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

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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 (Brochu 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\textsuperscript{1071} (BL-2980), hh\textsuperscript{115} (BL-5338), Egfr\textsuperscript{2} (BL-2768), Egfr\textsuperscript{296} (BL-6501), hh\textsuperscript{113} (BL-1684), UAS-rasv12 (BL-4847), UAS-DN-Egfr (BL-5364) and UAS-DN-TCF (BL-4784). The ems-Ga4, sal-Ga4, arm-Ga4, 69B-Ga4, UAS-lacZ, UAS-Ci\textsuperscript{28} (referred to as UAS-Ci-DN), UAS-AbdBm, en\textsuperscript{r}, lig\textsuperscript{775} and AbdB\textsuperscript{58} strains are described elsewhere (Brand and Perrimon, 1993; Castelli-Gair, 1998; Castelli-Gair et al., 1994; Gustavson et al., 1996; Hépker 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-spit\textsuperscript{2} fly strains were obtained from S. Kerridge; the UAS-wg, en-Ga4 and wg\textsuperscript{1114} strains were from A. Martinez-Arias. The UAS-GFP (EGFP variants) lines are from C. Desplan.

Cuticle preparations, immunostaining and whole-mount in situ hybridisation

Embryo collection, cuticle 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 was 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\textsuperscript{1114}), hh (hh\textsuperscript{237}) and Egfr (Egfr\textsuperscript{296}). 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 Egfr 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. 1A,I). rho expression, which marks cells secreting an active form of the Egfr ligand (Lee et al., 2001), occurs in all primordia of tracheal pits, in A8 as in 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 Egfr 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
Hox-resetting of intrasegmental patterning cues

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.
leads to the loss of differentiated filzkörpers (Fig. 4E-G). Blocking either pathway in spiracular chamber cells does not perturb stigmatophore morphogenesis, but specifically time on. Blocking either pathway in spiracular chamber cells for each pathway, counteracting Wg (DN-TCF), Egfr (DN-Egfr) or Hh [DN-Cubitus interruptus (Ci)] signalling from that primordia specification, were used to express DN molecules drivers, which promote expression approximately 1 hour after detected from late stage 11 only (Fig. 4B,D). These two already expressed at early stage 11 (Fig. 4A,C), GFP is complete posterior spiracles are present in thermosensitive alleles of Dh, IJ35 (Fig. 6A), and rho expression in the spiracle-specific cell cluster is lost (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. 6E). 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
Hox-resetting of intrasegmental patterning cues

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 be 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 SpiS (the mature form of Spi)
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 were 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 stigmatophore 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
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\^2 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. 2E).
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 fulfills 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...
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.

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

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Hox-resetting of intrasegmental patterning cues 3101