *rho* and *wg* patterns at mid-stage 11 suggests a regulation by AbdB. In *AbdB* mutants, *rho* expression in the spiracle-specific cell cluster is lost (Fig. 6A), and *wg* transcription does not evolve towards an A8-specific pattern (Fig. 6B). In embryos expressing AbdB ubiquitously, ectopic posterior spiracle formation in the trunk can be identified as ectopic sites of Cut accumulation. In such embryos, *rho* and *wg* are induced in trunk segments following patterns that resemble their expression in A8: *rho* in a cluster that overlaps the Cut domain (Fig. 6D), and *wg* in few cells abutting ectopic Cut-positive cells (Fig. 6E). These transcriptional responses to loss and gain of function of AbdB indicate that the Hox protein controls the A8-specific expression patterns of *wg* and *rho*. The *lines* gene (*lin*), which is known to be required for Cut and Sal activation by AbdB (Castelli-Gair, 1998), also controls *wg* and *rho* patterns respecification (see Fig. S1 in the supplementary material).

In contrast to *wg* and *rho*, *hh* does not adopt an A8-specific expression pattern at mid-stage 11 (Fig. 1D). At that stage, *hh* expression pattern is not affected upon *AbdB* mutation (Fig. 6C). The *hh* stripe in A8 lies posterior and adjacent to spiracular chamber cells and overlaps stigmatophore cells (Fig. 1E,F), suggesting that Hh signalling may participate in the regulation of *rho* and *wg* transcription by AbdB. In support of this, we found that the AbdB-dependent aspects of *rho* and *wg* transcription patterns are missing in *hh* mutant embryos (Fig. 6F,G). Thus, inputs from both Hh and AbdB are required to remodel Wg and Egfr signalling in A8.

The dependence of *wg* and *rho* A8 expression patterns on

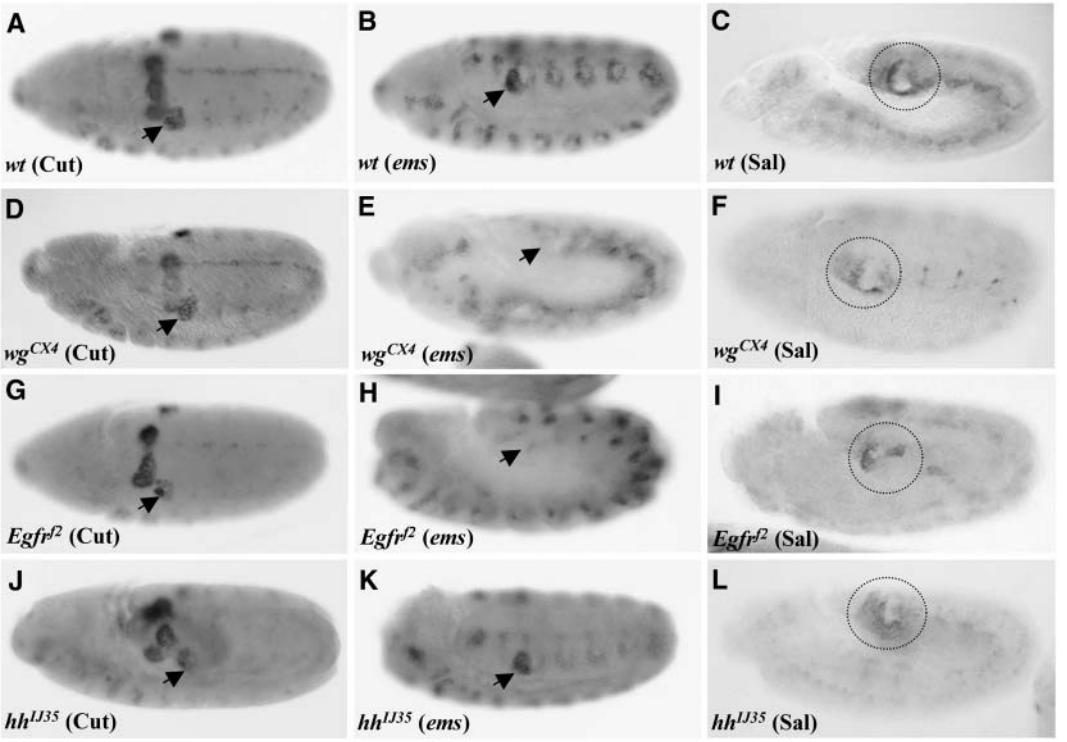

Fig. 3. Wg, Hh and Egfr signalling are dispensable for posterior spiracle primordia specification. Expression at stage 11 of Cut (A,D,G,J), *ems* (B,E,H,K) and Sal (C,F,I,L) in wild-type (A-C) or *wg* (D-F), *Egfr* (G-I) and *hh* (J-L) mutant embryos. Arrows and circles indicate the sites of posterior spiracle formation. Cut and Sal expression is maintained in mutant contexts, while *ems* transcription is absent in *wg* and *Egfr* mutant embryos.

Hh, and the loss of *ems* expression in *wg* and *rho* but not in *hh* mutants, suggest that transcription of *ems* requires Wg and Egfr signalling prior to *wg* and *rho* pattern respecification by AbdB and Hh. To explore this point further, we comparatively analysed the time course of *ems*, *wg* and *rho* expression. Embryos bearing an *ems-lacZ* construct stained for β-Gal and for *wg* or *rho* transcripts show that *ems* expression precedes *wg* pattern respecification (Fig. 6H,I), and occurs at the same time as *rho* acquires an A8-specific pattern (Fig. 6J,K). Importantly, we never detected A8-specific *rho* clusters before the onset of *ems* expression. Thus, *ems* transcription starts before *wg* and at the same time as *rho* pattern respecification, supporting that signalling by Wg and Egfr is required prior to mid-stage 11. These observations also indicate that respecification of the *wg* pattern occurs slightly later than that of *rho*, which could not been concluded from changes in embryo morphology.

Local sources of Wg and Egfr signals are independently required for posterior spiracle organogenesis

To determine whether signalling by Wg and Egfr from local sources is important for posterior spiracle organogenesis, we forced the production of Wg and Spi^{S} (the mature form of Spi)

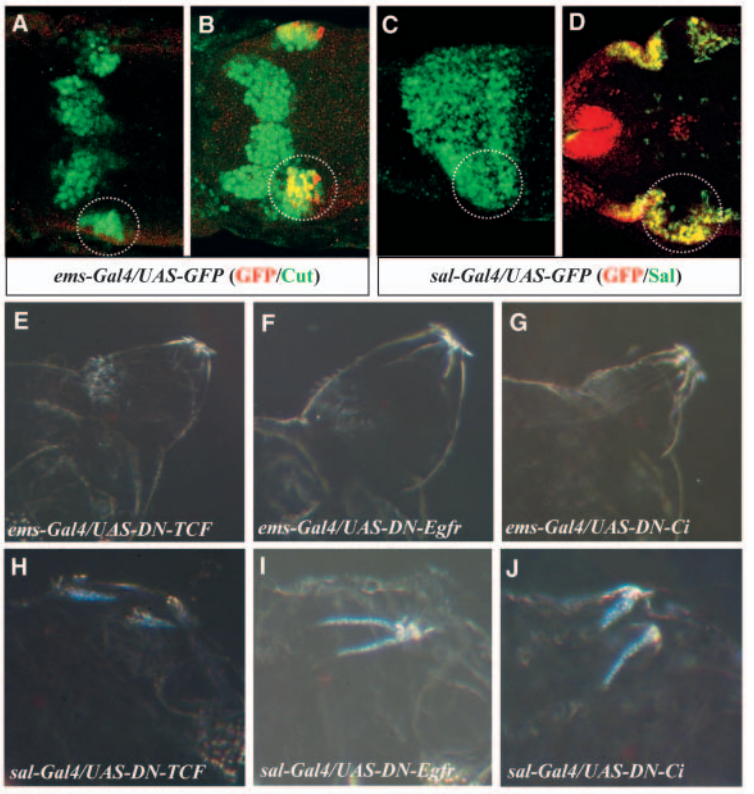

Fig. 4. Wg, Hh and Egfr signalling are required in spiracular chamber and stigmatophore cells for posterior spiracle organogenesis. (A-D) *ems-Gal4* or *sal-Gal4* promote expression at late stage 11. *ems-Gal4/UAS-GFP* (A,B) and *sal-Gal4/UAS-GFP* embryos (C,D), immunostained in red for GFP, and in green for Cut (A,B) and Sal (C,D) at early (A,C) or late (B,D) stage 11. GFP is detected in late stage 11 embryos, roughly 1 hour after the onset of Sal and Cut protein accumulation in the dorsal A8 ectoderm.(E-J) Effects of DN-TCF (E,H), DN-Egfr (F,I) and DN-Ci (G,J) expression driven by *ems-Gal4* or *sal-Gal4*. Blocking either signalling by *ems-Gal4* impairs filzkörper formation, and not that of stigmatophore (E-G). Reciprocally, blocking either signalling by *sal-Gal4* impairs stigmatophore development, leaving filzkörper differentiation unaffected (H-J). The stigmatophore phenotype resulting from the loss of Hh signalling (J) is weaker than those resulting from the loss of Wg and Egfr signalling (H,I).

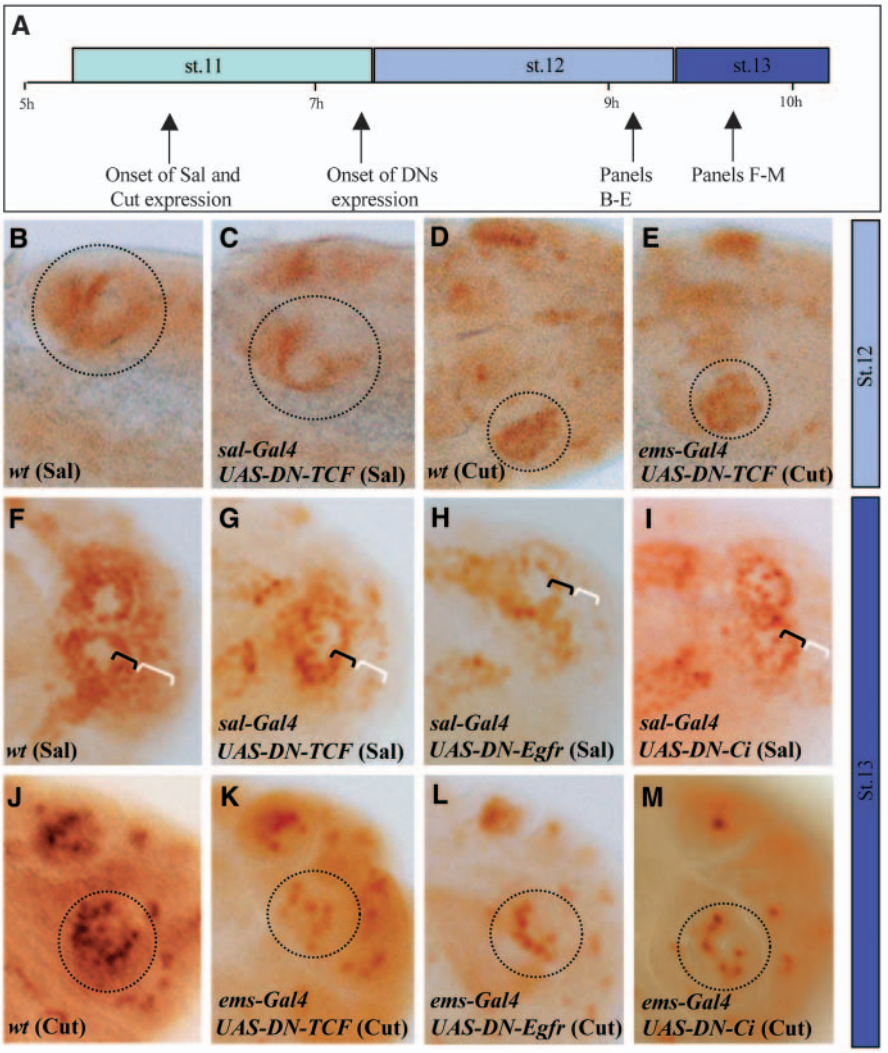

Fig. 5. Wg, Hh and Egfr signalling are required for maintenance of Cut and Sal expression in the posterior spiracle. (A) Schematic representation of the delayed effects of DN molecules expression on Sal and Cut expression, and correspondence between embryonic stages and times of development at 25°C. (B-E) Sal expression in *sal-Gal4/UAS-DN-TCF* (C) and Cut expression in *ems-Gal4/UAS-DN-TCF* (E) in late stage 12 embryos are not affected compared with expression in wild-type embryos (B,D). (F-I) Anti-Sal staining at stage 13. Wild-type embryos (F), and embryos carrying *sal-Gal4* and *UAS-DN-TCF* (G), *UAS-DN-Egfr* (H) or *UAS-DN-Ci* (I). Black brackets indicate cells close to the spiracular opening and white brackets indicate more distant cells, in which Sal is no longer detected upon signalling inhibition. (J-M) Anti-Cut staining at stage 13. Wild-type embryos (J), or embryos carrying *ems-Gal4* and *UAS-DN-TCF* (K), *UAS-DN-Egfr* (L) or *UAS-DN-Ci* (M). The discontinuous lines encircle spiracular chamber cells, where Cut expression is significantly reduced upon signalling inhibition.

ligands from domains broader than normal in A8 dorsal ectoderm. This was performed after posterior spiracle specification, using the *ems-Gal4* and *sal-Gal4* drivers. We observed that ectopic signalling results in abnormally shaped posterior spiracles: stigmatophores are reduced in size and filzkörpers do not elongate properly (Fig. 7A-D′). Ectopic signalling from all presumptive stigmatophore cells results in stronger defects than those produced when ectopic signals emanate from all spiracular chamber cells (compare Fig. 7C-D′ with 7A-B′). This can be correlated to the fact that *sal-Gal4* drives expression in a pattern that more strongly diverges from the wild-type situation than *ems-Gal4* does. Thus, restricted delivery of Wg and Spi^S signals is required for accurate posterior spiracle organogenesis.

We next asked whether, downstream of Hh, the Wg and Egfr pathways provide separate inputs for posterior spiracle organogenesis. Two sets of experiments where conducted. First, in embryos respectively mutant for *Egfr* or *wg*, *wg* and *rho* acquire A8-specific patterns (see Fig. S2A,B in the supplementary material). Second, epistasis experiments performed by forcing in spiracular or stigmatophores cells the activity of one pathway while inhibiting the other indicate that loss of one pathway could not be rescued by the other (see Fig. S2C-H in the supplementary material). Thus, Egfr and Wg pathways do not act as hierarchically organised modules, but provide independent inputs for posterior spiracle organogenesis.

AbdB controls A8-specific expression of *hh* at stage 12 and de novo expression of *engrailed* at stage 13

The expression of the posterior compartment selector gene *engrailed* (*en*) until stage 12 follows a striped pattern identical in all trunk segments (Fig. 8B). Later on, En adopts a pattern that is specific to A8: it is no longer detected in the ventral part of the segment; and, dorsally, the En stripe has turned to a circle of cells that surround the future posterior spiracle opening (Fig. 8C) and express the stigmatophore marker Sal (not shown). The transition from a striped to a circular pattern depends on AbdB (not shown) (Kuhn et al., 1992). This transition could result either from a migration of *en* posterior cells towards the anterior, or from transcriptional initiation in cells that were not expressing *en* before stage 12, and that can therefore be defined as anterior compartment cells.

To distinguish between the two possibilities, *en-Gal4/UAS-lacZ* embryos were simultaneously stained with anti β-Gal and anti-En antibodies. If circle formation results from cell migration, one would expect β-Gal and En to be simultaneously detected in all cells of the circle as the two proteins are already co-expressed in the posterior compartment stripe earlier on. Conversely, if the circle results from de novo expression, one would expect anterior cells in the circle to express En before β-Gal, as β-Gal production requires two rounds of transcription/translation compared with one for En (Fig. 8A). We found that cells from the anterior part of the circle express En but not β-Gal in stage 13 embryos (Fig. 8C), which demonstrates that de novo expression of En occurs in

anterior compartment cells. Further supporting En expression in anterior compartment cells, we found that precursors of anterior spiracle hairs that do not express En at stage 12 do so at stage 13 (see Fig. S3 in the supplementary material). En function in A8 is essential for posterior spiracle development, as stigmatophores do not form in *en* mutants (Fig. 8D), and are restored if En is provided in stigmatophore cells (Fig. 8E).

We also found that although identical in all abdominal segments at stage 11, *hh* transcription adopts an A8-specific pattern from stage 12 onwards: transcripts are then localised only at the anterior border of the En stripe (Fig. 8G). This expression of *hh* is lost in *AbdB* mutants (Fig. 8H) and still occurs in *en* mutants (Fig. 8I). The uncoupling of *hh* transcription from En activity in the dorsal A8 ectoderm correlates with the distinct phenotypes seen for *en* mutants, which do differentiate filzkörper like structures (Fig. 8D), and for *hh* mutants, which do not (Fig. 2E).

Discussion

Multiple and dynamic functional interactions between AbdB and signalling activities

Data presented in this paper allow us to distinguish four phases in functional interactions between AbdB and signalling by Wg, Hh and Egfr during posterior spiracle formation. The first phase corresponds to the specification of presumptive territories of the organ. The signalling activities are not involved in this AbdB-dependent process, as they are not required for the induction of the earliest markers of spiracular chamber and stigmatophore cells, Cut and Sal, in the dorsal ectoderm of A8.

The second phase, which immediately follows primordia specification, concerns the regulation of AbdB target genes activated slightly later. Inputs from the Hox protein and the Wg and Egfr pathways are then simultaneously needed, as seen for transcriptional initiation of the *ems* downstream target. This function of Wg and Egfr signalling precedes and does not require the reallocation of signalling sources in A8-specific patterns, as impairing A8-specific expression of *wg* and *rho* by loss of *hh* signalling does not affect *ems* expression. Within the third phase, AbdB and Hh activities converge to reset *wg* and *rho* expression patterns. The three phases take place in a narrow time window, less than 1 hour during stage 11, and could only be distinguished by studying the functional requirements of Wg, Hh and Egfr for transcriptional regulation in the posterior spiracle.

We refer the fourth phase as an organogenetic phase. Data obtained using DN variants to inhibit the pathways in cells already committed to stigmatophore or filzkörper fates, indicate that Wg, Egfr and Hh pathways are required for organ formation after specification and early patterning of the primordia. Their roles are then to maintain AbdB downstream targets expression in posterior spiracle cells as development proceeds, as shown for Cut and Sal at stage 13.

Hox control of morphogenesis: conferring axial properties to intrasegmental patterning cues

A salient feature of AbdB function during posterior spiracle development is to relocate Wg and Egfr signalling sources in the dorsal ectoderm at mid-stage 11. *wg* and *rho* then adopt expression patterns that differ from expressions in other abdominal segments, conferring axial properties unique to A8 to otherwise segmentally reiterated patterning cues. Resetting Wg and Egfr signalling sources into restricted territories is of functional importance for organogenesis, as revealed by the morphological defects that result from the delivery of Wg or Spi^S signals in all spiracular chamber or stigmatophore cells after the specification phase. During stage 12, AbdB also relocates the Hh signalling source by inducing En-independent expression of *hh* in the dorsal ectoderm. Thus, later than Wg and Egfr signalling, the Hh signal also acquires properties unique to A8. In generating this pattern, AbdB plays a fundamental role in uncoupling *hh* transcription from En activity, providing a context that prevents anterior compartment En-positive cells to turn on *hh* transcription (compare Fig. 8C with Fig. 1G,H), and that allows *hh* expression in the absence of En in other cells (Fig.

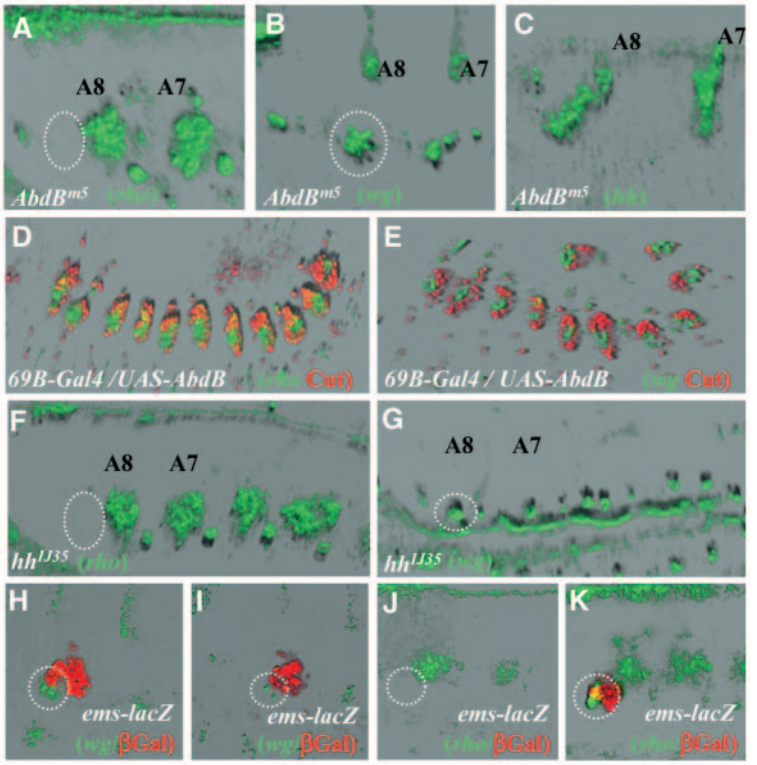

Fig. 6. AbdB and Hh signalling control A8-specific patterns of *wg* and *rho*. (A-C) In situ hybridisation (green) to *rho* (A), *wg* (B) and *hh* (C) transcripts in mid-stage 11 *AbdB* mutant embryos. *rho* and *wg* fail to adopt A8-specific patterns (circles; compare A with Fig. 1P and B with Fig. 1J). (D,E) *69B-Gal4/UAS-AbdB* embryos stained in red for Cut protein and in green for *rho* (D) or *wg* (E) RNA. The expression of *wg* and *rho* in all trunk segments mimics expression found in wild-type A8. However, *wg* is expressed in more than three cells (compare with Fig. 1M). (F,G). In situ hybridisation (green) to *rho* (F) or *wg* (G) transcripts in mid-stage 11 hh^{1J35} mutant embryos. As in *AbdB* mutant embryos, *rho* and *wg* patterns do not acquire A8-specific properties (circles). (H-K) Magnified views of *ems-lacZ* embryos stained for β-Gal (red) and *wg* (H,I) or *rho* (J,K) transcripts (green). β-Gal is detected before (H) and after (I) *wg* has adopted an A8-specific pattern. The transition of the *rho* expression pattern occurs slightly later than that of *wg*, as β-gal is not detected prior to *rho* expression (J), but concomitantly with its expression in the posterior dorsal cell cluster (K).

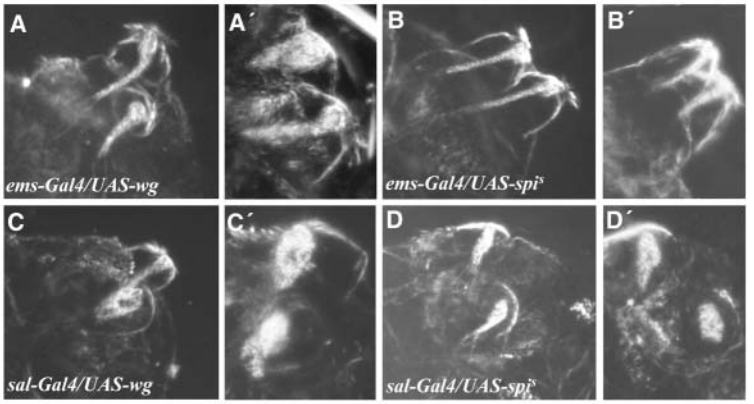

Fig. 7. Local requirements of Wg and Egfr signalling for posterior spiracle organogenesis. Effects of Wg and Spi^S expression in spiracular chamber or stigmatophore cells on the morphology of the posterior spiracle. *ems-Gal4/UAS-wg* (A,A′) or *ems-Gal4/UAS-spi^S* (B,B′) and *sal-Gal4/UAS-wg* (C,C′) or *sal-Gal4/UAS-spi^S* (D,D′). In all genotypes, embryos harbour filzkörpers elongation defects and reduced sized stigmatophores. Some variability in filzkörper defects is seen and illustrated in A′,B′,C′,D′, which show extreme phenotypes for each genotypes. Filzkörpers and stigmatophore defects are more pronounced when Wg and Spi^S expression is driven by *sal-Gal4*.

8I). Slightly later, at stage 13, AbdB modifies the expression of the posterior selector gene *en*, initiating de novo transcription in anterior compartment cells. In these cells, En fulfils different regulatory functions than in posterior cells, as discussed above for *hh* regulation. Changes in En expression and function can be interpreted as a requisite to loosen AP polarity in A8 and gain circular coordinates required for stigmatophore formation

AbdB function during posterior spiracle morphogenesis suggests that Hox-induced reorganisation of positional information may be central for shaping cellular fields during organogenesis. A recent report on limb morphogenesis supports this view: early colinear restriction of 5′ Hoxd genes provides initial asymmetry to the nascent limb bud and controls posterior expression of Sonic Hedgehog at the zone of polarising activity (Zakany et al., 2004). Subsequent to this initial phase, the expression of the same 5′ Hoxd genes acquires a reverse colinear polarity that is necessary for generating the distal limb structures. Thus, in this extreme case, Hox-controlled reorganisation of positional cues results in the modification of Hox gene coordinates themselves.

Finally, our study provides a further link between segmentation and segment identity specification, which was recently revealed by the findings that the Hox proteins Ubx and AbdA use the products of the segment polarity genes *en* and *sloppy paired* as repressive co-factors to inhibit the expression of the limb promoting gene *Distalless* (Gebelein et al., 2004). Our data strengthen the idea that the establishment of segment polarity and of segment identity are functionally linked, and extend the concept further: Hox genes not only use

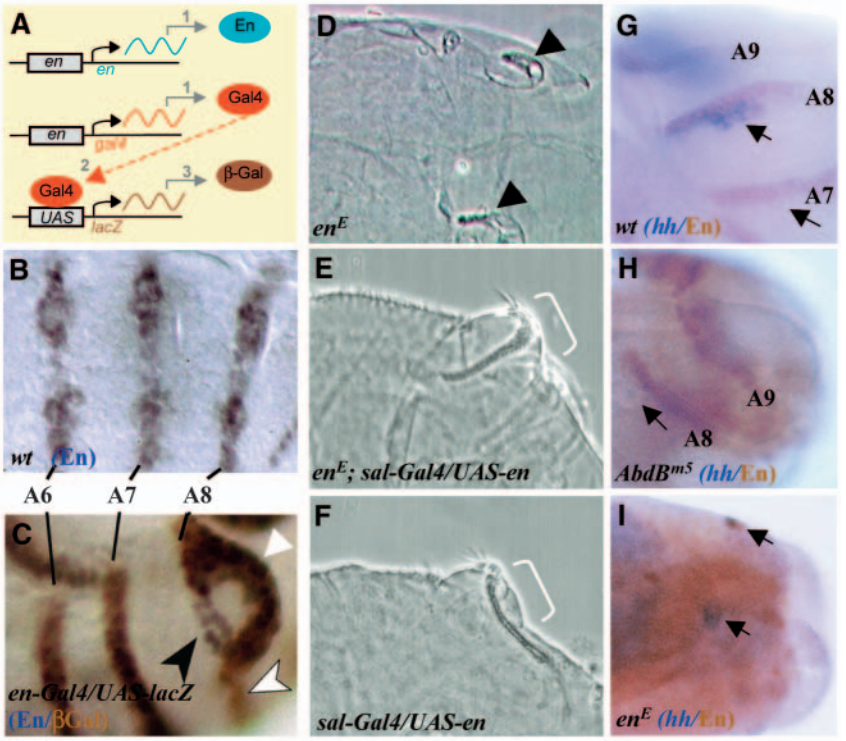

Fig. 8. A8-specific regulation of *hh* and *en* at stages 12 and 13. (A) Schematic representation of the 'delay experiment' used to detect de novo En expression in the anterior compartment of A8. En production requires a single step (1) but that of β-Gal requires three steps (1-3). The *en* introns, the small size of which does not induce a significant delay in En production, have not been represented. (B) Wild-type expression of En in abdominal segments at stage 11, shown here for A6-A8. In order to align the segments with those of a stage 13 embryo that has completed germ-band retraction, the magnified view shown in B comes from a stage 11 embryo oriented with anterior towards the left. (C) Stage 13 *en-Gal4/UAS-lacZ* embryo stained in blue for En and brown for β-gal. En and β-gal are co-expressed in A6 and A7. In A8, co-expression only occurs in cells lying in the posterior part of the stigmatophore (white arrow), while anterior stigmatophore cells express En but not yet β-gal (black arrowhead). Cells located ventrally in A8 do not express En, but β-gal is still detected because of the transcriptional delay and β-gal stability (white arrowhead, out of focus). (D-F) En is required for stigmatophore development. Cuticle of an *en^E* embryo (D) shows loss of stigmatophores, while filzkörper-like structures are still present (black arrows). *en^E* embryos bearing the *sal-Gal4* and *UAS-en* transgenes display developed stigmatophores (E, white bracket) comparable in size with those observed in embryos containing the two transgenes but in an otherwise wild-type context (F, white bracket). (G-I) *hh* is expressed in an A8-specific pattern from stage 12 onwards (G). In A8, but not in more anterior segments (arrow in A7), *hh* (blue) is expressed in cells (arrow in A8) that border the En segmental stripe (brown). This expression is lost (arrow) in *AbdB* mutants (H) and still occurs in *en* mutants (I).

intrasegmental positional cues for laying down segment identity (Gebelein et al., 2004), but they also impinge on segment polarity genes by conferring axial properties on their products.

We thank K. Mathews (Bloomington stock centre), S. Kerridge, A. Martinez-Arias, R. Holmgren, E. Sanchez Herrero and C. Desplan for fly strains; R. Shuh and H. McNeill for anti-Spalt and anti-Mirror antibodies; the Developmental Study Hybridoma Bank (DSHB, University of Iowa) for providing anti-AbdB, anti-Cut and anti-En antibodies; and S. Kerridge, A. Saurin and C. Zaffran-Maurel for comments on the manuscript. We also thank M. Affolter in whose laboratory part of the work was performed, and H. McNeill for drawing our attention on Mirror expression. This work was supported by the 'Centre National de la Recherche Scientifique' (CNRS), grants from 'la Ligue Nationale Contre Le Cancer (équipe labellisée La Ligue)', 'l'Association pour la Recherche contre le Cancer' (ARC), The Royal Society, The Welcome Trust, the 'Minesterio de education y ciencia (BFU 2004 0 96) and ARC and EMBO long term fellowships to S. Merabet.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/13/3093/DC1

References

Alexandre, C., Lecourtois, M. and Vincent, J. (1999). Wingless and Hedgehog pattern Drosophila denticle belts by regulating the production of short-range signals. *Development* **126**, 5689-5698.

Boube, M., Llimargas, M. and Casanova, J. (2000). Cross-regulatory interactions among tracheal genes support a co-operative model for the induction of tracheal fates in the Drosophila embryo. *Mech. Dev.* **91**, 271-278.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Brodu, V., Elstob, P. R. and Gould, A. P. (2002). abdominal A specifies one cell type in Drosophila by regulating one principal target gene. *Development* **129**, 2957-2963.

Castelli-Gair, J. (1998). The lines gene of Drosophila is required for specific functions of the Abdominal-B HOX protein. *Development* **125**, 1269-1274.

Castelli-Gair, J., Greig, S., Micklem, G. and Akam, M. (1994). Dissecting the temporal requirements for homeotic gene function. *Development* **120**, 1983-1995.

Chen, Y., Knezevic, V., Ervin, V., Hutson, R., Ward, Y. and Mackem, S. (2004). Direct interaction with Hoxd proteins reverses Gli3-repressor function to promote digit formation downstream of Shh. *Development* **131**, 2339-2347.

DiNardo, S., Heemskerk, J., Dougan, S. and O'Farrell, P. H. (1994). The making of a maggot: patterning the *Drosophila* embryonic epidermis. *Curr. Opin. Genet. Dev.* **4**, 529-534.

Dubrulle, J., McGrew, M. J. and Pourquie, O. (2001). FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation. *Cell* **106**, 219-232.

FlyBase Consortium (2003). The FlyBase database of the Drosophila genome projects and community literature. *Nucleic Acids Res.* **31**, 172-175.

Gebelein, B., McKay, D. J. and Mann, R. S. (2004). Direct integration of Hox and segmentation gene inputs during Drosophila development. *Nature* **431**, 653-659.

Gellon, G. and McGinnis, W. (1998). Shaping animal body plans in development and evolution by modulation of Hox expression patterns. *BioEssays* **20**, 116-125.

Gieseler, K., Wilder, E., Mariol, M. C., Buratovitch, M., Berenger, H., Graba, Y. and Pradel, J. (2001). DWnt4 and wingless elicit similar cellular responses during imaginal development. *Dev. Biol.* **232**, 339-350.

Graba, Y., Aragnol, D. and Pradel, J. (1997). *Drosophila* Hox complex downstream targets and the function of homeotic genes. *BioEssays* **19**, 379-388.

Grienenberger, A., Merabet, S., Manak, J., Iltis, I., Fabre, A., Berenger, H., Scott, M. P., Pradel, J. and Graba, Y. (2003). Tgf{beta} signaling acts on a Hox response element to confer specificity and diversity to Hox protein function. *Development* **130**, 5445-5455.

Gustavson, E., Goldsborough, A. S., Ali, Z. A. and Kornberg, T. B. (1996). The Drosophila *engrailed* and *invected* genes: Partners in regulation, expression and function. *Genetics* **142**, 893-906.

Hepker, J., Wang, Q. T., Motzny, C. K., Holmgren, R. and Orenic, T. V. (1997). *Drosophila cubitus interruptus* forms a negative feedback loop with *patched* and regulates expression of Hedgehog target genes. *Development* **124**, 549-558.

Hu, N. and Castelli-Gair, J. (1999). Study of the posterior spiracles of Drosophila as a model to understand the genetic and cellular mechanisms controlling morphogenesis. *Dev. Biol.* **214**, 197-210.

Immergluck, K., Lawrence, P. A. and Bienz, M. (1990). Induction across germ layers in Drosophila mediated by a genetic cascade. *Cell* **62**, 261-268.

Isaac, D. D. and Andrew, D. J. (1996). Tubulogenesis in *Drosophila*: A requirement for the *trachealess* gene product. *Genes Dev.* **10**, 103-117.

Jack, J. and DeLotto, Y. (1995). Structure and regulation of a complex locus: The *cut* gene of Drosophila. *Genetics* **139**, 1689-1700.

Jones, B. and McGinnis, W. (1993). The regulation of *empty spiracles* by *Abdominal-B* mediates an abdominal segment identity function. *Genes Dev.* **7**, 229-240.

Joulia, L., Bourbon, H. M. and Cribbs, D. L. (2005). Homeotic proboscipedia function modulates hedgehog-mediated organizer activity to pattern adult Drosophila mouthparts. *Dev. Biol.* **278**, 496-510.

Knosp, W. M., Scott, V., Bachinger, H. P. and Stadler, H. S. (2004). HOXA13 regulates the expression of bone morphogenetic proteins 2 and 7 to control distal limb morphogenesis. *Development* **131**, 4581-4592.

Krumlauf, R. (1994). *Hox* genes in vertebrate development. *Cell* **78**, 191-201.

Kuhn, D. T., Sawyer, M., Packert, G., Turenchalk, G., Mack, J. A., Sprey, T. E., Gustavson, E. and Kornberg, T. B. (1992). Development of the D. melanogaster caudal segments involves suppression of the ventral regions of A8, A9 and A10. *Development* **116**, 11-20.

Kuhnlein, R. P. and Schuh, R. (1996). Dual function of the region-specific homeotic gene spalt during Drosophila tracheal system development. *Development* **122**, 2215-2223.

Kuhnlein, R. P., Frommer, G., Friedrich, M., Gonzalez-Gaitan, M., Weber, A., Wagner-Bernholz, J. F., Gehring, W. J., Jackle, H. and Schuh, R. (1994). spalt encodes an evolutionarily conserved zinc finger protein of novel structure which provides homeotic gene function in the head and tail region of the Drosophila embryo. *EMBO J.* **13**, 168-179.

Lee, J. R., Urban, S., Garvey, C. F. and Freeman, M. (2001). Regulated intracellular ligand transport and proteolysis control EGF signal activation in Drosophila. *Cell* **107**, 161-171.

Lewis, E. B. (1978). A gene complex controlling segmentation in *Drosophila*. *Nature* **276**, 565-570.

Llimargas, M. and Casanova, J. (1997). ventral veinless, a POU domain transcription factor, regulates different transduction pathways required for tracheal branching in Drosophila. *Development* **124**, 3273-3281.

Martinez-Arias, A. and Lawrence, P. A. (1985). Parasegments and compartments in the *Drosophila* embryo. *Nature* **313**, 639-642.

Marty, T., Vigano, M. A., Ribeiro, C., Nussbaumer, U., Grieder, N. C. and Affolter, M. (2001). A HOX complex, a repressor element and a 50 bp sequence confer regional specificity to a DPP-responsive enhancer. *Development* **128**, 2833-2845.

McGinnis, W. and Krumlauf, R. (1992). Homeobox genes and axial patterning. *Cell* **68**, 283-302.

Merabet, S., Catala, F., Pradel, J. and Graba, Y. (2002). A green fluorescent protein reporter genetic screen that identifies modifiers of Hox gene function in the Drosophila embryo. *Genetics* **162**, 189-202.

Panganiban, G. E., Reuter, R., Scott, M. P. and Hoffmann, F. M. (1990). A Drosophila growth factor homolog, decapentaplegic, regulates homeotic gene expression within and across germ layers during midgut morphogenesis. *Development* **110**, 1041-1050.

Ponzielli, R., Astier, M., Chartier, A., Gallet, A., Therond, P. and Semeriva, M. (2002). Heart tube patterning in Drosophila requires integration of axial and segmental information provided by the Bithorax Complex genes and hedgehog signaling. *Development* **129**, 4509-4521.

Reuter, R., Panganiban, G. E., Hoffmann, F. M. and Scott, M. P. (1990). Homeotic genes regulate the spatial expression of putative growth factors in the visceral mesoderm of Drosophila embryos. *Development* **110**, 1031-1040.

Sanchez-Herrero, E., Vernos, I., Marco, R. and Morata, G. (1985). Genetic organization of Drosophila bithorax complex. *Nature* **313**, 108-113.

Sanson, B. (2001). Generating patterns from fields of cells: Examples from Drosophila segmentation. *EMBO Rep.* **2**, 1083-1088.
Sanson, B., White, P. and Vincent, J. P. (1996). Uncoupling cadherin-based adhesion from *wingless* signalling in Drosophila. *Nature* **383**, 627-630.
Tabata, T., Eaton, S. and Kornberg, T. B. (1992). The Drosophila *hedgehog* gene is expressed specifically in posterior compartment cells and is a target of *engrailed* regulation. *Genes Dev.* **6**, 2635-2645.
Warga, R. M. and Kimmel, C. B. (1990). Cell movements during epiboly and gastrulation in zebrafish. *Development* **108**, 569-580.
Wiellette, E. L. and McGinnis, W. (1999). Hox genes differentially regulate Serrate to generate segment-specific structures. *Development* **126**, 1985-1995.
Zakany, J., Kmita, M., Alarcon, P., de la Pompa, J. L. and Duboule, D. (2001). Localized and transient transcription of Hox genes suggests a link between patterning and the segmentation clock. *Cell* **106**, 207-217.
Zakany, J., Kmita, M. and Duboule, D. (2004). A dual role for Hox genes in limb anterior-posterior asymmetry. *Science* **304**, 1669-1672.