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Ultrabithorax and the control of cell morphology in *Drosophila* halteres

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SUMMARY

The *Drosophila* haltere is a much reduced and specialised hind wing, which functions as a balance organ. *Ultrabithorax (Ubx)* is the sole *Hox* gene responsible for the differential development of the fore-wing and haltere in *Drosophila*. Previous work on the downstream effects of *Ubx* has focused on the control of pattern formation. Here we provide the first detailed description of cell differentiation in the haltere epidermis, and of the developmental processes that distinguish wing and haltere cells. By the end of pupal development, haltere cells are 8-fold smaller in apical surface area than wing cells; they differ in cell outline, and in the size and number of cuticular hairs secreted by each cell. Wing cells secrete only a thin cuticle, and undergo apoptosis within 2 hours of eclosion. Haltere cells continue to secrete cuticle after eclosion. Differences in the shape of wing and haltere cells reflect differences in the architecture of the actin cytoskeleton that become apparent between 24 and 48 hours after puparium formation. We show that Ubx protein is not needed later than 6 hours after puparium formation to specify these differences, though it is required at later stages for the correct development of campaniform sensilla on the haltere. We conclude that, during normal development, Ubx protein expressed before pupation controls a cascade of downstream effects that control changes in cell morphology 24-48 hours later. Ectopic expression of Ubx in the pupal wing, up to 30 hours after puparium formation, can still elicit many aspects of haltere cell morphology. The response of wing cells to Ubx at this time is sensitive to both the duration and level of Ubx exposure.

Key words: *Drosophila melanogaster*, *Ubx*, Hox genes, Cell shape, Pupal development, Wing, Haltere, Morphogenesis

INTRODUCTION

Much remains to be learned about the molecular processes that shape differentiating cells, and how these basic mechanisms are modified in different regions of the body to mould specific details of body architecture. In this paper, we define an attractive system for the study of this question – the transformation of a *Drosophila* wing cell into a haltere cell under the control of the *Hox* gene *Ultrabithorax*.

Halteres are the reduced and highly modified hind wings borne on the third thoracic segments of Diptera (true flies). These club-shaped appendages bear a complex array of sensory organs in their basal part (Fig. 1A). They play a crucial role in maintaining balance during flight and motion (Pringle, 1948; Chan et al., 1998).

Ultrabithorax is the primary genetic switch that controls the differences between the wing and haltere of *Drosophila* (Lewis, 1978). No Hox gene is expressed in the wing epithelium, but Ubx protein is expressed in the haltere throughout development (White and Wilcox, 1984). Mutations that block Ubx expression in the developing haltere lead to a complete homeotic transformation of the haltere into a wing (Bender et al., 1983). Conversely, *Ubx* mutations that cause ectopic expression of Ubx protein in the developing wing cause transformations of varying penetrance into haltere tissue (White and Akam, 1985; González-Gaitán et al., 1990).

Ubx is responsible for the differences between fore and hind wings, not just in Diptera, but also in moths, beetles, and probably all winged insects (Denell et al., 1996; Weatherbee et al., 1999). Therefore the specialised characteristics of the Dipteran haltere probably evolved, in large measure, by changes in the regulation of Ubx downstream target genes. Some of these targets modify the growth and early patterning of the haltere during the larval period, when both wing and haltere are developing as imaginal discs (sacs of largely undifferentiated cells that lie within the larval body). Growth of the haltere disc ceases at about 10,000 cells, the wing disc at 50,000. Primordia for the marginal bristles and territories for the veins are specified in the wing during the larval period. These patterning events are suppressed in the haltere (Weatherbee et al., 1998).

The onset of metamorphosis is followed by a complex series of morphogenetic movements that transform the folded, single layered wing disc into a flat, bi-layered wing blade (for review, see Fristrom and Fristrom, 1993). During later stages of pupal development wing cells assume a complex shape to form the scaffold upon which the hairs and other cuticular structures will be secreted. This process of cell differentiation has been characterised in some detail (Mitchell et al., 1983; Fristrom et al., 1993, Eaton et al., 1996), but the differentiation of haltere epidermis has never been described. We show here that the differences between individual wing and haltere cells are substantial.

As a first step to establishing how *Ubx* controls these differences, we have examined when they are generated during pupal development, and when Ubx protein acts to induce them, both during normal development and following ectopic expression. We find that *Ubx* is required only during the latest stages of larval development and possibly the first few hours in the puparium to elicit a program of differentiation in the haltere that will shape cell morphology during the following 4 days of pupal development. From 6 hours after puparium formation (APF), the orchestration of this complex process can proceed almost normally in the absence of Ubx protein. However, many morphological features of the wing can still be modified by Ubx protein to resemble those in the haltere for the first 30 hours after pupation.

MATERIALS AND METHODS

Fly strains

Cell morphology was analysed in Oregon-R flies or in a strain expressing nuclear green fluorescent protein under the control of a Ubiquitin promoter (*UbqnGFP*), (Davis et al., 1995). The *apterous lac-Z* reporter construct was used to mark dorsal territories in the halteres (Díaz-Benjumea and Cohen, 1993).

Ubx^{1}, a null allele of the *Ubx* locus (Lindsley and Zimm, 1992), was used in the clonal analysis experiment, recombined with the *FRT82* insertion (Xu and Rubin, 1993). The *HsUbx* strain used for ectopic Ubx expression was the homozygous viable *HSU42* insertion described by González-Reyes and Morata (1990).

Clones expressing Ubx ectopically were generated using the *AyGAL4 25* flip-out cassette (Ito et al., 1997), the *yw P{ry+; hsFLP}* flippase source (Golic and Lindquist, 1989) and the *UAS UbxIa1* insertion (H. Reed and M. A., unpublished), which expresses the Ia isoform (Kornfeld et al., 1989) of the Ubx protein under the control of the UAS promoter (Brand and Perrimon, 1993). These clones were marked with both GFP, by means of the *UAS-GFP S65T* and by the *yellow* mutation (Ito et al., 1997). The *AyGAL4 25* construct contains a cytoplasmic actin promoter that drives GAL4 expression after flippase mediated recombination. All flies were cultured at 25°C on yeast/glucose medium unless otherwise stated.

Cell morphology analysis

Pupae were collected over 4 hour periods, aged at 25°C to the appropriate time and dissected in phosphate-buffered saline, pH 7.2 (PBS). Dissected pupae were fixed overnight at 4°C in 4% paraformaldehyde in PBT (PBS+0.3% Triton X-100), after piercing the pupal case to allow penetration of fixative. Wings and halteres were hand peeled to remove the pupal membrane enclosing the tissue. The tissue was incubated for 2 hours at room temperature in rhodamine-conjugated phalloidin (Molecular Probes; 0.33 mM in PBT), or with the membrane dye FM1-43 (Molecular Probes; 16 mM in PBS). After staining, tissues were washed for 30 minutes in PBS and mounted in Vectashield (Vector) for confocal microscopy.

Electron microscopy and thick sections

Wings and halteres from adult flies were collected 2 hours after eclosion, fixed for 4 hours in 2.5% glutaraldehyde in 0.1 M sodium cacodylate, 5% sucrose buffer at pH 7.2, washed, and fixed in 1% osmium tetroxide and 1% uranyl acetate. After dehydration samples were mounted in Araldite, and sections of 60-90 nm were cut with a Reichert Ultramicrotome OMU2. Sections were stained with uranyl acetate and lead citrate and examined with a Phillips EM 300 electron microscope. 5 μm thick sections were stained with 1% methylene blue in 1% boric acid.

Ubx clonal analysis

Clones of homozygous *Ubx* mutant cells were generated in the haltere using the FLP/FRT system (Xu and Rubin, 1993). Flies of the genotype *w hsFLP; FRT 82B Ubx*^{1} *e/FRT82B* were heat shocked at 37°C for 1.5 hours at 24 hours before puparium formation (BPF), 6 hours BPF, 6 hours APF and 18 hours APF.

Induction of ectopic Ubx expression

Flies with 0, 1, or 2 copies of the *HsUbx* construct (*+/TM6b*, *+/HsUbx* and *HsUbx/HsUbx*, respectively) were raised at 25°C. Newly forming pupae (0-4 hours APF) were selected and transferred to fresh vials. 45 minute heat shocks were administered by placing vials containing the pupae into a 37°C water bath. Heat shocks were applied between 12 and 42 hours APF. Before 12 hours APF, heat shocks lead to complete developmental arrest, and so could not be studied. To maintain Ubx expression for periods longer than 6 hours, heat shocks were administered as a series of single pulses of 45 minutes, spaced at 6 hour intervals.

After the heat shock, flies were left to develop at 25°C, dissected at 56-60 hours APF and stained with rhodamine phalloidin. In some cases we also analysed the cuticle morphology of wings and halteres dissected from pharate adults (*HsUbx* flies do not eclose after heat shock). These different observation techniques provide similar results,

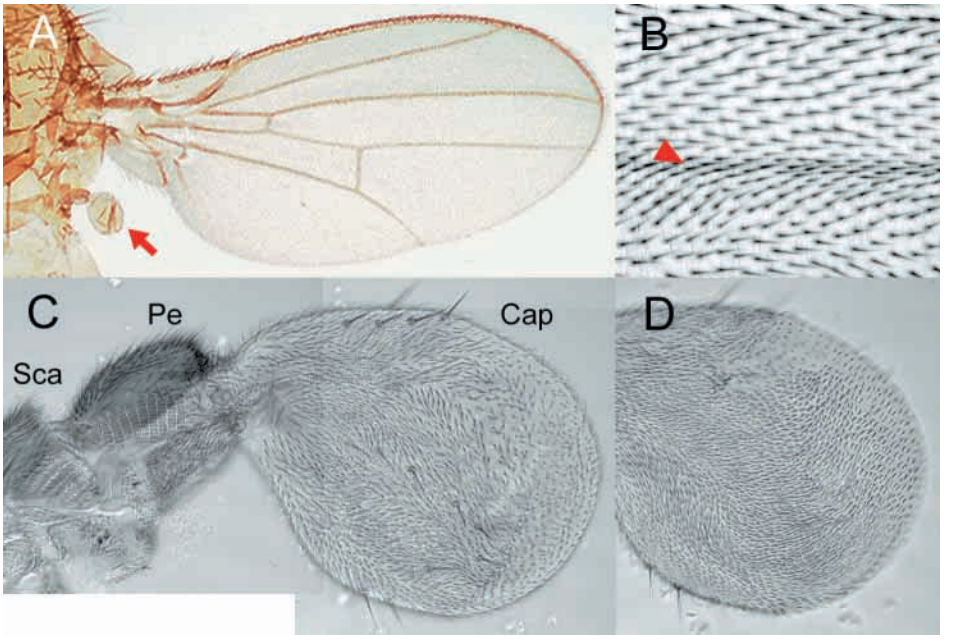

Fig. 1. Wing and haltere adult cuticle morphology. (A) Adult fly thorax showing its two dorsal appendages, the mesothoracic wing and the small metathoracic haltere (arrow). (B) The transparent adult wing cuticle is secreted by flat cells that produce a single hair pointing distally. The vein cells (arrowhead) have a more reduced apical surface and are also pigmented. (C,D) The haltere is made of three distinct pieces of cuticle, from proximal to distal, the scabellum (Sca), the pedicellus (Pe), containing a conspicuous array of campaniform sensilla, and the capitellum (Cap). The haltere cuticle surface is covered by short and thin hairs densely packed.

confirming that any developmental delay caused by the treatments is not responsible for the phenotypes observed at 56-60 hours APF. In the control group without the *HsUbx* construct, heat shock slows development, but does not cause significant malformations in the overall shape of wings or in wing cell morphology.

Clones expressing Ubx ectopically were generated at different times during larval development by heat shocking flies of the *yw hsFLP; AyGAL4 25 UAS-GFP S65T/UAS UbxIa1* genotype for 15 minutes in a 37°C water bath. Animals pupating 0-4 hours and 44-48 hours after heat shock were allowed to develop at 25°C. Clones were observed at 36 or 48 hours APF in fixed pupal wings stained with rhodamine phalloidin or were analysed in fully developed flies, before eclosion. GFP could be detected in the wing cells underneath the cuticle before eclosion. The pupal case was removed and flies were left in distilled water with 0.3% Triton X-100 for about 10 minutes, to facilitate wing expansion. Flies were then fixed for 25 minutes in 1:1 heptane:glutaraldehyde 0.75% in PBS, with constant agitation. Wings were washed in PBS, and immediately mounted in Vectashield (Vector) for fluorescence microscopy.

Clones were also induced in flies of the same genotype just after an egg collection of 24 hours. These early clones were detected in the adult body as patches marked with the *yellow* mutation.

Immunostaining

Immunostainings were performed according to (Fristrom et al., 1993) in pupal tissues using the anti-Ubx FP3.38 monoclonal antibody 1/100 (White and Wilcox, 1984), the rabbit anti-PS2 serum, 1/1000 (Bloor and Brown, 1998) and the mouse anti-β-gal (Promega), 1/1000. Secondary antibodies used were anti-mouse and anti-rabbit IgG conjugated to Cy5 or FITC (Jackson Labs). Nuclei were counter stained with propidium iodide.

RESULTS

By the end of pupal development, epithelial cells of the wing blade and the haltere capitellum differ dramatically. The most obvious differences are that each wing cell secretes an 8 fold larger area of cuticle than a typical haltere cell, and each wing cell produces a single long hair, while haltere cells bear 2, 3 or 4 hairs, each much smaller (Figs 1, 2). These, and other less obvious features (see below) reflect differences in the organisation of the cytoskeleton of the cells at the time when cuticle is secreted.

To characterise the morphological differences between differentiating wing and haltere cells, we stained pupal imaginal discs with rhodamine-conjugated phalloidin and examined them by confocal microscopy. Phalloidin binds specifically to actin microfilaments (Wulf et al., 1979), and so reveals cell outlines and microfilament bundles that underlie differentiating hairs. We have restricted our observations to the distal regions of the appendages, avoiding the complexity of the proximal hinge regions, and have considered only the typical wing epithelial cells, not the cells of the wing veins.

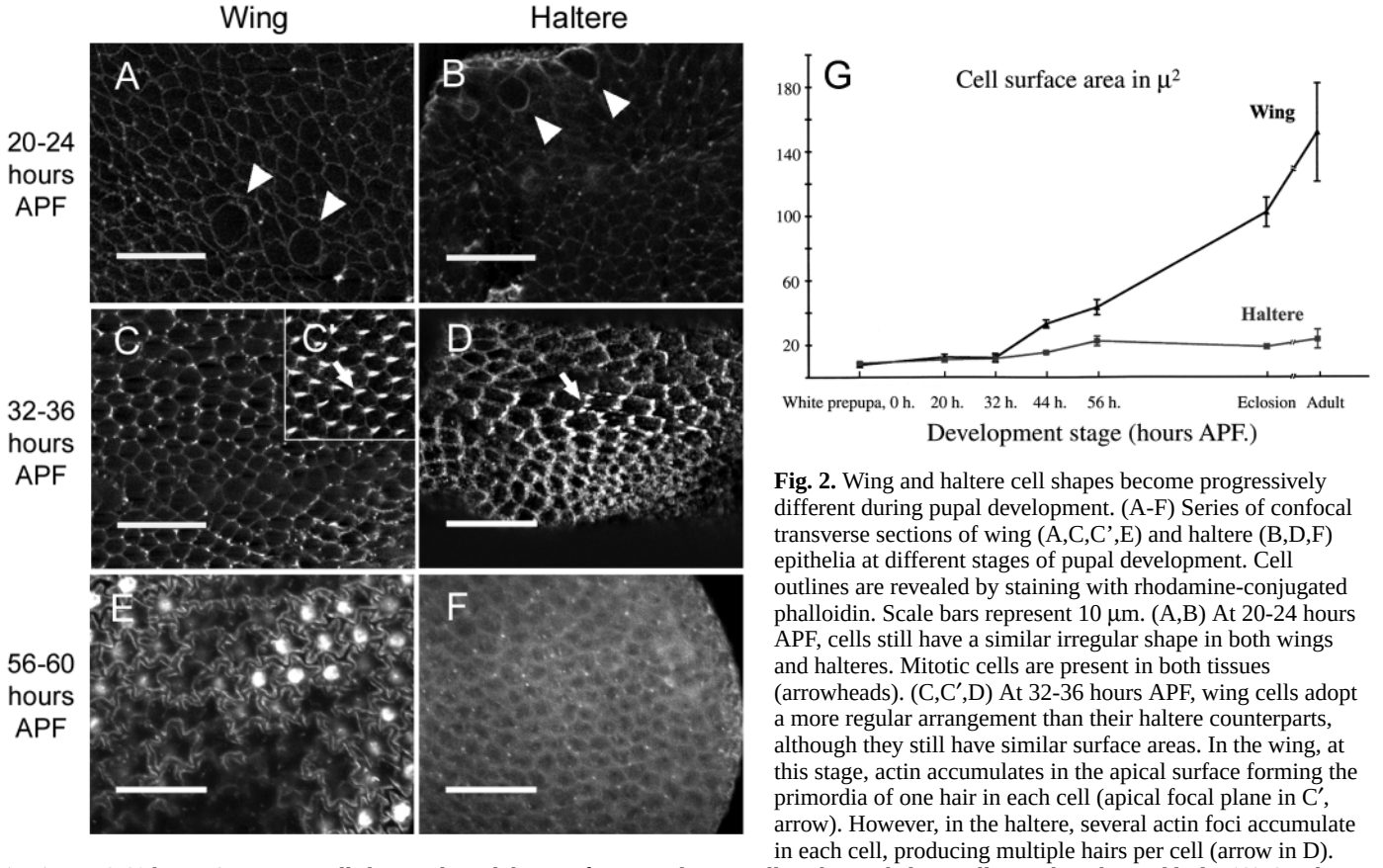

Fig. 2. Wing and haltere cell shapes become progressively different during pupal development. (A-F) Series of confocal transverse sections of wing (A,C,C',E) and haltere (B,D,F) epithelia at different stages of pupal development. Cell outlines are revealed by staining with rhodamine-conjugated phalloidin. Scale bars represent 10 μm. (A,B) At 20-24 hours APF, cells still have a similar irregular shape in both wings and halteres. Mitotic cells are present in both tissues (arrowheads). (C,C′,D) At 32-36 hours APF, wing cells adopt a more regular arrangement than their haltere counterparts, although they still have similar surface areas. In the wing, at this stage, actin accumulates in the apical surface forming the primordia of one hair in each cell (apical focal plane in C′, arrow). However, in the haltere, several actin foci accumulate in each cell, producing multiple hairs per cell (arrow in D). (E,F) By 56-60 hours APF, wing cells have enlarged their surface area dramatically, whereas haltere cells area has changed little. (G) Graph comparing the surface areas of wing (triangles) and haltere cells (squares) at different times during development. Estimates of cell areas were obtained in each case by counting the number of cells present in a square of known area. At least ten independent measurements were taken in each case and the average value was plotted. Error bars indicate the standard deviation of the mean calculated for each data set. After eclosion (96 hours APF), wing cells expand further before cuticle hardening.

Wing and haltere cell morphology diverge during pupal development

The size and shape of cells in wings and halteres are very similar at the time of puparium formation (not shown), and for the first day of pupal development (Fig. 2A,B). During this period, the folded discs evert, and the pseudostratified epithelium that is characteristic of the imaginal discs transforms into a columnar epithelium. In discs 20-24 hours APF we observe cells in both the wing and the haltere that are undergoing mitosis (Fig. 2A,B). These mitotic cells round up at the apical surface of the epithelium and appear as holes in the more regular array of non-dividing cells.

Differences between the wing and haltere epithelia become evident by 32-36 hours APF. In the wing, cells form a well organised hexagonal array (Fig. 2C). Single hairs appear in every cell as brightly stained actin projections (Fig. 2C′ insert), see also Wong and Adler (1993). In the haltere, cells have more variable shapes. Hair development is slightly delayed with respect to the wings, but by this stage we already observe multiple actin foci forming at the distal apical margin of many cells (Fig. 2D).

By 56-60 hours APF, both epithelia have acquired many features of the fully differentiated tissue. Wing cells have increased in surface area, and have acquired a star like shape, interdigitating with their neighbours (Fig. 2E). In the centre of each wing cell, on the apical side, a conspicuous cytoplasmic projection called the hair pedestal, harbours a single hair (Figs. 2E and 3A,C,E) (Fristrom et al., 1993). In contrast, haltere cells enlarge very little (compare panels 2D and 2F). They are cuboidal, almost rectangular in surface outline, and each carries between 1 and 4 short hairs, pointing to the distal part of the appendage (Fig. 3B,D,F).

Between 56 hours APF and the time of eclosion (about 96 hours APF), the surface area of the wing epithelial cells continues to expand by a factor of almost three, while the area of cells in the haltere changes very little (Fig. 2G). There is a further increase in the surface area of the wing cuticle after eclosion, as the wing is inflated before tanning of the cuticle defines the final shape and size of the mature wing.

Wing cells secrete only a thin cuticle (0.2 μm). Within the 2 hours immediately following adult eclosion, the intervein cells in the wing epithelium undergo apoptosis and disappear (Fig. 3G) (Johnson and Milner, 1987). We find that haltere cells secrete a much thicker cuticle (>0.6 μm). They are still alive 2 hours after eclosion, and appear to be actively secreting cuticle (Fig. 3H).

Basal adhesion is differentially regulated in wings and halteres

Although wing and haltere cells have similar shapes and sizes until 24 hours APF, their basal adhesion properties are already different by 6 hours APF. Cells of the dorsal wing surface become apposed to cells of the ventral surface immediately after pupariation (Fristrom et al., 1993). The two layers separate again during head eversion (11 hours APF) but reappose at 20 hours APF, remaining tightly attached until the end of development (Fristrom et al., 1993). Haltere sections of different stages stained with phalloidin show that the halteres remain hollow throughout pupal development and post-eclosion development (Fig. 4A-D).

In the wing, integrins are necessary for the process of apposition (for review, see Gotwals et al., 1994). They encode transmembrane receptors that bind extracellular matrix proteins. Dorsal wing cells express the α1 and β integrin subunits, which together form the integrin PS1 heterodimer.

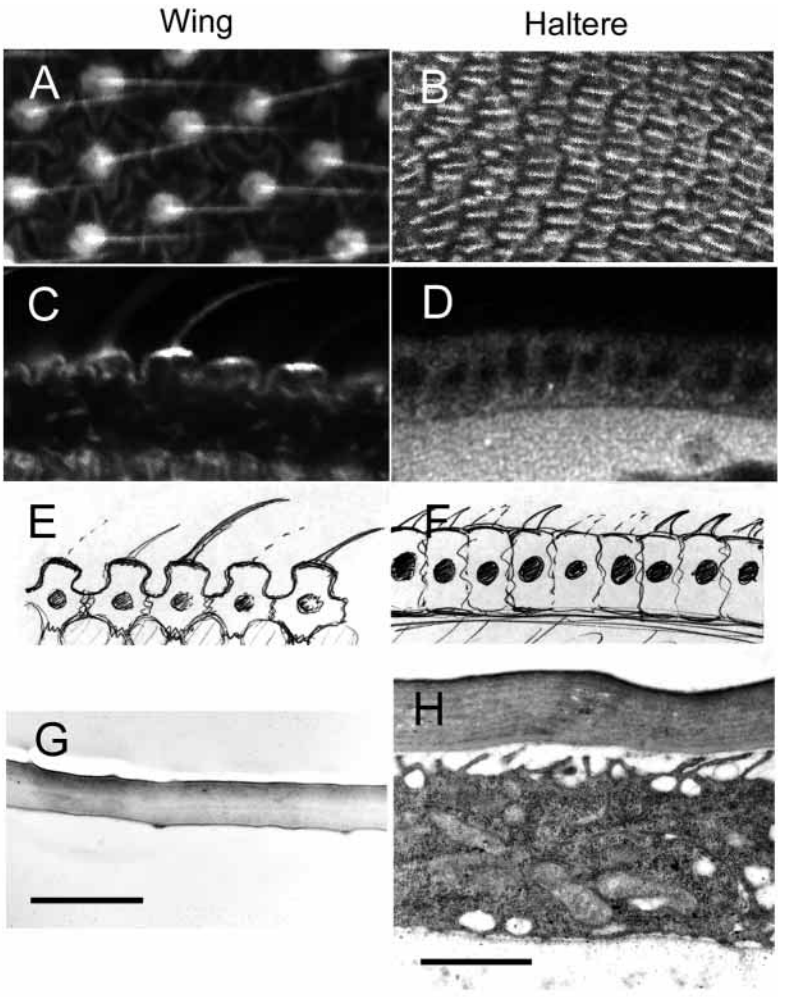

Fig. 3. Cell morphology in wing (A,C,E,G) and haltere (B,D,F,H) cells. Apical transverse sections (A,B) or longitudinal sections (C-F) of wings and halteres aged 56-60 hours APF, stained with rhodamine phalloidin (A-C) or the membrane dye FM1-43 (D). At this stage, the flat wing cells show their characteristic hair pedestal, accumulating actin in the base of the hairs, and showing a star-like lateral contour. Cells make close basal contacts with the cells in the opposite surface (visible at the bottom of C). The haltere cells are cuboidal and produce multiple short hairs on their rectangular apical face. Their basal side faces the lumen of the haltere. The cartoons in E and F illustrate schematically the differences between the two tissues. (G,H) Transmission electron micrographs of ultra thin sections of wing (G) and haltere (H), fixed 2 hours after eclosion. The wing is made of two thin layers of cuticle tightly apposed. The cells that secreted it have disappeared after undergoing apoptosis. The haltere cells are still present at this stage and are metabolically active, secreting a thick cuticle from their apical side by means of cytoplasmic extensions. Scale bars represent 1 μm.

Ventral cells express the α2 and β subunits, forming the PS2 heterodimer.

We have examined whether the adhesion differences between wing and haltere are correlated with changes in the pattern of expression of integrin PS2. This does not seem to be the case. Stainings with an anti-PS2 antibody show that by 6 hours APF, PS2 is present in the majority of the ventral cells, although these are not apposed to the dorsal epithelium (Fig. 4E). At 40 hours APF, cells retract from the centre of the haltere, leaving a hollow cavity. At this stage, in the haltere capitellum, PS2 is again associated with contacts between ventral cells and the central haltere matrix, rather than with dorsal cells (Fig. 4F). In proximal regions, PS2 is associated with basal contacts between dorsal and ventral cells (Fig. 4F), as occurs in the wing, and with basal contacts involving only ventral cells.

Ultrabithorax requirements during haltere cell differentiation

We have shown above that many of the differences between wing and haltere cells first become apparent between 24 and 48 hours APF. To determine when the action of Ubx protein is required to specify these differences, we have generated clones of cells homozygous for the Ubx^1 null mutation at different times during the late larval and early pupal period.

In essence, these experiments repeat those carried out more than 20 years ago by Morata and García-Bellido (1976). They showed that *Ubx* mutant clones induced in the haltere up to 16 hours BPF developed as wing epithelium, not haltere. However, clones induced after 16 hours BPF showed no obvious transformation. They interpreted this result as being most likely due to the persistence of Ubx protein inherited from the clone mother cell in its descendants throughout the period of cell differentiation (Morata, 1975).

In our experiments, we have directly determined how long Ubx protein persists after clone induction. We used an anti-Ubx antibody to distinguish homozygous *Ubx* null clones and their twin wild-type clones from the background of heterozygous *Ubx* cells in the imaginal discs.

Using FLP recombinase to induce clones, we find that Ubx protein does not persist at detectable levels for more than 12 hours after clone induction. When the recombinase is induced by a heat shock at 6 hours BPF, single cell null clones and their twin spots are clearly visible by 6 hours APF (Fig. 5A,B). The null clones contain no detectable residual Ubx protein. These clones remain in the disc until at least 44 hours APF (not shown), but no abnormalities can be detected in the final cuticle (Fig. 5C). We presume that they differentiate as normal haltere cells (see also Morata and García-Bellido, 1976).

When clones are induced in exactly the same way, but earlier in development, 24-28 hours BPF, the *Ubx* null cells produce

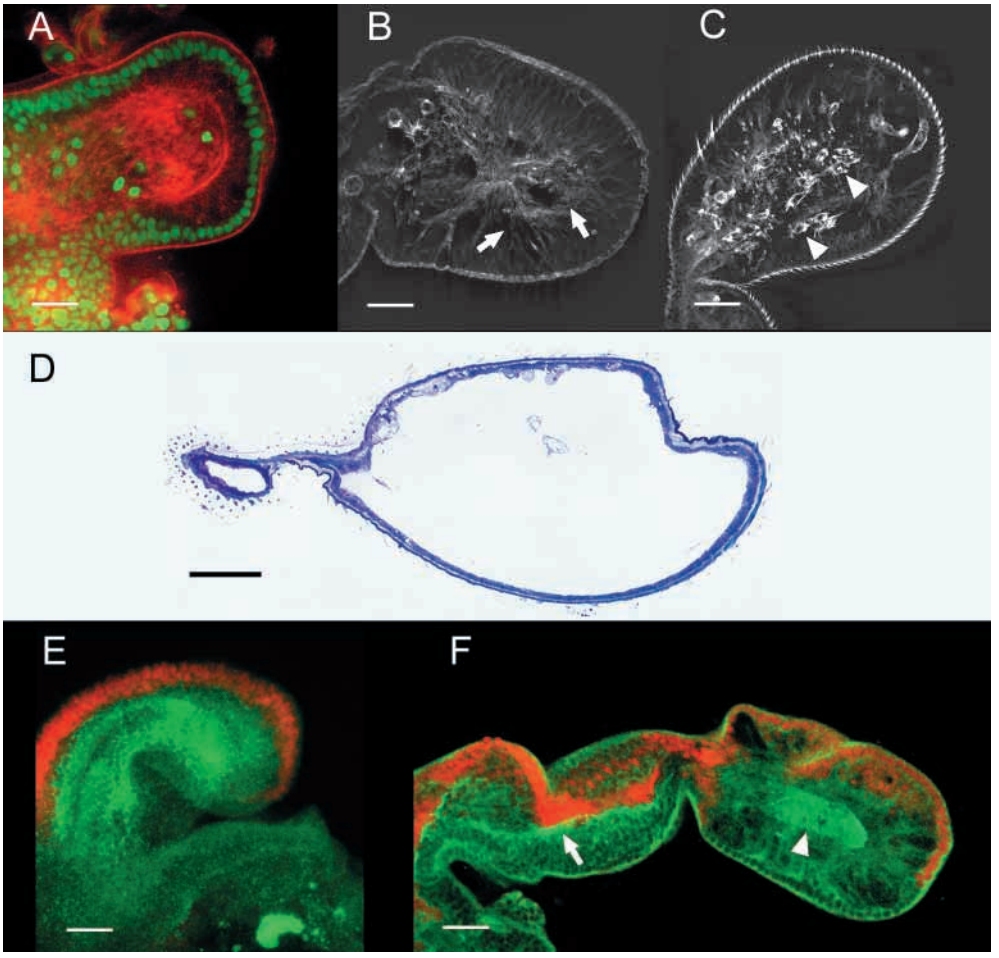

Fig. 4. Basal adhesion and PS2 expression during haltere pupal development. (A-C) Haltere confocal longitudinal sections stained with rhodamine phalloidin. Nuclei are also marked in A with nuclear GFP. (D) Longitudinal thick section of a haltere stained with toluidine blue, dissected 2 hours after eclosion. (E,F) Confocal pictures of halteres immunostained with anti-PS2 (green, membrane) and β-gal expression driven by the *apterous lac-Z* dorsal cells marker (red, nuclear). (A) 6 hours APF. The haltere capitellum is hollow and is filled with a loose matrix patrolled by haemocytes. At this stage, PS2 is strongly expressed by most of the ventral cells in the capitellum (E) and accumulates at the basal side of the epithelium, despite the absence of contacts with the dorsal surface. (B) By 20-24 hours APF, haltere cells have elongated. Long basal projections contact the central matrix (arrows). (C) At 44-48 hours APF, the haltere cells retract along their apical/basal axis, leaving behind a space containing only haemocytes (arrowheads). PS2 expression at this stage is associated with contacts between opposite surfaces in the pedicellus region (arrow), and with the matrix in the capitellum centre (arrowhead) (F). In the adult (D), cells have flattened and retracted completely from the central space, leaving a hollow cavity within the haltere. Scale bars represent 10 μm.

thicker, longer and more widely spaced hairs than the neighbouring haltere cells, suggesting that they are partially transformed to the wing cell type (Fig. 5D).

We conclude that, in normal development, Ubx protein acts prior to or immediately after puparium formation, and that, in most respects, cell differentiation in the pupal haltere is independent of the direct effects of *Ubx*.

Wing cells remain sensitive to Ubx during pupal development

The results above suggest that Ubx protein is not normally required during most of the pupal period. However, if Ubx is ectopically expressed in wing cells after pupation, it can still elicit many aspects of the haltere phenotype (Figs 6, 7).

We have used a heat-shock inducible *Ubx* construct to express the Ia isoform of Ubx protein at different times during pupal development. Three parameters have been controlled in these experiments: the onset of Ubx expression (determined by the time of first heatshock), the duration of Ubx expression (determined by the number of consecutive heat shocks), and the levels of Ubx protein produced (determined by the number of *HsUbx* genes in each cell).

Using this transgene in embryos, heat shocks of 1 hour produce levels of Ubx protein similar to the maximum endogenous levels seen in parasegment 6. These levels are reached within half an hour of the end of the pulse, and persist for about 6 hours before declining (González-Reyes and Morata, 1990). We have monitored the expression of Ubx in pupal wings 2 hours after a heat shock pulse of 45 minutes. Under these conditions, levels of Ubx protein in the wings of flies heterozygous for the *HsUbx* insertion (1 copy *HsUbx*) are similar to those seen in wild-type halteres of the same age. Flies homozygous for the *HsUbx* insertion (2 copies *HsUbx*) produce significantly higher levels of expression than those seen in normal halteres (data not shown). Thus, we have assayed in our experiments the effects of expressing Ubx levels typical of the haltere, and also of expressing significantly higher levels.

Different heat shock regimes lead to very different degrees of transformation. Cell morphologies range from those typical of the normal wing, to something resembling haltere tissue (Fig. 6). Anterior wing cells, close to the margin, are in all cases more transformed than posterior cells, with a smooth gradient of transformation seen across the wing, even though the heat shock construct provides rather uniform levels of Ubx expression throughout the wing (data not shown).

In animals carrying a single copy of the transgene, cell morphology is not dramatically affected (Fig. 6C,E,G,I), although the formation of hair pedestals is disturbed by early pulses (12-24 hours APF and 18-30 hours APF; Fig. 6C,E). Much more dramatic effects can be seen in flies carrying two copies of *HsUbx*. Late heat shocks (30 to 42 hours APF) prevent the formation of hair pedestals and significantly inhibit cell expansion, but are unable to produce the duplication of the cell hairs normally seen in the haltere (Fig. 6J). Earlier heat shocks (24 to 36 or 18 to 30 hours APF) also cause cells to produce short and multiple hairs, and reduce cell expansion more dramatically (Fig. 6F,H). However, even in the most extreme cases (12 to 24 hours APF), transformed cells are not identical to typical haltere cells (compare Fig. 6B and D).

When comparing the effects of different heat shock regimes,

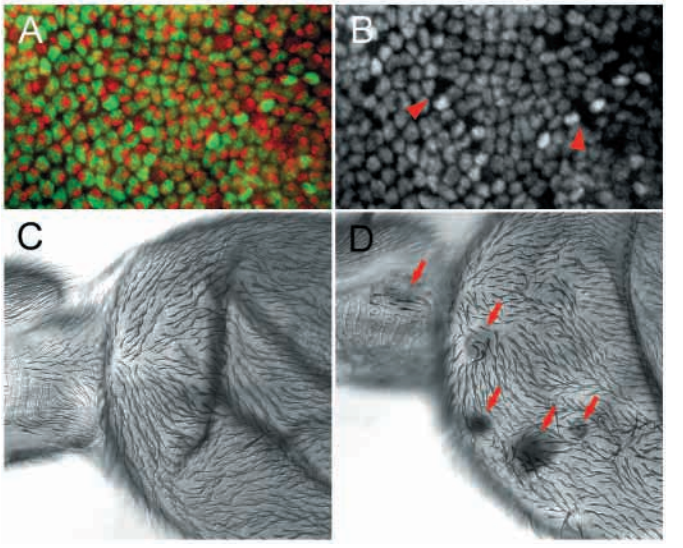

Fig. 5. *Ubx* clonal analysis during pupal development. (A,B) Expression of Ubx protein in halteres dissected 6 hours APF and containing Ubx^{1} mutant clones induced 6 hours BPF. (A) Ubx protein (green) is absent from single nuclei (counter stained with propidium iodide, red). (B) Same picture as A, showing only the green channel. For each cell lacking Ubx (arrowheads), a twin cell can be identified that accumulates higher levels of Ubx than the surrounding $Ubx^{-/+}$ cells. (C) Adult cuticle of a haltere containing *Ubx* mutant clones induced 6 hours BPF, as in A,B. No morphological abnormalities can be detected in the cuticle, despite the high frequency of clones induced. (D) Some mutant Ubx^{-} clones induced 28-24 hours BPF do show cuticle transformations (arrows). Hairs are longer, thicker and more spaced out than in the rest of the haltere. Null clones are homozygous for the *ebony* marker, which makes them darkly pigmented (though this marker is not fully autonomous).

several consistent trends can be highlighted. Early pulses always have greater effects than late ones, provided that the length of the cell exposure to Ubx is equal (compare Fig. 7D with F, and 7E with G). Long periods of Ubx expression have stronger effects than short ones (compare 7D and H), in some cases even if the short pulses were initiated earlier than the long ones (data not shown). After identical treatments, two copies of the *HsUbx* construct produce stronger morphological effects than a single copy (compare Fig. 7D with E, and 7F with G). Ubx overexpression has no effects on cell morphology if induced later than 20-24 hours APF in animals carrying a single copy of *HsUbx* (Fig. 7F). This limit is extended to at least 32-36 hours APF in the case of the flies bearing two copies of *HsUbx* (not shown).

We conclude that the commitment of cells to acquire a certain morphology is affected by the presence of Ubx during pupal stages; that the system is sensitive to Ubx protein levels and also to the length of time that Ubx is acting within the cells.

Other features of wing development are also affected by the expression of Ubx in the pupa. Most treatments result in a marked reduction in wing size. This is largely due to inhibition of the cell expansion that takes place in normal wings after 44-48 hours APF. Treatments given before 20-24 hours APF prevent cell adhesion between the two wing surfaces, at least in the anterior part of the wing, leaving a hollow cavity filled with haemocytes. Treatments given before 12-18 hours APF

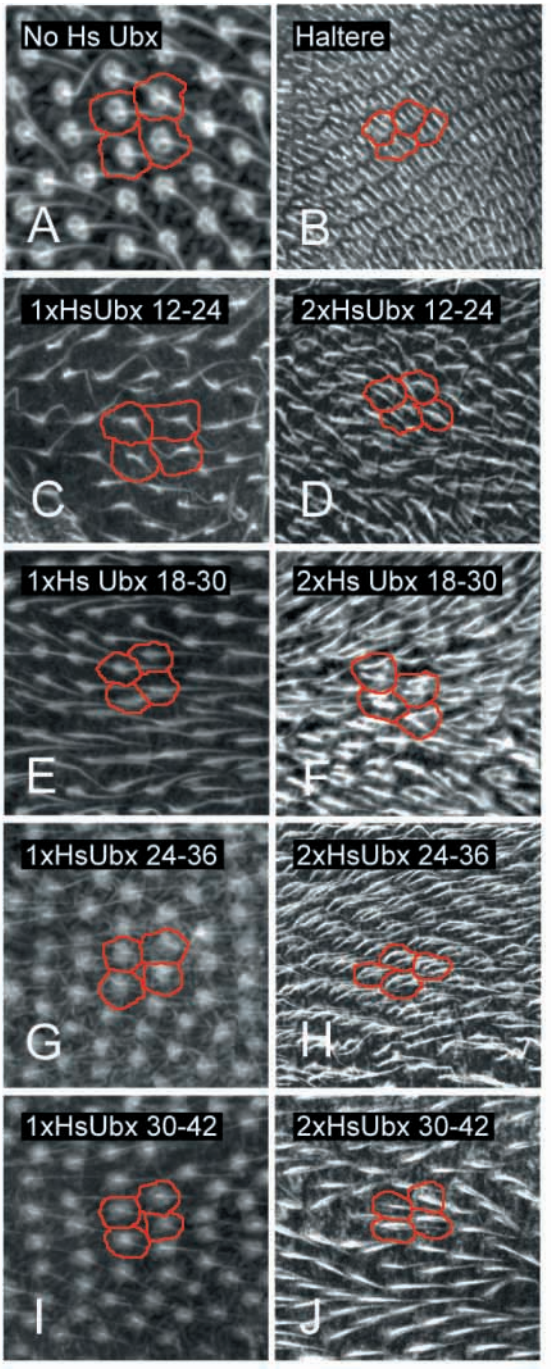

Fig. 6. Confocal images of the cellular surface from wings (A,C-J) or from a haltere (B) aged 56-60 hours APF and stained with rhodamine phalloidin. All the pictures correspond to the most anterior region of the wing, between the margin and the second longitudinal vein. (A,B) Control *HsUbx* non heat shocked wing and haltere, respectively. (C,E,G,I) Wings carrying one copy of the *HsUbx* transgene treated at different times during pupal development. Early treatments (C,E) preclude pedestal formation but do not elicit the formation of multiple hairs. Treatments given more than 24 hours APF have little effect on cell morphology (G,I). (D,F,H,J) Wings of flies carrying two copies of *HsUbx*, treated as in C,E,G,I. Early treatments produce cells that are much smaller than those of normal wings, carrying multiple hairs, resembling haltere cells (D), although the transformation is not complete (compare to B). Later *HsUbx* treatments produce intermediate phenotypes (F,H). Induction after 24-36 hours is unable to induce hair cell duplications (J), although it impairs cell expansion and suppresses the formation of hair pedestals.

also abolish the differences in cell phenotype that characterise wing veins and interveins (data not shown). Bristle morphology is affected in the treated wings, although neither the number nor the position of bristles is altered.

Ectopic expression of Ubx in clones produces cell morphology phenotypes

To confirm the results of the heat shock experiments, and to test whether the effects of *Ubx* are cell autonomous, we have generated flies that express Ubx ectopically in clones in the wing. These experiments used a construct activated by FLP recombinase to drive the expression of GAL4 in clones (Ito et al., 1997), which in turn can activate both UbxIa and GFP. They proved much more difficult to interpret than the heat shock experiments, and highlight some difficulties with the GAL4-UAS system. The Ubx protein levels produced varied a great deal between cells of different clones and even within the same clone, for reasons that we do not understand. Ubx and GFP were not always co-expressed, and the expression of detectable Ubx was delayed, sometimes by more than 24 hours after heat shock induction of FLPase (not shown).

Clones induced at 0-4 hours APF show no obvious phenotype. Clones induced 48 hours BPF are sometimes transformed to resemble haltere cells, but this transformation is not fully penetrant (see legend to Fig. 8). Not all clones show any transformation, and even in those that do, not all cells are transformed (Fig. 8C). However, we do not believe that this can be taken as evidence of non-autonomy. Levels of Ubx protein vary markedly between clones, and between cells within clones, even many hours after clone induction.

As a control, we also generated clones much earlier, during embryogenesis. These clones gave rise to large patches of tissue in the wing that differentiated as tissue very similar to the normal haltere (Fig. 8D,E). Thus this GAL4 system of ectopic Ubx expression can drive the differentiation of typical haltere tissue, but the activation of target genes is unreliable when the expression of GAL4 is induced at late stages.

Many of the 48 hours BPF clones expressing Ubx ectopically have fewer cells than controls (an average of 4 cells per clone, instead of 24 in controls), indicating that Ubx may also be affecting the rate of cell division, or having toxic effects when present at high levels. When the same experiments were repeated with a different *UAS Ubx* line expressing higher levels of Ubx protein, few clones were observed in adult wings, and apoptotic cells were observed in both wing and haltere discs, suggesting that very high levels of Ubx protein kill cells.

Ubx protein is required during pupal development for the shaping of campaniform sensilla on the haltere

Although cells of the haltere epithelium are independent of *Ubx* from the time of puparium formation onwards, specific sense organs on the haltere require Ubx during this time for normal development. These include the campaniform sensilla in the dorsal pedicellus, which are homologous to those on the dorsal radius of the wing (Cole and Palka, 1982). On the haltere

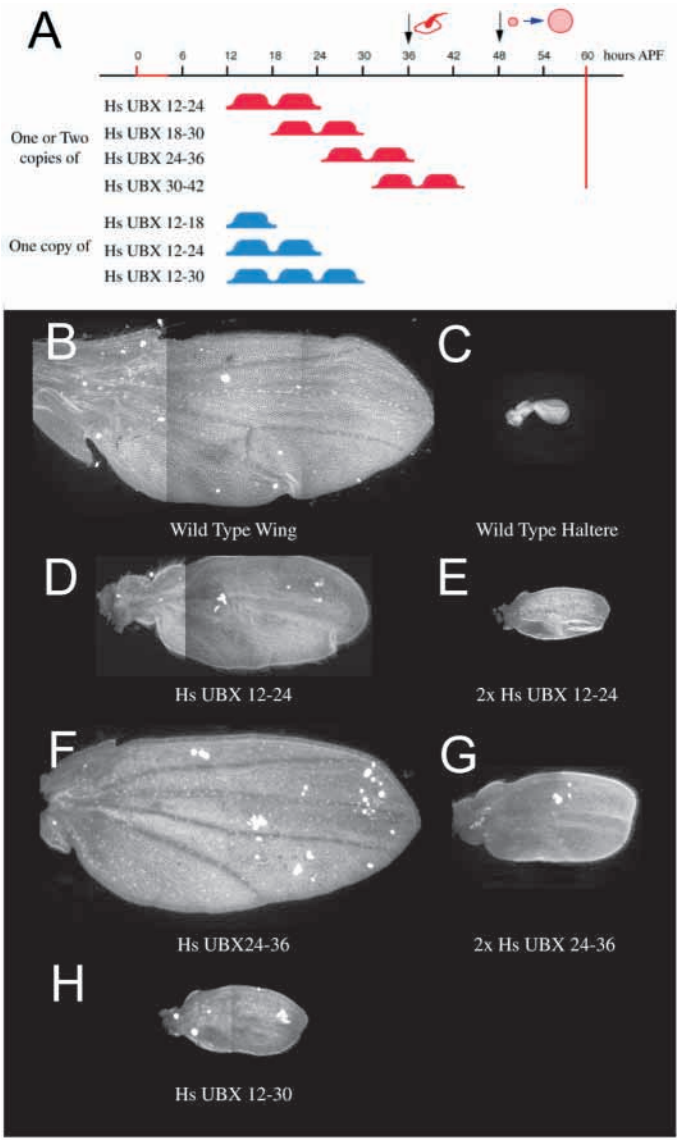

Fig. 7. Effects of different heat shock Ubx treatments on overall wing morphology. All the wings and the haltere, stained with rhodamine phalloidin, were dissected at 56-60 hours APF and photographed using the same magnification. (A) Cartoon showing the schedule of heat shock Ubx pulses used in this study. Each single peak of Ubx expression is achieved after a 45 minute heat shock pulse at 37°C, and Ubx expression is assumed to last for 6 hours. Continued expression during longer periods was achieved by administering a series of pulses spaced by 6 hours at 25°C. Hair formation starts at about 36 hours APF and the phase of cell expansion begins at about 48 hours APF. (B) Wild-type wing, (C) wild-type haltere. (D,F,H) Wings carrying one copy of the *HsUbx* insertion, heat shocked at 12 to 24 hours APF (D), 24 to 36 hours APF (F) and 12 to 30 hours APF (H). (E,G) Same experimental conditions as in (D,F,H) but with flies carrying two copies of the *HsUbx* insert. Wings heat shocked from 12 to 30 hours APF could not be recovered in this case.

these sensilla are covered by a 'pavilion' derived from the socket. This structure is absent in the corresponding wing sensilla (Fig. 9A,B, after Cole and Palka, 1982 and Keil, 1997). *Ubx* null mutant clones induced 6 hours APF transform the haltere sensillum to resemble that in the wing. No

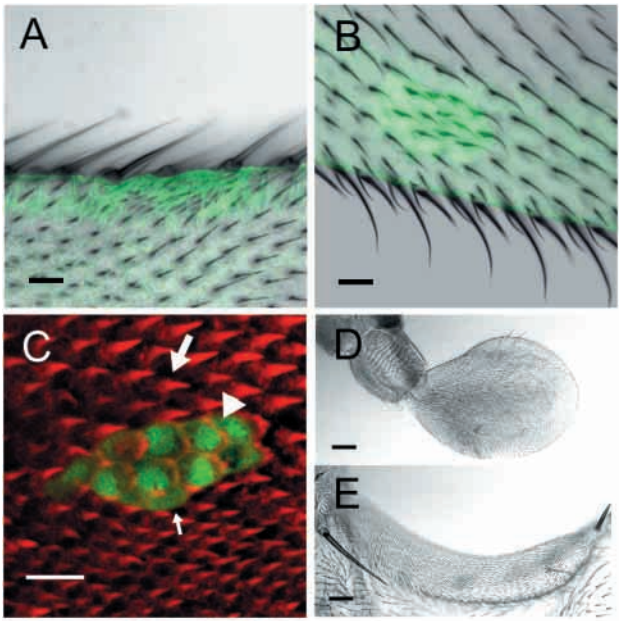

Fig. 8. Effects of Ubx overexpression in clones. (A-C) Wing clones expressing Ubx and GFP (green) induced at 44-48 hours BPF. Adult clones (A,B) can be identified under the adult cuticle, prior to eclosion. (A) About 34% of the observed clones differentiate pigmented cuticle, displaying duplicated or triplicated shortened hairs, like this clone beside the wing margin. (B) In 22% of the clones, cell expansion is partially inhibited and cell hair spacing is reduced, as in this case. A large percentage of the clones (43%), do not show any obvious mutant phenotype (not shown). (C) A similar clone stained with rhodamine phalloidin (red) and marked with GFP (green), from a wing dissected at 32-36 hours APF. Several actin foci per cell can be identified in the mutant cells (arrowhead), as opposed to the single forming hair seen in the neighbouring tissue cells (arrow). Notice two cells belonging to the clone with single hairs, showing that the phenotypes are not fully penetrant (small arrow). (D) Cell morphology in the wild-type haltere cuticle. (E) Early clone expressing Ubx, induced during embryogenesis, showing the complete transformation of the cells to haltere histotype (compare with D). Scale bars, 10 μm in A-C and 40 μm in D,E.

transformations are observed when FLP recombinase is induced later (18 hours APF), but this may simply be because there are no subsequent cell divisions to segregate null clones.

Conversely, heat-shock induction of Ubx in the wing as late as 30-42 hours APF transforms the wing type of sensillum into a sensillum with a pavilion (Fig. 9F,G). Late heat shocks (18-30 hours APF) are better at inducing this transformation than early ones (12-24 hours APF, data not shown). Clearly Ubx is required in these cells much later than in the capitellum of the haltere, and at a later stage in the process of cell differentiation.

DISCUSSION

The contrasting patterns of cell differentiation in the wing and haltere provide a good model for studying the links between *Hox* genes and the process of morphogenesis. The expression of a single transcription factor, the product of the *Hox* gene *Ubx*, controls the formation of two very different cell morphologies. In this work we have described these differences

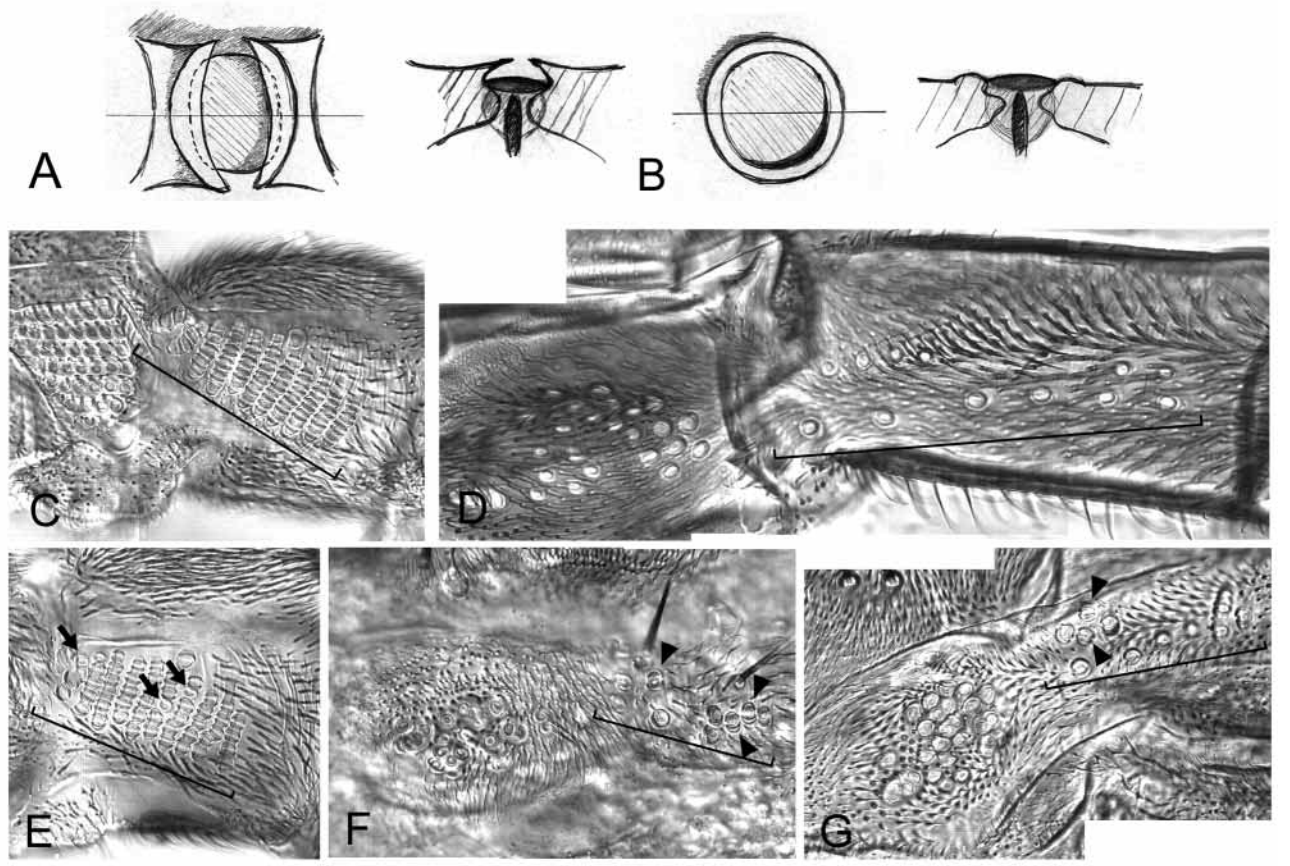

Fig. 9. *Ubx* affects the morphology of the campaniform sensilla in the haltere pedicellus. (A,C) In the dorsal pedicellus of the haltere (between brackets in C,E), the domes of the campaniform sensilla are covered by a pavilion, derived from the socket cell. This structure is absent in the sensilla located in the dorsal radius of the wild-type wing (B,D). The dorsal radius is indicated by brackets in D,F,G. (E) *Ubx* mutant clones induced 6 hours APF prevent the formation of the pavilion (arrows). (F) Dorsal radius of a fly carrying two copies of *HsUbx*, heat shocked from 12 to 24 hours APF. The sensilla are now clustered together and some of them are covered by a pavilion (arrowhead). (G) Dorsal radius of a fly of the same genotype heat shocked from 30 to 42 hours APF. Some sensilla are covered by pavilions (arrowheads). The schematic drawings of the campaniform sensilla are after Keil (1997).

in detail for the first time, defined when they arise, and when during this process Ubx protein is actually required. These studies are necessary pre-requisites to defining the gene network controlled by *Ubx* that is responsible for the observed changes in cell behaviour.

Wing and haltere cells differ in their basal adhesion properties almost immediately after puparium formation. However, the differences in cell morphology between the two tissues do not became apparent until the end of the last round of mitosis, about 24 hours APF. At this time, cells of both wing and haltere epithelia undergo a major cytoskeletal reorganisation, forming actin foci at their apical surfaces (32-36 hours APF). These will become the primordia of the growing cell processes, around which cuticular hairs are secreted. Wing cells develop a single hair, but haltere cells develop multiple foci at their surface, and hence multiple wing hairs. Later, (44-48 hours APF), wing cells flatten dramatically, acquiring a star-like shape and producing a conspicuous hair pedestal. This transition does not occur in the haltere, where cells remain cuboidal.

Ubx is expressed at high levels in the pupal haltere, and our expectation was that Ubx protein present after pupation would be essential to specify these differences between wing and haltere cells in normal development. This would be similar to the situation in the *Drosophila* third leg, where Ubx protein acts to suppress hair development during a narrow window between 18 and 28 hours APF (Stern, 1998), shortly before the hairs are formed. However, our data suggest that the control of cell morphogenesis in the haltere is different. Clonal analysis shows that cells of the haltere epithelium can differentiate normally even if they lack a functional *Ubx* gene throughout pupal development (Morata and García-Bellido, 1976). We have ruled out the possibility that this result is due to the persistence of Ubx protein in the clone daughter cells, by showing that, when clones are induced 6 hours BPF, no Ubx protein can be detected in the mutant cells 12 hours after clone induction. This suggests that the half life of Ubx protein in these cells is at most a few hours, or perhaps that cells eliminate all Ubx protein after mitosis and synthesise it de novo after each division.

We therefore conclude that Ubx protein present before pupation is sufficient to control the normal differences in behaviour that distinguish wing and haltere cells much later, between 24 and 48 hours APF. We suggest that these differences are primed by gene products transcribed before pupation. These may include molecules that control cell morphogenesis directly,

which are accumulated in the larval or prepupal period but activated once metamorphosis starts. They may also include an intermediate tier of transcription factors that control the transcription of morphogenetic molecules during the pupal period. No such targets of Ubx have yet been described. Several genes are known to be differentially transcribed in the wing and haltere (Weatherbee et al., 1998), but these are concerned with the control of growth and pattern formation. They are not known to play a role in cell differentiation.

Non-autonomous effects of *Ubx* could provide an alternative explanation for the failure of small *Ubx*$^-$ clones to show any mutant phenotype. Such clones are surrounded by wild-type cells expressing Ubx protein. However, previous work (Morata and García-Bellido, 1976) and our own observations of clones induced earlier have failed to report any rescue of mutant cells at clone boundaries, making such non-autonomous effects unlikely. Hart and Bienz (1996) have also examined whether ectopic expression of Ubx in the wing is capable of activating endogenous *Ubx* genes in neighbouring cells. They found no evidence for non-autonomous effects. In analogous experiments we have made small Ubx expressing clones using the Flip-out GAL 4 system. The clones display transformations similar to those obtained after ubiquitous ectopic expression of Ubx. However, we have never observed abnormal morphologies in cells neighbouring Ubx-expressing clones.

Any model for the action of Ubx in the haltere must account for its ability to modify wing cells towards a haltere morphology during the first 30 hours of pupal development, at a time when the absence of Ubx protein would not alter normal haltere development. Ubx must actively promote haltere development by eliminating functions needed for wing cell morphogenesis, or producing activities that competitively direct cells towards the behaviour seen in halteres. However, the transformations obtained with ectopic Ubx expression are never complete, suggesting that some of the early steps in wing cell morphogenesis cannot be reversed by late Ubx expression.

Our results show clearly that wing cell morphology is sensitive to the level of Ubx protein present in the cell, and to the length of time during which the protein is present, at least during the pupal period. This confirms and extends previous observations showing that the response of wing cells to Ubx expression is graded, and not an all or none response (Morata, 1975; Hart and Bienz, 1996).

In our experiments, we noted that exposure to ectopic Ubx protein reduces the size of the haltere as well as the wing (data not shown). This sensitivity to Ubx protein levels is likely to be of physiological relevance, because haltere size and morphology are both sensitive to the number of copies of the *Ubx* gene present in otherwise wild-type flies. Animals with one functional *Ubx* gene have enlarged halteres, whereas flies carrying *Ubx* duplications have smaller ones (Smolik-Utlaut, 1990). This emphasises the fact that relatively small (i.e. 2×) variations in the level of homeotic gene expression may be relevant to normal development, and important for the evolution of quantitative differences in morphology (Stern, 1998). In the haltere, these differences give rise to different cell morphologies, whereas in the legs, similar small differences lead to a threshold response – the production of cuticular hairs is completely repressed if levels of Ubx protein exceed a certain threshold.

We have suggested that the differential expression of *Ubx* prior to pupation programs haltere and wing cells for proper morphogenesis in the pupa. The program evoked in each appendage may be triggered by the hormonal system that co-ordinates metamorphosis, without any direct input from the *Hox* genes. We do not suggest that this is a universal property of the *Hox* genes. We have shown that at least one cell type in the haltere behaves differently (the campaniform sensilla), as does patterning of the legs (Stern, 1998; M. Rozowski and M. A., unpublished data). However, there is at least one other case documented where there seems to be an analogous separation between the requirement for homeotic gene expression, and the appearance of segment-specific patterns of cell behaviour. During the patterning of the embryonic nervous system, the *Ubx* and *abd-A Hox* genes establish the differences between thoracic and abdominal neuroblasts. Function of these genes is required before neuroblast segregation, early in embryogenesis (Prokop et al., 1998). However, segment-specific differences in the fates of these cells will become apparent during neuroblast proliferation and differentiation, in late embryogenesis and during larval life. At this time, Ubx and Abd-A proteins are not expressed in the neuroblasts, which appear to be committed already to an appropriate segment-specific behaviour (Prokop et al., 1998).

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