Differential Activity of *Drosophila* Hox Genes Induces Myosin Expression and Can Maintain Compartment Boundaries

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Abstract

Compartments are units of cell lineage that subdivide territories with different developmental potential. In *Drosophila*, the wing and haltere discs are subdivided into anterior and posterior (A/P) compartments, which require the activity of Hedgehog, and into dorsal and ventral (D/V) compartments, needing Notch signaling. There is enrichment in actomyosin proteins at the compartment boundaries, suggesting a role for these proteins in their maintenance. Compartments also develop in the mouse hindbrain rhombomeres, which are characterized by the expression of different Hox genes, a group of genes specifying different structures along their main axis of bilaterians. We show here that the *Drosophila* Hox gene *Ultrabithorax* can maintain the A/P and D/V compartment boundaries when Hedgehog or Notch signaling is compromised, and that the interaction of cells with and without *Ultrabithorax* expression induces high levels of non-muscle myosin II. In the absence of *Ultrabithorax* there is occasional mixing of cells from different segments. We also show a similar role in cell segregation for the Abdominal-B Hox gene. Our results suggest that the juxtaposition of cells with different Hox gene expression leads to their sorting out, probably through the accumulation of non-muscle myosin II at the boundary of the different cell territories. The increase in myosin expression seems to be a general mechanism used by Hox genes or signaling pathways to maintain the segregation of different groups of cells.

Introduction

During animal development there is a progressive subdivision of the organism into distinct groups of cells that will form different organs and structures. In this process, the cells normally acquire different cellular affinities, which allow both to keep a coherent group of cells with the same fate and to distinguish them from surrounding cells with different identity [1].

The development of the *Drosophila* wing imaginal disc is a good model to study cell segregation. Wing and haltere imaginal discs are subdivided, early in development, into an anterior (A) and a posterior (P) compartment [2]. The selector gene *engrailed* (*en*) is expressed in the P compartment and induces the expression of the Hedgehog (*hh*) signaling molecule. Cells from the P compartment, transcribing *en* and *hh*, do not mix with cells from the A compartment, lacking the expression of both genes. The boundary separating the two compartments forms a straight border, the line of minimal contact, named the antero-posterior (A/P) compartment boundary [2–4].

This strict lineage segregation can be compromised in two ways. First, posterior cells lacking *en* (and its cognate gene *in*), can penetrate into the A compartment [3,5]; reciprocally, if *en* is ectopically expressed in anterior cells, they can move to the P compartment [3]. Second, anterior cells mutant for *smoothed* (*smo*), an obligatory component of the Hh signaling pathway, can cross into the P compartment [6,7]. Although a complete mixing of A and P cells requires changes in the activity of both En and the Hh pathway, eliminating the response to the Hh signal causes a more complete response and predominates over the mechanism depending on changes in *en* [3].

Wing and haltere imaginal disc are further subdivided into dorsal (D) and ventral (V) compartments. Dorsal cells transcribe *apterous* (*ap*), which regulates the expression of *Serrate* (*Ser*), a ligand of *Notch* (*N*), whereas ventral cells express another *N* ligand, *Delta* (*Dl*). *N* is active at both sides of the dorso-ventral (D/V) compartment boundary, and it is required to maintain the segregation of D and V cells [8]. Experiments that compared the behavior of cells mutant for *N* or *ap* at the D/V boundary [9–12] suggested that *ap* has an instructive role, and *N* a permissive one, in defining the D/V boundary [12,13]. However, an alternative model proposed that *N* signaling is sufficient to separate D and V cells by creating a “fence” [10,14,15].

Segregation between distinct populations of cells also occurs in rhombomeres of the chick vertebrate hindbrain [16]. Rhombomeres have distinguishable cell lineages and express unique combinations of Hox genes [17,18]. These genes specify the main axis in bilaterians [19], and are required to maintain the correct architecture of rhombomeres in the mouse hindbrain [20]. In
**Materials and Methods**

**Genetics**

Most of the mutations and constructs are described in Flybase. Other constructs used are UAS-dsUbx [25], UAS-OUbx [26], sph-GFP [27], baz-GFP and zip-GFP [28]. In the experiments with the Gal4/Gal80\(^{\text{TS}}\) system [29] the larvae were changed from 17°C to 29°C at the early third larval instar and kept at 29°C for 24h.

**Clonal Analysis**

Clones of the following genotypes were induced at 24–48 h and 48–72 h (smo, smo Ubx and Abd-B clones) or 48–72 h and 72–96 h (Ubx clones) after egg laying with a one-hour heat-shock given at 37°C.

| Genotype                        | n | Do not cross | Other cases* |
|---------------------------------|---|--------------|--------------|
| smo\(^{\text{in}}\) wildtype haltere disc | 10 | 5             | 2            |
| smo\(^{\text{in}}\) bx\(^{\text{TM2}}\) haltere disc | 12 | 0             | 7            |
| smo\(^{\text{in}}\) wildtype II leg disc | 18 | 0             | 11           |
| smo\(^{\text{in}}\) wildtype III leg disc | 11 | 6             | 1            |

*Small clones or clones where the crossing or not crossing was not evident.

**Immunochemistry**

Antibody staining was done according to standard protocols. The antibodies used are: mouse anti-Ubx at 1/10 [30], rabbit anti-

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*Table 1. Number of clones crossing or respecting the A/P boundary in haltere, second and third leg disc in wildtype and mutant combinations.*

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**Figure 1. Hedgehog signaling and Ultrabithorax provide specific cell affinities to the cells.** In Figures 1 and 2 anterior compartments (A) of the imaginal discs are to the left and posterior ones (P) to the right. (A) Ubx mutant clones, marked by the absence of arm-lacZ expression (in green), are round and tend to segregate from the surrounding tissue. (B) An Ubx-expressing clone (arrow), marked with yellow and induced in the second thoracic segment also segregates from the rest of the notum. (C, C') A smo clone in the anterior compartment of the wing pouch, marked by the absence of GFP signal (in green), penetrates into the posterior compartment, which is marked by en-lacZ expression (in red). (D) hh-lacZ expression in the haltere disc. (E–E') A smo clone in the anterior compartment of the haltere pouch, marked as in C also penetrates into the posterior compartment, marked with hh-lacZ (in red, E'). Merged image in E'. Scale bars are 30 μm in A, D, E' and 60 μm in C'.

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GFP (1:200, Invitrogen), mouse anti-β-galactosidase (1:100, Cappel) and rabbit anti-β-galactosidase (1:2000, Cappel). TRITC-phalloidin is from Sigma.

Adult Cuticle Analysis
It was done following standard procedures.

Results and Discussion
The Drosophila Ultrabithorax (Ubx) Hox gene determines the development of the third thoracic segment (T3), and Ubx mutants transform this segment into the second thoracic one (T2) [31]. Ubx is expressed in the haltere discs, which will form the dorsal adult T3, but not in the wing disc (but for the peripodial membrane), which develops into the dorsal T2 [32]. As observed in the adult

Figure 2. Ultrabithorax can maintain the A/P boundary in the absence of Hedgehog signaling. In all the panels of this Figure, the clones are marked by the lack of GFP in green, and the posterior compartment (to the right) is marked by either hh-lacZ or en-lacZ reporters in red. (A–B’’) Wing (A–A’’) and haltere (B–B’’) discs showing anterior clones double mutant for smo and Ubx that invade the posterior compartment. (C–C’’) smo clone induced in the anterior compartment of a bx2/TM2, Ubx130 haltere disc. See that it does not cross the compartment boundary. Note that a few cells in the A compartment weakly express hh-lacZ. (D–D’’) An anterior smo clone induced in the third leg disc cross the A/P compartment boundary. (E–E’) A similar clone induced in the second leg disc does not cross the boundary. Scale bars are 30 µm except in A’’ (60 µm).
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Figure 3. Ultrabithorax can maintain a smooth D/V boundary in the absence of Notch signaling. (A, B) ap-Gal4 UAS-GFP wing (A) and haltere (B) discs, showing the smooth boundary between dorsal (D, in green) and ventral (V) compartments. (C, D) In ap-Gal4 UAS-GFP/apU6035 wing (C) and haltere (D) discs, this boundary is uneven. Note in D a group of dorsal cells in the ventral compartment (arrow). (E, F) In ap-Gal4 UAS-GFP/apU6035, UAS-Ubx/tub-Gal80U wing discs (E), or in haltere discs of ap-Gal4 UAS-GFP/apU6035, DI109 UAS-dsUbx/+ larvae (F), the straight D/V boundary is restored. See that the dorsal compartment in E is slightly reduced and that in F slightly enlarged. Scale bars are 40 µm in A, C and 30 µm in B, D, E and F.
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Figure 4. Differences in amount of Ultrabithorax between adjacent cells induce accumulation of spaghetti-squash, zipper and bazooka.

(A–C”) Z-stacks of Ubx clones induced in the haltere disc, marked by the absence of arm-lacZ (in grey in A, B and C, in blue in A”, B” and C”), showing a ring of sqh-GFP, zip-GFP or baz-GFP (in green in A’, B’ and C’, respectively), and higher levels of F-actin (in red in A”, B” and C”) around the clones. Merged images in A”, B” and C” show a sagittal section of the clone shown in C”–C” (arrows). Note the invagination of the clone and the accumulation of baz-GFP and F-actin in the border of the clone (arrowheads). (D–D”) Haltere disc of the bx3 hh-lacZ/TM2, Ubx130 genotype, showing accumulation of sqh-GFP (in green in E, arrowhead) and F-actin (in red in E”) at the A-P boundary, where compartments with (P compartment) and without (A compartment) Ubx abut (Ubx expression in grey in E and in blue in E”). Merged image in E””. (F–F”) In bx3 hh-lacZ/TM6B haltere discs, by contrast, there is no accumulation of either sqh-GFP (in green in F) or F-actin (in red in F”) at the A-P boundary; ß-galactosidase expression is in grey in F. (G) abx bx3 pbx/TM2, Ubx130 adult showing a fusion of the T2 and T3 (transformed into the T2) segments (arrow). Scale bars are 10 μm in A”, B” and C”, and 30 μm in E”” and F””.

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[33], cells lacking Ubx expression in the haltere disc do not mix with Ubx-expressing cells. Ubx mutant clones induced in this disc are round, with smooth borders and segregate from the surrounding epithelium (Fig. 1A). This segregation is also evident in clones expressing Ubx ectopically (Fig. 1B). These observations confirm that Ubx activity provides specific cell affinities.

**Ultrabithorax can Maintain the Antero-posterior Compartment Boundary in the Absence of Hh Signaling**

Anterior clones mutant for smo, when abutting the A/P compartment boundary of the wing disc, frequently cross into the P compartment [6,7] (Fig. 1C, C'). In the haltere disc, the A/P boundary is not so straight as in the wing disc (Fig. 1D), but we have also observed a similar behavior of many anterior smo clones (Fig. 1E–E' and Table 1; see also Fig.1d in Ref. 7).

The observation that either Ubx activity or Hh signaling can provide the cells with particular cell affinities, and that Hh is needed to maintain the separation of cells from A and P compartments, suggested the possibility that Ubx activity may be sufficient to maintain compartment boundaries. Anterior clones double mutant for smo and Ubx, if induced close to the A/P boundary of wing or haltere discs, can penetrate into the P compartment (Fig. 2A–B'). This seems to suggest that differences in Ubx activity cannot compensate for the absence of Hh signaling.

However, in this experiment both A and P compartments express Ubx, and Ubx* cells from both compartments may equally reject the mutant cells. We wondered if a different activity of Ubx in A and P compartments could be sufficient to separate A and P cells when Hh signaling is compromised. To answer this question we induced anterior smo− clones in bithorax (bx) haltere discs, in which only the P cells express Ubx [31,32]. These clones, when abutting the P compartment, respect the A/P boundary [Fig. 2C–C' and Table 1]. The comparison of the distribution of smo clones induced in wildtype and bx discs suggests that the different Ubx activity in A and P cells significantly contributes to maintain the A/P compartment boundary in the absence of Hh signaling.

We decided to study if the same result applies to the second and third leg discs. The rationale for these experiments is that Ubx is expressed in both compartments of the third leg disc but only in the P compartment of the second leg disc [34,35]. The results presented in Fig. 2D–E' and in Table 1 show that most smo− clones induced in the A compartment of the third leg disc cross into the P compartment, whereas similar clones induced in the second leg disc respect the boundary. As also observed in the haltere disc, a few smo− clones in the third leg disc do not readily cross into the P compartment (Table 1). This may be due to Ubx being present at higher levels in the P compartment of this disc than in the A compartment [32,36], or represent a coincidental event. Collectively, the data strongly suggest that Ubx can maintain the A/P boundary in the absence of Hh signaling.

**Ultrabithorax Maintains a Smooth Dorso-ventral Boundary in the Absence of Notch Signaling**

Dorsal and ventral cells of the wing and haltere discs are separated by a straight D/V boundary (Fig. 3A, B). When ap expression is substantially reduced (ap-Gal4 UAS-GFP/apUGO35 discs), and N signaling, therefore, compromised, the boundary in the wing [12] (Fig. 3C) and haltere (Fig. 3D) disc is uneven. However, if we express Ubx in the dorsal compartment of an ap− wing disc (ap-Gal4 UAS-GFP/apUGO35, UAS-Ubx/tub-Gal80'),

**Figure 5. Differences in the amount of different Hox genes cause accumulation of sqh-GFP in imaginal discs.** (A–A') Z-stack of an Abd-B mutant clone induced in the genital disc and marked by the absence of lacZ expression (in blue in A') showing increased sqh-GFP expression around it (in green, A, A'). (B, B') Z-stack of a control Abd-B clone similarly marked but induced in the wing disc, showing there is no increase in sqh levels. (C) In ap-Gal4 UAS-GFP/apUGO35; UAS-Abd-B/tub-Gal80' wing discs, the D/V boundary is smooth (compare with Fig. 3C). (D) A similar result is obtained in ap-Gal4 UAS-GFP/apUGO35; UAS-OUbx/tub-Gal80'.

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lack of N signaling. These results suggest that strong differences in compartment, the smooth D/V border is largely restored in both Ubx larvae; n = 11), and therefore keep Ubx activity only in the dorsal compartment, the smooth D/V border is largely restored in both discs (Fig. 3E, F). These results suggest that strong differences in Ubx expression between dorsal and ventral compartments (that is, an on/off Ubx situation) can maintain a smooth D/V boundary in discs lacking N signaling.

Differences in Ultrabithorax Expression Induce Accumulation of Myosin

It has been proposed that N signaling creates a “fence” that prevents cells from the D and V compartments from mixing [10,14,15]. Accordingly, elevated levels of F-actin and of the regulatory chain of non-muscle myosin II, encoded by the gene spaghetti-squash (sqh), are observed at this boundary in the wing disc [14,15]. Significantly, a role for actomyosin in maintaining the A/P boundary in the wing disc [37] and in the embryo [38] has been described. Moreover, absence of zipper (zip), encoding the non-muscle myosin II heavy chain, prevents the maintenance of a normal D/V boundary in the wing disc [15].

To check if these proteins might also play a role in the separation of cells with different Ubx expression, we induced Ubx clones in the haltere disc and observed the expression of sqh-GFP and of zip-GFP. Most of these clones are surrounded by a ring of sqh-GFP, zip-GFP and phalloidine staining (Fig. 4A–B′′′). A lower level of bazooka (baz), a gene required to establish apico-basal cell polarity in Drosophila [39], was also reported at the wildtype D/V boundary of the wing disc [14], but in cells surrounding Ubx mutant clones baz-GFP expression is also increased (Fig. 4C–D′′′). Similar results are seen at the A/P compartment boundary of bx3 hh-lacZ/TM2, Ubx130 mutant clones, and in bx3 hh-lacZ/TM6B control discs the higher expression of sqh-GFP is not observed (Fig. 4E–F′′′). These results suggest that when the A and P compartments have different Ubx expression, the higher levels of myosin II at the Ubx+/Ubx− border may contribute to the maintenance of the A/P boundary in the absence of Hh signaling.

Although haltere and wing imaginal discs are physically separated throughout development, cells of different discs (as wing and haltere discs) form a continuous layer of adult cuticle during pupation. The different affinities in imaginal discs provided by Hox genes may prevent mixing of cells when this fusion takes place. In agreement with this idea, adult flies defective for Ubx occasionally present abnormal contralateral fusion of the T2 and T3 segments (Fig. 4G; G. Morata, personal communication).

Different Hox Genes Can Induce High sqh Levels

To see if other Hox genes may also induce cell segregation, we induced Abd-B mutant clones and look for myosin expression in the genital disc, where this gene is required [40]. As shown in Fig. 5A, A′, increased expression of sqh is observed surrounding these clones, but not in control Abd-B clones induced in the wing disc, where Abd-B is not required (Fig. 5B, B′). Consistently with these results, the expression of Abd-B in the dorsal compartment of the wing pouch is sufficient to maintain a straight D/V boundary when A signaling is compromised (Fig. 5C; n = 11). Moreover, the expression of an onycophoran Ubx (UAS-OUbx) [26] is also
Drosophila

Ubx

Hox gene

Deformed

Hh and N signaling

Ubx

23]. We have shown here that Ubx is sufficient to maintain to a large extent the A/P and D/V boundaries in the absence of Hh and N signaling (see Fig 6 for a summary of results). The mechanism whereby Ubx sorts out cells may be similar to that used by Hh and N signaling, and probably involves the accumulation of myosin where cells expressing and lacking Ubx are juxtaposed. This prevents territories with different properties to mix freely, and helps to get coherent patches of cells with distinct fates.

The sorting out of cells with distinct Hox activity in Drosophila has been reported before [24,33,40,42–44], and in the case of the Hox gene Deformed a possible function in cell segregation has been assigned to such activity [24]. We have observed some cases that show that Ubx is needed to maintain segregation of cells from different segments during pupation. It is possible that Drosophila Hox genes may have a function in cell segregation during this pupal stage, where cells from different discs and histoblast nests fuse to develop the adult cuticle. The mechanism of segregation seems to rely on the confrontation of cells with different Hox function and not on the absolute levels of Hox expression. This implies that Hox activity in neighboring cells may be checked through proteins at the cell membrane whose expression or levels must be controlled by Hox genes. In the embryo, the Hox gene Abd-B has been shown to regulate molecules like cadherins [45], and such proteins may mediate segregation between adjacent cells with distinct Hox input.

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In vertebrates, cells from different rhombomeres are also almost completely prevented from freely mixing [17]. As we have shown here for Drosophila, it has been proposed that the tension provided by the activity of actomyosin molecules, controlled by Hox genes, could prevent mixing of cells in the vertebrate’s rhombomeres [46]. Hox-directed cell segregation, therefore, prevents cells with different Hox code to intermingle, and therefore the appearance of homeotic transformations. This function of Hox genes may be an old one in evolution, required in animals in which development of different body regions is not coupled to the mechanisms of segmentation [47]. In Drosophila, this role of Hox genes may not be needed in cells that are physically separated during most of development (as in imaginal discs and histoblasts from different segments) or superseded by the activity of proteins like Engrailed and Hedgehog, but the maintenance of different affinities by Hox genes and signaling pathways through myosin accumulation may be a general mechanism to segregate cell populations in different species.

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Author Contributions

Conceived and designed the experiments: JRC LFdN ES. Performed the experiments: JRC LFdN ES. Analyzed the data: JRC LFdN ES. Wrote the paper: ES.
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