**Polycomb-dependent Ultrabithorax Hox gene silencing induced by high Ultrabithorax levels in Drosophila**

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The Ultrabithorax (Ubx) gene of *Drosophila* specifies the third thoracic and first abdominal segments. Ubx expression is controlled by several mechanisms, including negative regulation by its own product. We show here that if Ubx expression levels are inappropriately elevated, overriding the auto-regulatory control, a permanent repression of Ubx is established. This continuous repression becomes independent of the presence of exogenous Ubx and leads to the paradoxical result that an excess of Ubx results in a phenotype of Ubx loss. The mechanism of permanent repression depends on Polycomb-group genes. Absence of endogenous Ubx transcription when Ubx levels are highly elevated probably activates Polycomb complexes on a Polycomb response element located in the Ubx major intron. This, in turn, brings about permanent repression of Ubx transcription. Similar results are obtained with the gene *engrailed*, showing that this mechanism of permanent repression may be a general one for genes with negative auto-regulation when levels of expression are transitorily elevated.

**KEY WORDS:** Hox, Ultrabithorax, Polycomb, Autoregulation, engrailed

**INTRODUCTION**

The Hox genes specify the anteroposterior (A/P) axis of bilaterians (Lewis, 1978; Duboule, 2007). They are expressed in defined domains along this axis and their mis-expression frequently causes gross alterations in the body plan. Therefore, Hox gene expression must be tightly regulated throughout development (reviewed by Carroll et al., 2001).

In *Drosophila*, the expression domains of Hox genes are set in the early embryo by the activity of gap genes (White and Lehmann, 1986; Harding and Levine, 1988; Irish et al., 1988; Casares and Sánchez-Herrero, 1996). After this initial regulation, Hox genes expression domains are maintained by two groups of genes: the Polycomb (Pc) group, which code for proteins that maintain the repression of Hox genes (and other genes); and the trithorax (trx) group, coding for proteins that maintain Hox transcription by preventing Pc silencing (reviewed by Schwartz and Pirrotta, 2007). Pc-group complexes bind to DNA in specific regions called Polycomb response elements (PREs) (reviewed by Müller and Kassis, 2006; Ringrose and Paro, 2007). Hox expression is also regulated by the Hox genes themselves: those expressed more posteriorly along the A/P axis downregulate the expression of those transcribed more anteriorly (Hafen et al., 1984; Struhl and White, 1985). Finally, some Hox genes control their own expression. For example, *Deformed* maintains its own transcription in cells of the epidermis and central nervous system (Kuziora and McGinnis, 1988; Lou et al., 1995). The opposite effect, negative regulation by its own product, has been described for the Ultrabithorax (Ubx) gene (Irvine et al., 1993).

*Ubx* expression in the embryonic epidermis extends from parasegment (PS) 5 to PS12. In the larval thorax, *Ubx* is expressed in imaginal discs of the third thoracic segment (haltere disc and third leg disc) and in the posterior compartment of the second leg disc (Beachy et al., 1985; White and Wilcox, 1985). In this region, *Ubx* mutations transform the third leg into the second one, and the haltere and metanotum (proximal part of the dorsal metathorax) into wing and mesonotum (corresponding region of the mesothorax), respectively (Lewis, 1963).

*Ubx* regulates negatively its own expression in the embryonic epidermis and imaginal discs (Irvine et al., 1993; Casares et al., 1997). Thus, increasing the amount of Ubx protein reduces *Ubx* transcription, and the opposite effect is seen with mutations that reduce its expression (Irvine et al., 1993). Changes in *Ubx* levels also modify the adult phenotype, as increasing *Ubx* dose reduces haltere size (Smolik-Utlaut, 1990; Irvine et al., 1993); conversely, heat-shock-induced *Ubx* expression occasionally brings about a very slight transformation of haltere into wing (Irvine et al., 1993). This transformation is particularly intriguing, as adding more *Ubx* seems to reduce *Ubx* activity.

We have studied this surprising result and have found that the increased expression of *Ubx* produces a strong *Ubx* mutant phenotype, but only if the high *Ubx* protein levels are transitorily present in the imaginal disc cells. The transient high *Ubx* expression causes a Pc-group-dependent permanent inactivation of *Ubx*, probably owing to the repression of *Ubx* endogenous transcription. A similar effect is also observed in engrailed (en), a gene required to specify the development of posterior compartments. The mechanism we have uncovered, therefore, seems to be at work in different genes showing negative auto-regulation in *Drosophila* development.

**MATERIALS AND METHODS**

**Genetics**

The Gal4/UAS system (Brand and Perrimon, 1993) was used with the following drivers: MS372-Gal4 (a gift from F. Jiménez), Ubx-Gal4SS2 (A. Sánchez and E.S.-H.), C-765-Gal4 (Guillén et al., 1995), sd-Gal4 (M.C. and G. Morata, unpublished), Ubx-Gal4M1, Ubx-Gal4M2, dpp-Gal4, en-Gal4, ptc-Gal4, ap-Gal4, hh-Gal4 (FlyBase; http://flybase.bio.indiana.edu) and with the following UAS constructs: UAS-Ubx (Castelli-Gair et al., 1994; Michelson, 1994), UAS-Ubx-HA (Ronshaugen et al., 2002), UAS-dsRNA-Ubx (Monier et al., 2005), UAS-ubd-d (Michelson, 1994), UAS-Abd-B (m) (Castelli-Gair et al., 1994), UAS-en (Guillén et al., 1995), UAS-UbxTri (Merabet et al., 2007), UAS-UbxAf (from Artemia...
franciscana) (Ronshaugen et al., 2002) and UAS-PcI-RNAi (Vienna Drosophila RNAi Center). The Pce and tr(3R) mutations, the Df(1)109 deletion, which eliminates Ubx, and the UAS-GFP reporter construct are described in FlyBase. P-lacZ lines inserted in the Ubx (Ubxlac1) (Casares et al., 1997), abd-A (HC71A1), Abd-B (HC199) (Bender and Hudson, 2000) and en (ryxho25) (Hama et al., 1990) were used as reporters of expression of the corresponding genes.

Clonal analysis
Clones expressing Ubx were induced in larvae of the following genotype: y w hs-flp; tub-Gal80sd/UAS-Ubx-HA; Ubxlac1 '/tub-flu-GFP, y >Gal4. The tub-flu-GFP, y >Gal4 construct has been previously described (Zecca and Struhl, 2002). The Gal80PcGal4 system (McGuire et al., 2003) was used to control the time of expression of the exogenous Ubx. Clones were marked by the absence of GFP expression and induced according to the following protocol: larvae at 48-72 hours of development, raised at 25°C, were heat-shocked for 50 minutes to induce recombination between the FRT sequences, transferred to 29°C for 24 hours, heat-shocked for 1 hour at 37°C, grown for 1 day at 29°C and pupation is delayed. After several days at 17°C, the discs were fixed and scored for clones. In this protocol: larvae at 48-72 hours of development, raised at 25°C, were heat-shocked for 1 hour at 37°C, grown for 25°C for 50 minutes to induce recombination between the FRT sequences, transferred to 29°C for 2 days (to allow Gal4 activity) and then transferred to 17°C to inactivate the Gal4 protein (McGuire et al., 2003). After several days at 17°C, the discs were fixed and scored for clones. In this and similar experiments with several temperature changes, the time of pupation is delayed.

Clones eliminating Pce function in a background in which Ubx permanent repression was established were induced according to this procedure: 24-48 hour larvae of the genotype sda-Gal4 hs-flp; UAS-Ubx; tub-Gal80sd; Ubx-GFP FRT2A hs-CD2 ri PceTS109 FRT2A, grown at 25°C, were transferred to 29°C for 24 hours, heat-shocked for 1 hour at 37°C, grown for 1 day at 29°C and transferred to 17°C for 2 days before fixation. The hs-CD2 ri FRT2A PceTS109 chromosome is described by Beuchle et al. (Beuchle et al., 2001).

Immunostaining
Imaginal discs were stained according to standard procedures. The antibodies used are mouse and rabbit anti-β-galactosidase (Cappel), mouse Mab4D9 anti-En (Patel et al., 1989), rat anti-haemagglutinin (Roche) and mouse anti-Ubx (White and Wilcox, 1984). Secondary antibodies are coupled to Red-X, Texas Red, FITC and Cy5 fluorochromes (Jackson ImmunoResearch).

In situ hybridization
Haltere discs were hybridized with a Ubx cDNA probe according to standard protocols (Wolff, 2000).

Adult cuticle analysis
Flies were kept in a mixture of ethanol: glycerol (3:1), cooked in 10% KOH at 60°C for 10 minutes, dissected, washed with water, dehydrated with ethanol and mounted in Euparal for inspection under a compound microscope.

X-gal staining and inverse PCR
X-gal staining was carried out as previously described (Wolff, 2000). The P-Gal4 line Ubx-Gal4552 was localized by inverse PCR (http://www.fruitfly.org/about/methods/inverse.pcr.html).

RESULTS
The induction of high Ubx levels in the haltere disc results in absence of the Ubx protein
Ubx represses its own expression in the embryonic epidermis, and in the haltere and third leg imaginal discs (Irvine et al., 1993). This is easily observed when expressing Ubx with the Gal4/UAS system (Brand and Perrimon, 1993) in haltere discs of larvae carrying a Ubx-lacZ reporter insertion, Ubx lacZ (Casares et al., 1997). In these discs, there is strong repression of β-galactosidase expression, indicating repression of the endogenous Ubx gene by the exogenous Ubx protein (Fig. 1B, compare with the lacZ expression in Ubx lacZ discs in Fig. 1A). Flies with increased Ubx expression present, in some Gal4/UAS combinations, a partial transformation of haltere into wing (Fig. 1D,E, compare with the wild type in Fig. 1C). A weak effect on haltere development has been described after expressing Ubx under heat-shock control (Irvine et al., 1993) but the transformations we observe are much stronger. Other regions of the third thoracic segment (metanotum and third leg) are also transformed into the second thoracic one (see below). By contrast, Ubx expression driven by other Gal4 lines do not transform the halteres into wings but reduce their size. This is similar to what has been previously described (Smolik-Utlaut, 1990; Irvine et al., 1993), and suggests that there may be some peculiarity in the Gal4 drivers that produce Ubx transformations when expressing Ubx.

These transformations are paradoxical, as providing an excess of Ubx protein results in a phenotype similar to that produced when eliminating the Ubx product. We wondered whether there could be, in addition to the repression of the endogenous Ubx gene, a dominant-negative effect caused by an excess of Ubx protein or whether, by contrast, total Ubx protein levels were reduced. To check this, we stained third instar haltere discs of the MS372-Gal4/UAS-Ubx combination, which produces Ubx transformations (Fig. 1E), with an anti-Ubx antibody. As shown in Fig. 1F, there are patches of cells that lack Ubx expression. Therefore, the Ubx phenotype is due to the absence of the Ubx protein.

High transient expression of exogenous Ubx accounts for the permanent absence of Ubx protein
A question posed by the previous experiments is why only some Gal4 lines elicit Ubx mutant transformations. It could be that these lines are repressed by Ubx. We studied whether this is the case with the MS372-Gal4 line, which drives expression in the haltere disc (Fig. 2A). To distinguish the contribution of endogenous and exogenous Ubx proteins to the Ubx pattern, we monitored the endogenous Ubx gene (Endo-Ubx) with the Ubx lacZ insertion and the exogenous one (Exo-Ubx) by using an antibody against haemagglutinin (HA) in larvae expressing a Ubx protein tagged with this epitope (Ubx-HA) (Ronshaugen et al., 2002). Several
High Ubx levels permanently repress Ubx

Fig. 2. Absence of endogenous (Endo–) and exogenous (Exo–) Ubx when expressing Ubx with certain Gal4 lines. (A–A′) Absence of Ubx protein (in red in A′) in UAS-GFP MS372-Gal4/UAS-Ubx Ubxlac1 haltere discs (GFP in green in A) correlates with the absence of Endo-Ubx, detected by the expression of Ubxact (A′, grey scale). Merged image in A′′. The inset indicates a region without GFP, Ubx or Endo-Ubx expression. (B–B′) Exo-Ubx and Endo and Ubx expression in UAS-Ubx-HA+/ +; MS372-Gal4 UAS-GFP/Ubxacr haltere discs. GFP is shown in B (in green), the Exo-Ubx protein is detected with an antibody against the haemagglutinin (HA) epitope (B, in red) and the Endo-Ubx with an antibody against the β-galactosidase protein (grey scale in B′). Merged image in B′′. Note the coincidence of Exo-Ubx and GFP expression but not of Endo-Ubx and the absence of Endo-Ubx in many cells, probably owing to the long stability of the β-galactosidase protein. The inset indicates a region without GFP, Exo-Ubx or Endo-Ubx expression. (C) Ubx expression in a tub-Gal80ts+; dpp-Gal4 UAS-GFP/UAS-Ubx haltere disc transferred from 17°C to 29°C and then back to 17°C during the larval period. Ubx is absent in most of the anterior compartment. Discs are oriented with the anterior compartment towards the left and the ventral compartment upwards.

Conclusions can be drawn when analyzing UAS-GFP/+; MS372-Gal4/UAS-Ubx Ubxacr haltere discs (GFP in green in A) correlates with the absence of Endo-Ubx, detected by the expression of Ubxact (A′, grey scale). Merged image in A′′. The inset indicates a region without GFP, Ubx or Endo-Ubx expression. (B–B′) Exo-Ubx and Endo and Ubx expression in UAS-Ubx-HA+/ +; MS372-Gal4 UAS-GFP/Ubxacr haltere discs. GFP is shown in B (in green), the Exo-Ubx protein is detected with an antibody against the haemagglutinin (HA) epitope (B, in red) and the Endo-Ubx with an antibody against the β-galactosidase protein (grey scale in B′). Merged image in B′′. Note the coincidence of Exo-Ubx and GFP expression but not of Endo-Ubx and the absence of Endo-Ubx in many cells, probably owing to the long stability of the β-galactosidase protein. The inset indicates a region without GFP, Exo-Ubx or Endo-Ubx expression. (C) Ubx expression in a tub-Gal80ts+; dpp-Gal4 UAS-GFP/UAS-Ubx haltere disc transferred from 17°C to 29°C and then back to 17°C during the larval period. Ubx is absent in most of the anterior compartment. Discs are oriented with the anterior compartment towards the left and the ventral compartment upwards.

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The idea is supported by the use of the Gal4/Gal80th system to control the time of activity of the driver (McGuire et al., 2003). In ptc-Gal4 UAS-GFP/+; UAS-Ubx/tub-Gal80th or tub-Gal80th+/ +; dpp-Gal4 UAS-GFP/UAS-Ubx haltere discs, when larvae were transferred from 17°C, the permissive temperature of the Gal80th protein, to 29°C, the restrictive one, and back again to 17°C, Ubx is absent in patches of cells in the anterior compartment, which exceptionally occupy a large domain (Fig. 2C and data not shown). It is known that the dpp expression domain is wider in early wing discs than in late ones, where its transcription is confined to cells abutting the anteroposterior boundary (Weigmann and Cohen, 1999). Assuming similar changes occur in the haltere disc, the transient Ubx expression driven by dpp-Gal4 (or ptc-Gal4) away from this boundary in early larvae may account for the absence of Ubx protein observed at late stages. Consistently, we see transformations of haltere to wing in dpp-Gal4/UAS-Ubx flies (not shown).

We have supposed that the transitory high expression of Ubx may repress its own transcription permanently. To check this idea, and to avoid complications using Gal4 lines with variable expression throughout development, we decided to use the ap-Gal4 line, which drives constant expression in the dorsal compartment of wing and haltere discs (Calleja et al., 1996) (Fig. 3A), and to temporally restrict its activity with the Gal4/Gal80th system. First, we studied Endo-Ubx expression when there is a continuous supply of Exo-Ubx. The dorsal compartment of ap-Gal4 UAS-GFP/+; UAS-Ubx Ubxlac1/+ haltere disc shows high Ubx levels (Fig. 3A′) and complete repression of Endo-Ubx (Fig. 3A′′). We next restricted the time when Exo-Ubx is synthesized. ap-Gal4 UAS-GFP/+; UAS-Ubx Ubxlac1/tub-Gal80th larvae (24-48 hours), grown at 17°C, were transferred to 29°C for 2 or 3 days to activate the Gal4 protein, and then grown at 17°C to suppress Gal4 activity. Haltere discs were fixed at different times after the temperature change and GFP and total Ubx protein monitored (hereafter, we refer to this protocol of temperature change as the standard protocol). We consistently observe three effects in these discs: first, a progressive decay of GFP and Ubx expression in the dorsal region, the latter at a faster rate, revealing absence of Gal4 activity after the temperature change (Fig. 3B-D′). Second, the Endo-Ubx expression is not restored. Even after 6 days at 17°C, when Exo-Ubx is no longer present, Endo-Ubx transcription has not resumed (Fig. 3D′′), indicating a permanent Ubx repression. In situ hybridization experiments with a Ubx probe showed that Ubx transcription is similarly repressed in the dorsal compartment (not shown). Finally, because there is no Ubx protein in the dorsal compartment of the haltere disc, this is transformed into the corresponding compartment of the wing disc, and increases its size significantly (Fig. 3C-D′). These results are not explained by Ubx needing an exceptionally long time to restore its expression. We used a UAS-dsUbxRNA construct (Monier et al., 2005) to prevent Ubx protein synthesis by RNA interference in the ap domain. If the larvae are kept at 29°C, there is no Ubx protein expression in the dorsal compartment (Fig. 3E), but if they undergo our standard protocol, Ubx expression in this compartment is almost completely restored after 4 days at 17°C (Fig. 3F). Such recovery is not observed in ap-Gal4 UAS-GFP/+; UAS-Ubx/tub-Gal80th larvae that underwent the same temperature changes, not even after 6 or 7 days at 17°C (Fig. 3C-D′ and data not shown). We conclude that a mechanism must prevent the restoration of Ubx transcription in the latter case.

In accordance with these results, we observe a different phenotype in halteres of flies expressing Ubx permanently or transiently. In ap-Gal4/UAS-Ubx flies, the halteres are reduced (Fig. 3G), as has been described previously when Ubx levels are increased (Smolik-Utlaut, 1990; Irvine et al., 1993). By contrast, if this increased expression is transient, a transformation of haltere into wing ensues (Fig. 3H). Similar phenotypic effects, under similar experimental regimes, are obtained when we use a scalloped-Gal4 (sd-Gal4) line, which also drives expression in the haltere disc (not shown). These experiments explain the contrasting effects obtained with different Gal4 lines when expressing Ubx. Those that reduce haltere size most probably maintain a fixed domain of expression throughout development, whereas those that show transformations of haltere to wing, like dpp-Gal4 or MS372-Gal4, vary their expression domains with time (Weigmann and Cohen, 1999) (data not shown).
The repression of Ubx is maintained cell-autonomously

We wanted to know whether the permanent repression of Ubx by its own product is a cell autonomous effect. To this aim, we induced clones expressing transiently the Ubx product and studied exogenous, endogenous and total Ubx expression in these clones after several temperature changes (see Materials and methods). We observe in these clones that Endo-Ubx expression is continuously repressed in all the cells that previously expressed Exo-Ubx (even several days after the exogenous product is no longer present), but not outside it (Fig. 3I-L). This suggests that the permanent Ubx repression is maintained cell autonomously.

Permanent repression of an Ubx-Gal4 line inserted close to a Polycomb response element

The distinct effect of lines with permanent or transient Ubx expression has one exception: one Ubx-Gal4 line (Ubx-Gal4SS2) produced very strong transformations of haltere to wing (Fig. 4A), metamoton into mesonotum (Fig. 4B) and third leg into second leg (Fig. 4C), even though it drives constant expression in the anterior haltere disc (Fig. 4D). By contrast, when we expressed Ubx under the control of two other Ubx-Gal4 lines, Ubx-Gal4M1 and Ubx-Gal4M3, the only effect we saw was a reduction of haltere size (not shown). When we co-expressed GFP and Ubx with the Ubx-Gal4SS2 line, we observed coincident absence of both proteins in large areas of this disc (Fig. 4E-G), indicating there is repression of both Ubx and the Gal4 driver. By contrast, in most UAS-GFP/+; Ubx-Gal4M1/tub-Gal80AP UAS haltere discs, grown for 2 days at 29°C during the larval period, there was strong GFP and Ubx expression in the pouch (not shown).

We guessed that the different effect of the Ubx-Gal4SS2 line may depend on its location within the Ubx gene. Ubx-Gal4M1 and Ubx-Gal4M3 have been mapped upstream and close to the Ubx transcription start site (de Navas et al., 2006). By contrast, we have located the Ubx-Gal4SS2 insertion to position 274.277 (coordinates according to Martin et al., 1985), very close to the Polycomb response element (PRE) of the bithorax (bx) region of Ubx (Orlando et al., 1998; Ringrose et al., 2003; Papp and Müller, 2006; Beisel et al., 2007) (Fig. 4H). We suspect that the particular position of the Ubx-Gal4SS2 insertion may account for its different morphological effect when expressing Ubx.

The permanent repression of Ubx depends on the Pc-group and trx-group genes

The continuous repression of Ubx and the location of the Ubx-Gal4SS2 insertion (with its particular properties) close to the bx PRE suggest that Pc-group genes may be part of the mechanism used for Ubx permanent repression. To verify this, we first
examined whether the partial transformation of halteres into wings observed in Ubx-Gal4SS.2 UAS-Ubx flies was modified in a Polycomb or trithorax heterozygous mutant background. In Ubx-Gal4SS.2 UAS-Ubx/Pc^3 flies there is a significant reduction in the penetrance (and expressivity) of the haltere to wing transformation when compared with the controls. An opposite effect is observed in Ubx-Gal4SS.2 UAS-Ubx/trx^2 flies (Fig. 5A). A second experiment made use of the UAS-Pcl-RNAi construct, which inactivates the Polycomblike (Pcl) gene, a member of the Pc group (Duncan, 1982). We compared the Ubx expression in third instar haltere discs of ap-Gal4 UAS-GFP/++; UAS-Ubx tub-Gal80^s/TM6B and ap-Gal4 UASGFP/++; UAS-Ubx tub-Gal80^s/UAS-PclRNAi larvae that went through the standard protocol of temperature changes. The area that lacks Ubx protein is much larger in the former than in the latter (Fig. 5B). Finally, we induced Pc mutant clones in haltere discs where permanent repression of Ubx had been established (see Materials and methods). In many of these clones we observed derepression of Ubx, strongly suggesting that Pc is required to maintain the Ubx silencing induced by high Ubx levels (Fig. 5C-C').

**The UbdA domain of Ubx may be needed for Ubx permanent repression**

A first step in the mechanism leading to Ubx lasting repression is suppression of Ubx transcription. We reasoned that, as the abdominal-A (abd-A) and Abdominal-B (Abd-B) Hox genes downregulate Ubx embryonic expression (Struhl and White, 1985), they may also cause permanent repression of Ubx in haltere discs. In MS372-Gal4/UAS-abd-A haltere discs, there are large areas of Ubx repression that resemble those observed in the same discs of MS372-Gal4/UAS-Ubx larvae (Fig. 6A, compare with Fig. 1F), and Ubx-Gal4SS.2/UAS-abd-A adults show a strong transformation of halteres into wings (Fig. 6B). As Abd-A can also make normal halteres (Casares et al., 1996; de Navas et al., 2006), these results strongly suggest that Abd-A can repress Ubx permanently in haltere discs.

A different result is obtained with Abd-B, as in MS372-Gal4/UAS-Abd-B adults there is no or weak transformation of halteres into wings (Fig. 6C). To study in detail whether Abd-B can permanently repress Ubx, we first studied Ubx expression in ap-Gal4 UAS-GFP/UAS-Abd-B(m); tub-Gal80^s/+ haltere discs of larvae grown at 29°C for 3 or more days. In the dorsal compartment of such discs, Ubx signal disappears or is strongly reduced (Fig. 6D-G). By contrast, if larvae of this genotype are grown according to our standard protocol, and the discs fixed 4 days after the last transfer to 17°C, only a small proportion of haltere discs show patches of cells lacking Ubx protein (Fig. 6H). Consistently, only a few flies in this experiment show transformations of halteres into wings. Our conclusion is that Abd-A and Abd-B can repress Ubx transcription in haltere discs, but that only Abd-A can consistently induce permanent Ubx repression.

Ubx and Abd-A proteins share common protein motifs, like the Hexapeptide (HX) and the Abd-A one, which the Abd-B protein lacks (Chan and Mann, 1993; Bürglin, 1994; Mann and Chan, 1996). To investigate whether any of these domains accounts for the differences we detected between Ubx and Abd-B, we expressed Ubx proteins lacking either the Hexapeptide (Ubx^HX) or the Ubd-A (Ubx^UbdA) domains (Merabet et al., 2007), and analyzed whether there is permanent repression of Ubx. Many Ubx-Gal4SS.2 UAS-Ubx^HX flies show transformations of haltere into wing and, occasionally, of metanotum into mesonotum (Fig. 6I). Consistently, in ap-Gal4 UAS-GFP/UAS-Ubx^HX; tub-Gal80^s/+ larvae that went through our standard protocol, we see permanent repression of Ubx signal in the dorsal compartment of the haltere disc (Fig. 6J). By contrast, in Ubx-Gal4SS.2/UAS-Ubx^UbdA flies, the halteres are not transformed into wings (Fig. 6K) and there is no permanent...
Fig. 5. The Pc group of genes are required for the permanent Ubx repression. (A) Histogram showing the percentage of halteres that are transformed into wings in UbxGal4SS.2 UAS-Ubx animals in different mutant backgrounds. U stands for UbxGal4SS.2 UAS-Ubx. In each pair of columns, we show the control (TM6B; left) and the experimental (right) percentages, indicated over each column. The number of halteres scored is indicated below each column. The heterozygosity for Pc^2 decreases the percentage (and the expressivity) of the transformations, whereas the heterozygosity for trx increases the number of transformations. (B) Histogram showing the average percentage of the area in the dorsal compartment of the haltere disc in which Ubx expression is absent. The red and blue columns correspond to average percentages in ap-Gal4 UAS-GFP/+; UAS-Ubx tub-Gal80+/TM6B and ap-Gal4 UAS-GFP/+; UAS-Ubx tub-Gal80+/UAS-PcRNAi haltere discs, respectively. The data are from larvae that completed embryonic development at 17°C, were incubated for 2 (left) and 3 (right) days at 29°C, and were then transferred to 17°C. Numbers of halteres studied are above the columns. Representative examples of Ubx staining in the haltere discs of the corresponding days and genotypes are shown below. (C-C’) Haltere disc of a sd-Gal4/his-flp; Ubx-Ubx/tub-Gal80^0; Ubi-GFP FRT2A/his-CD2 n Pcb^{T109} FRT2A larva that went through temperature changes to establish Ubx permanent repression (see Materials and methods) and in which several Pc mutant clones (marked by the absence of GFP, in green in C) were induced. There is derepression of Ubx in the clones (in red, C’), showing that Pc is required to maintain permanent repression. The weak derepression in some clones may be related to the position of the clone. There are also cells in which Ubx has not been repressed (asterisk). Merged image in C’. Discs are oriented with the anterior compartment towards the left and the ventral compartment upwards.

repression of Ubx in ap-Gal4 UAS-GFP/UAS-Ubx^{CubD4}; tub-Gal80^*/+ haltere discs that underwent the standard treatment (Fig. 6L). After 5 days at 29°C, we see suppression of the Ubx^{lacI} reporter in most cells of the haltere disc (Fig. 6M) and if the larvae are then transferred to 17°C they give rise to adults with wild-type halteres (not shown). These results suggest that the UbdA domain may not be essential for Ubx repression, but for permanent Ubx silencing. However, the Ubx^{CubD4} protein downregulates, but does not suppress, Ubx^{lacI} expression after 3 days at 29°C (not shown), indicating that in our standard protocol the absence of silencing may be due to lack of complete Ubx repression. We have also observed that expressing the Ubx protein from the crustacean Artemia franciscana, with a different C-terminal domain from that of the Drosophila Ubx protein (Galant et al., 2002; Ronshaugen et al., 2002), also leads to permanent repression of Ubx (in Ubx-Gal4^{SS.2} UAS-Ubx-Af flies there is transformation of halteres into wings). Out of the domains tested, the UbdA may be the only motif in the Ubx protein required for Ubx permanent repression.

The en grailed gene is also permanently repressed by high levels of its protein

We have observed similar results to those of Ubx with the engrailed gene, which is required to determine the posterior identity of Drosophila segments and is also regulated negatively by its own product in the imaginal discs (Guillén et al., 1995; Simmonds et al., 1995; Tabata et al., 1995). Increased expression of the en gene driven by different Gal4 lines causes transformations of posterior-to-anterior compartments in the notum and the wing resembling those observed in weak en mutants (Guillén et al., 1995; Simmonds et al., 1995; Tabata et al., 1995; Tabata et al., 1995; Casares et al., 1997). This

DISCUSSION

The Ubx and en selector genes are negatively regulated by their own products in imaginal discs (Irvine et al., 1993; Guillén et al., 1995; Simmonds et al., 1995; Tabata et al., 1995; Casares et al., 1997). This
is probably a compensation mechanism to cope with protein level fluctuations during development, which could interfere with the correct expression of target genes that are highly sensitive to Ubx concentration (Tour et al., 2005). High levels of Ubx in the haltere disc, for example, reduce haltere size (Smolik-Utlaut, 1990). This negative regulation demands a precise control of protein levels, which is of high importance to fly development because, as we have seen, if such fine-tuning does not take place, the two genes are permanently inactivated.

This continuous repression is dependent on the Pc-group of genes. Several publications have demonstrated that transcription through a PRE in regulatory domains of the abd-A or Abd-B genes is probably a compensation mechanism to cope with protein level fluctuations during development, which could interfere with the correct expression of target genes that are highly sensitive to Ubx concentration (Tour et al., 2005). High levels of Ubx in the haltere disc, for example, reduce haltere size (Smolik-Utlaut, 1990). This negative regulation demands a precise control of protein levels, which is of high importance to fly development because, as we have seen, if such fine-tuning does not take place, the two genes are permanently inactivated.

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results in inefficient Pc silencing, ectopic expression of the corresponding gene and homeotic transformations (Bender and Fitzgerald, 2002; Hogga and Karch, 2002; Rank et al., 2002; Schmitt et al., 2005). We assume that the phenomenon we have observed (Pc-dependent permanent repression of Ubx) makes use of a converse mechanism, that is, assembling of Pc complexes at the PRE following absence of transcription. There is a PRE located in the large Ubx intron (Chiang et al., 1995; Orlando et al., 1998) and it was suggested that Ubx transcription through this PRE may contribute to inactivation of Pc-complexes (Papp and Müller, 2006). Our results support this view. If Ubx transcription is suppressed, as it is when we force high Ubx levels, Pc-group complexes may become active at this PRE and repress the Ubx region (Fig. 8). How Ubx transcription may inactivate Pc-complexes activity is not clear. It has been proposed that transcription through a PRE may prevent binding of the complexes to the DNA (Bender and Fitzgerald, 2002; Hogga and Karch. 2002; Rank et al., 2002; Schmitt et al., 2005). However, recent experiments have demonstrated that Pc-group proteins are bound to the bx PRE in haltere discs, where Ubx is transcribed (Papp and Müller, 2006). Nevertheless, binding of some of these proteins is reduced in the haltere disc, when compared with the wing disc, suggesting that Ubx transcription may reduce this binding (Papp and Müller, 2006). Whether affecting Pc-group proteins binding or activation, our results favor the view that Ubx transcription is a requisite to prevent Ubx silencing and that absence of transcription leads to permanent repression.

The results obtained with the Ubx-Gal4SS.2 line, inserted close to the bx PRE, may be relevant to this hypothesis. When this line directs expression of Ubx, there is repression of both Ubx and the Gal4 driver in the anterior haltere imaginal disc. We assume that, following repression of Ubx transcription, the Pc-group complexes at the PRE ‘close’ the chromatin in nearby DNA (reviewed by Müller and Kassis, 2006), thus repressing the Ubx-Gal4SS.2 driver (Fig. 8). This contrasts with what happens with other Ubx-Gal4 lines, located far from the PRE (de Navas et al., 2006) and in which the inactivation of the driver occurs more rarely. Our hypothesis is that, in the latter, there is repression of the Gal4 line by the exogenous Ubx product. This repression reduces Ubx protein levels, relieves endogenous Ubx repression, and the subsequent Ubx transcription through the PRE prevents Pc-mediated silencing. It is possible that Ubx does not repress the Ubx-Gal4SS.2 line as efficiently as the other Ubx-Gal4 lines owing to its position close to the PRE. Alternatively, the Pc-group complexes may not completely silence Ubx-Gal4SS.2 and Ubx-Gal4MM insertions.

Although repression of transcription is a requisite for establishing permanent repression in the Ubx gene, it may not be sufficient. We have shown that abd-A, but not Abd-B, consistently achieve Ubx silencing in the haltere disc, although both genes repress Ubx transcription. This different behavior of Ubx/Abd-A and Abd-B proteins depends neither on the C-terminal region, which contains a conserved block of glutamines and alanines (Galant et al., 2002; Ronshaugen et al., 2002), nor on the HX motif, but may depend on the presence of the UbdA domain. The UbxUbdA protein can partially transform wings into halteres (not shown) and downregulates wing disc-specific targets (Merabet et al., 2007), but is unable to establish permanent Ubx repression under our standard protocol conditions. It is possible that the lack of permanent repression we observe when expressing Abd-B or UbxUbdA may be due to these proteins allowing very low levels of Ubx transcription, enough to prevent Pc-mediated permanent repression. In fact, the UbxUbdA protein needs to be present for a long time (5 days at 29°C in our experiments) to achieve complete repression of the endogenous Ubx. Alternatively, the UbdA domain may be necessary for the Ubx protein to collaborate with the establishment of Pc silencing.

If this requirement of the Ubx protein to establish Pc-dependent Ubx repression is true, it may be needed only in structures where Ubx is transcriptionally active, such as the haltere disc. Obviously, in segments anterior to PS5, repression of Ubx by Pc-group proteins cannot depend on Ubx. In this context, it is relevant to mention a specific case of Ubx repression: that occurring in the posterior wing disc. The Ubx promoter is ectopically expressed in the posterior compartment of the larval wing disc in mutations (like bx or abx mutations) that eliminate Ubx expression in this compartment (Irvine et al., 1993). This suggests there is Ubx early expression in this domain that is subsequently shut off by the Ubx protein itself (Irvine et al., 1993) and that the repression is maintained by Pc-group genes. We have found there is indeed Ubx expression in the posterior compartment of the incipient wing disc (in stage 12 embryos) and that this expression disappears from the wing disc at later embryonic stages (stage 16; see Fig. S2 in the supplementary material). Therefore, in the mature posterior wing disc there is a Pc-dependent permanent repression of Ubx that was set after a transient Ubx protein expression, similar to the mechanism we have shown. Such early expression may confer specific properties to cells that initially express Ubx. Thus, in mutations that inactivate partially a
**References**


