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Regeneration can take place across *Drosophila* compartments or segments with different *Hox* gene expression

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ABSTRACT Regeneration in *Drosophila* has primarily been studied in imaginal discs, which are organized into distinct compartments defined by strict lineage boundaries. While cells typically respect these borders, during regeneration cells can cross them to restore the original organ specification, which is determined by Hox genes. Although differences in Hox gene expression are known to segregate cell populations, the role of such differences as a potential barrier to regeneration remains unclear. We investigated this by analyzing two experimental settings: the analia (derived from the Hox-compound genital disc) and haltere discs with mutations in *Ultrabithorax* regulatory regions (*bithorax* or *postbithorax*). Our findings demonstrate that Hox gene differences are not an absolute impediment to regeneration across segments or compartments. However, we observed occasional regenerative limits, which were non-specifically enhanced in the *postbithorax* background.

KEYWORD: Hox, regeneration, Drosophila.

LABURPENA *Drosophila*n, birsorkuntza disko imaginaletan aztertu izan da nagusiki. Egitura horiek leinu-muga zorrotzek mugatutako konpartimentu bereizietan antolatzen dira. Zelulek muga horiek errespetatu ohi dituzten arren, birsortze-prozesuan, zeharkatu egin ditzakete, organoaren jatorrizko espezifikazioa (*Hox* geneek ezarria) berrezartze aldera. Jakin badakigu *Hox* geneen adierazpenean dauden desberdintasunek zelula-populazioen bereizketa eragiten dutela; alabaina, oraindik ez dago argi desberdintasun horiek birsorkuntza mugatzen duen hesi gisa joka dezaketen ala ez. Hori ikertzeko, bi egoera esperimental aztertu genituen: analia (*Hox* geneek zehaztutako disko genital konposatutik eratorria) eta *Ultrabithorax* genearen eskualde erregulatzaileetan mutazioak (*bithorax* edo *postbithorax*) zituzten halterio-diskoak. Gure emaitzek erakusten dutenez, *Hox* geneetako desberdintasunak ez dira erabateko eragozpena segmentu edo konpartimentuen arteko birsorkuntzarako. Hala ere, zenbait birsortze-muga hauteman genituen tarteka; muga horiek gehiago agertu ziren, modu ez-espezifikoan, *postbithorax* mutazioaren testuinguruan.

HITZ-GAKOAK: Hox, birsorkuntza, Drosophila

Introduction

Regeneration is a process whereby an organism restores the structure and function of a part of the body that has been lost or damaged. Regeneration has been studied in different organisms, among them *Drosophila melanogaster*, in which the wing imaginal disc has been the main target of investigation (Smith-Bolton *et al.,* 2009; Bergantiños *et al.,* 2010; Worley *et al.,* 2012).

One key question in regeneration studies is the origin of the cells that repair the damaged organ. Different experiments in

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Drosophila imaginal discs have demonstrated that cells from outside the injured domain contribute to its regeneration. For example, cells from the wing disc hinge contribute to the regenerated wing pouch (Smith-Bolton *et al.,* 2009; Herrera *et al.,* 2013; Verghese and Su, 2016). At a more local scale, cells from an intervein region of the wing pouch may be re-specified to replace vein cells that have undergone cell death (Repiso *et al.,* 2013).

The wing disc, like other discs, is first divided into anterior (A) and posterior (P) compartments (García-Bellido *et al.,* 1973). These are units of cell lineage separated from very early in development, so it was expected that regeneration would be carried out only by cells from the injured compartment. Previous reports have indeed shown a compartment-specific regeneration following targeted cell death in one of these cell lineage units (Bosch *et al.,* 2008; Bergantiños *et al.,* 2010). However, more recent experiments demonstrated that a damaged posterior compartment could be restored by anterior cells migrating into the posterior territory, changing compartment identity, and rebuilding the injured tissue (Herrera and Morata, 2014).

Specification of imaginal discs, and in general of different segments along the anteroposterior (A/P) axis, is provided by Hox genes (Lewis, 1978; Rezsohazy *et al.,* 2015). The Hox gene *Antennapedia* is expressed in the wing disc (Paul *et al.,* 2021), whereas *Ultrabithorax* (*Ubx*) differentiates the homologous haltere disc from the wing disc (Lewis, 1963). Several studies have indicated that Hox genes can induce cell segregation and separate cell populations (García-Bellido, 1968; Morata and García-Bellido, 1976; Estrada and Sánchez-Herrero, 2001; Gandille *et al.,* 2010; Curt *et al.,* 2006; Prin *et al.,* 2014; Tiberghien *et al.,* 2015). Therefore, it is unknown if regeneration across segments or compartments, as described in the wing disc (Herrera and Morata, 2014), can also occur when the apposed cell populations possess different homeotic information.

This question can be addressed analyzing mutations causing compartment-specific Hox gene expression, or in discs, like the genital disc, expressing more than one Hox gene. Previous studies in which the genital disc was mechanically fragmented proved its ability to regenerate (Ursprung, 1962; Bryant, 1975a; Bryant, 1975b; Littlefield and Bryant, 1979). However, these experiments could not delimit precisely the point of fragmentation, and therefore the question of whether differences in Hox gene expression affected regeneration remained unresolved.

We have addressed this issue analyzing regeneration in adult derivatives of the genital disc and in haltere discs carrying *Ubx* regulatory mutations. Both model systems exhibit distinct compartmentalization defined by differential Hox expression. Our results are consistent with transgression of segment boundaries during regeneration in adult male analia and in *bithorax* (*bx*) mutant haltere discs, but also show extensions and duplications of anterior haltere and wing disc tissue in a *postbithorax* (*pbx*) mutant background. These effects may reflect an unspecific increase in the frequency of such exceptional regenerative events occurring in an *Ubx* wildtype background.

Results

The analia regenerates after cell death induced by expression of pro-apoptotic genes

The genital disc is made by the fusion of the A8, A9 and A10 abdominal segments (Nöthiger *et al.,* 1977; Schübpach *et al.,* 1978), which express the Hox gene *Abdominal-B* (*Abd-B*) in the A8 and A9 and *caudal* (*cad*) in the A10 (MacDonald and Struhl, 1986; Mlodzik and Gehring, 1987; Freeland and Kuhn, 1996; Casares *et al.,* 1997; Estrada and Sánchez-Herrero, 2001; Morata and Moreno, 1999; Gorfinkiel *et al.,* 2003; Foronda *et al.,* 2006) (Fig. 1A-D''). Although *cad* is not a Hox gene, it bears several features in common with Hox genes: it has a homeobox, it represses *Abd-B*, which is expressed just anterior to *cad* in the embryo and genital disc (thus following the Hox gene rule of transcriptional down-regulation along the A/P axis), and specifies a particular structure, the analia (Moreno and Morata, 1999). The restricted expression of *cad* and *Abd-B* to A10 and A9 (Fig. 1B', B'', D', D''), and their specification of analia and genitalia, respectively, prompted us to analyze if the regeneration of the analia may use cells coming from the A9 primordium.

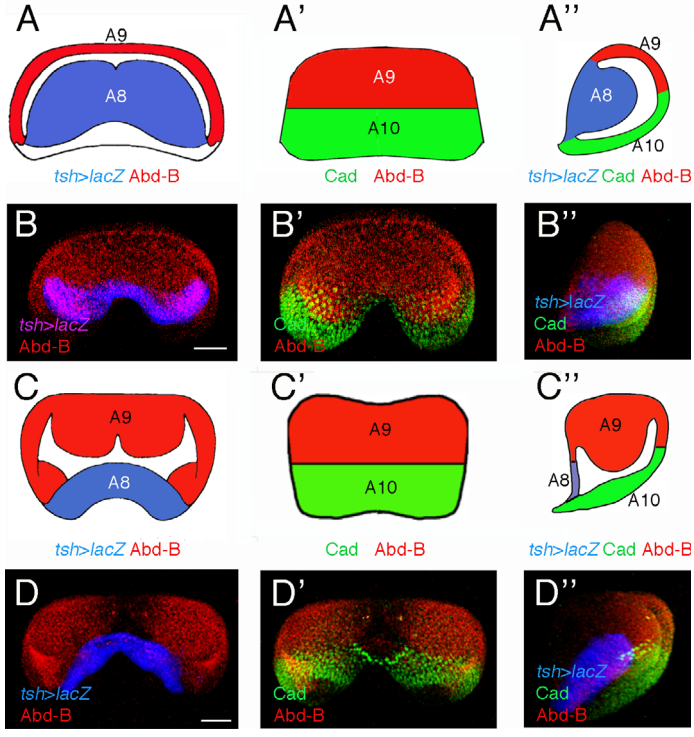


Fig. 1. Segments in the genital disc. The female (A-B'') and male (C-D'') genital disc segments are represented with drawings (A-A'', C-C'') and their gene expression shown (B-B'', D-D''). The orientation of the discs is ventral (A-D), dorsal (A'-D') and lateral (A''-D''). In the female disc the A8 is bigger than the A9 while in the male is the opposite, and in the A8 of both sexes there is expression of genes like *teashirt* (*tsh*) (Gorfinkiel *et al.,* 2003). *Abd-B* is expressed in both A8 and A9 and *cad* in A10. Scale bars are 50 µm.

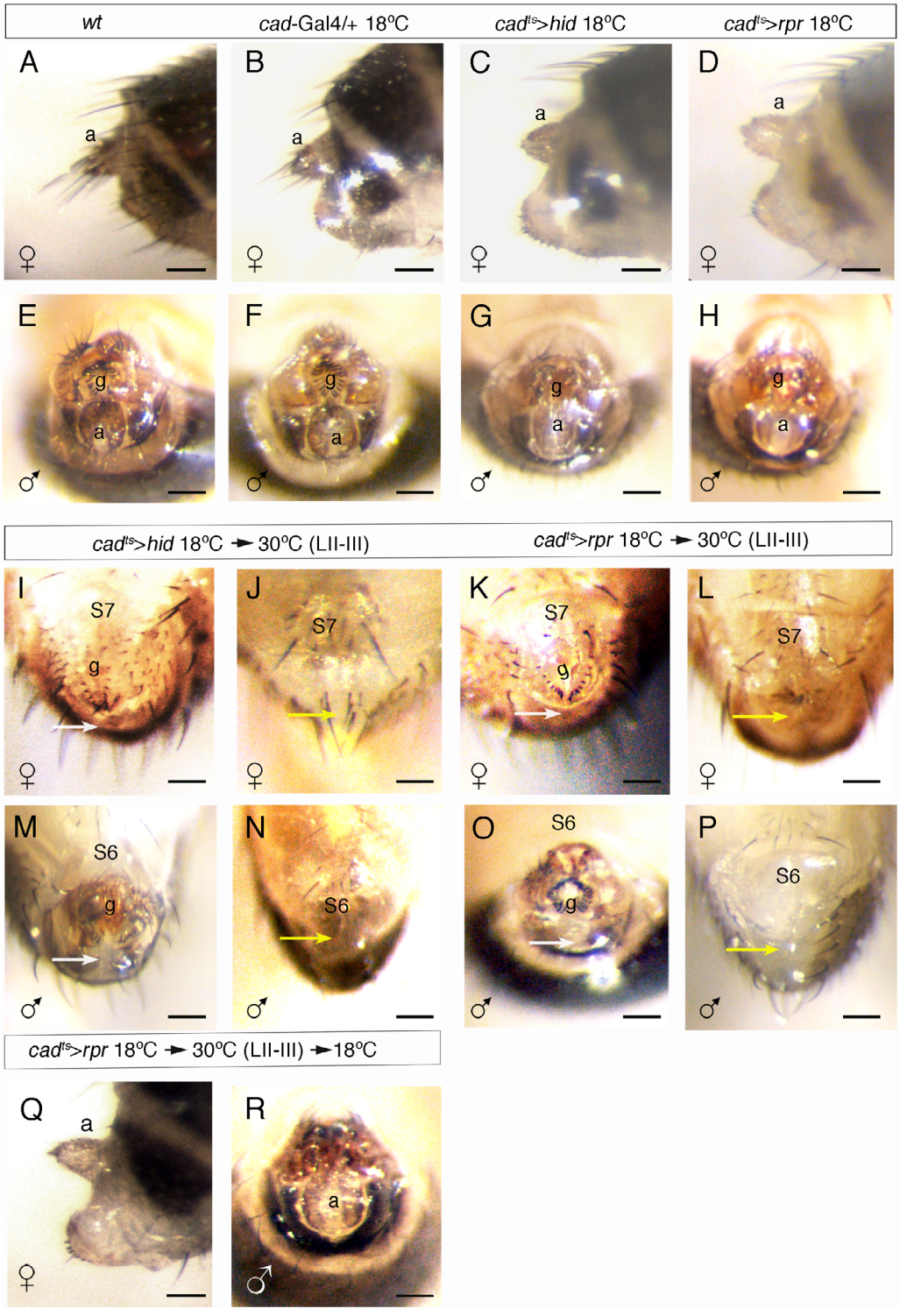


Fig. 2. Development of genitalia and analia in males and females after cell death induced in the analia. (A-H) Lateral (A-D) and ventral (E-H) views of the posterior abdominal segments and analia (a) of females (A-D), and genitalia (g) and analia of males (E-H), of *wt* (A, E), *cad*-Gal4/+ at 18ºC (B, F), *cad*-Gal4/UAS-*hid tub*-Gal80^{ts} at 18ºC (C, G) and *cad*-Gal4 *tub*-Gal80^{ts}/UAS-*rpr* at 18ºC (D, H). (I-P) Ventral views of the posterior part of females (I-L) and males (M-P) of *cad*-Gal4/UAS-*hid tub*-Gal80^{ts} (I, J, M, N) and *cad*-Gal4 *tub*-Gal80^{ts}/UAS-*rpr* (K, L, O, P) shifted from 18ºC to 30ºC at late second and early or late third instar. (Q, R) Female (Q) and male (R) *cad*-Gal4 *tub*-Gal80^{ts}/UAS-*rpr* analia after cell death induction and regeneration, showing the "wt" phenotype in females and normal analia in males. White arrows indicate absence of analia and the yellow arrows, of genitalia and analia. S6 and S7, sixth and seventh sternites, respectively. LII-III indicates the shift to 30ºC was done in the second-third larval period. The > symbol in this and subsequent Figures represents the Gal4/UAS combination. Scale bars are 100 µm.

TABLE 1

REGENERATION NUMBERS AFTER CELL DEATH INDUCED IN THE CAUDAL DOMAIN EXPRESSING THE PROAPOPTOTIC GENE *HID*. THE CROSS IS *CAD*-GAL4/*CYO, TB* X UAS-*HID TUB*-GAL80^{TS}/*CYO, TB*. TREATMENTS ARE 24H AT 18ºC, 5 OR 6 DAYS (D) AT 18ºC AND 30H OR 40H AT 30ºC BEFORE SHIFTING BACK THE LARVAE TO 18ºC. "WT" INDICATES THE ANALIA PHENOTYPE OF FEMALES AT 18ºC WITH *TUB*-GAL80^{TS} (SEE TEXT). NO A/NO A + G MEANS ABSENCE OF ANALIA OR OF GENITALIA AND ANALIA, RESPECTIVELY

	Phenotype	***cad > hid*** **Number of individuals and percentage**	
		24h-6d-30h	**24h-5d-40h**
Females	Regenerated: wt	423/545 (77.6%)	68/92 (73.9%)
	Partially regenerated	26/545 (4.7%)	10/92 (10.8%)
	No regenerated (No A/No A+G)	96/545 (17.6%)	14/92 (15.2%)
Males	Regenerated: wt	203/432 (46.9%)	31/113 (27.4%)
	Partially regenerated	16/432 (3.7%)	10/113 (8.8%)
	No regenerated (No A/No A+G)	213/432 (49.3%)	72/113 (63.7%)

We used the UAS/Gal4/Gal80^{ts} system (McGuire *et al.,* 2003) to achieve temporal control of the expression of the pro-apoptotic genes *head involution defective* (*hid*) or *reaper* (*rpr*) under *cad*-Gal4 control, and studied subsequent tissue regeneration (Smith-Bolton *et al.,* 2009; Bergantiños *et al.,* 2010; Worley *et al.,* 2012). Surprisingly, *cad*-Gal4 *tub*-Gal80^{ts} UAS-*hid* or *cad*-Gal4 *tub*-Gal80^{ts} UAS-*rpr* females exhibited analia with very thin and near transparent bristles when grown at the permissive temperature of 18ºC (Fig. 2C, D, compare with the wildtype and *cad*-Gal4/+ females grown at the same temperature, Fig. 2A, B). Males with the same genotype and temperature, by contrast, showed normal analia, as the controls do (Fig. 2E-H). This indicates that, as in other body parts (Fig. S1), the effect of cell death on bristle development cannot be completely suppressed by *tub*-Gal80^{ts} at 18ºC. Animals in which cell death was induced at the third larval period lack analia (Fig. 2I, K, M, O), or both genitalia and analia if induction was done earlier in development (Fig. 2J, L, N, P). This indirect effect on genitalia is probably due to the damage-induced disruption of the interaction between the genitalia and analia primordia (Gorfinkiel *et al.,* 2003). This effect on genital development was more frequently observed in males, perhaps because genitalia and analia primordia are contiguous in the male genital disc.

After the cell death and regeneration protocol (see Methods), females were able to recover the analia phenotype observed when grown at 18ºC (referred to heretofore as "wt", see Fig. 2C, D, Q), whereas males fully recovered the wildtype analia (Fig. 2R). We also noted that, when inducing apoptosis with *hid*, females regenerate the analia (the "wt" phenotype) more frequently than males (see Table 1).

To determine to which extent the treatment induced cell death and subsequent regeneration in the larval A10 primordium, we checked apoptosis with an anti-cleaved Dcp-1 antibody and trace the lineage of cells expressing *cad* with the flip-out technique (Struhl and Basler, 1993) in larvae of the genotype *cad*-Gal4/UAS-*hid tub*-Gal80^{ts}; UAS-*flp act*>stop>*lacZ*/+. At 18ºC, since the Gal80^{ts} protein inhibits Gal4 activity, minimal cell death and no marked cells were observed (Fig. 3A-A''), whereas after a 24h pulse at 30ºC the abnormal genital discs exhibited massive expression of activated Dcp-1, roughly coincident with the *cad*-Gal4 cell lineage (Fig. 3B-B"). The activation of the c-Jun N-terminal kinase (JNK) pathway (Bergantiños *et al.,* 2010; Herrera *et al.,* 2013), detected with the TRE-DsRed reporter (Chatterjee and Bohmann, 2012), and of Wg signaling, monitored with a BRV-B-GFP reporter (Harris *et al.,* 2016), are restricted, in controls, to a small region (stalk) connecting the genital disc with larval tissue (Macías *et al.,* 2004; Rousset *et al.,* 2010). However, when cell death is induced, their activity domains expand into the A10 segment (Fig. S2A-A", B-B'').

Contribution of neighboring cells to analia regeneration

We first analyzed *Abd-B* and *cad* expression in the male and female genital discs to ascertain whether cells cross the A9/A10 boundary during regeneration of the analia primordium. As shown in Fig. S3A-A''', B-B''', immunostaining revealed a slight overlap between *Abd-B*-expressing and *cad*-expressing cells at the A9/A10 boundary, both in male and female genital discs, consistent with previous reports (Moreno and Morata, 1999). This overlap obscures whether cells from one primordium contribute to the regeneration of the other. Moreover, the *cad*-Gal4 strain does not precise coincide with endogenous Cad signal, as *cad-Gal4* expression is absent in a small, anterior subset of *cad*-expressing cells (Fig. S3C-C'''). These combined limitations hampers detecting if cells from the A9 segment, expressing *Abd-B*, change segment position and Hox expression to regenerate the A10 segment.

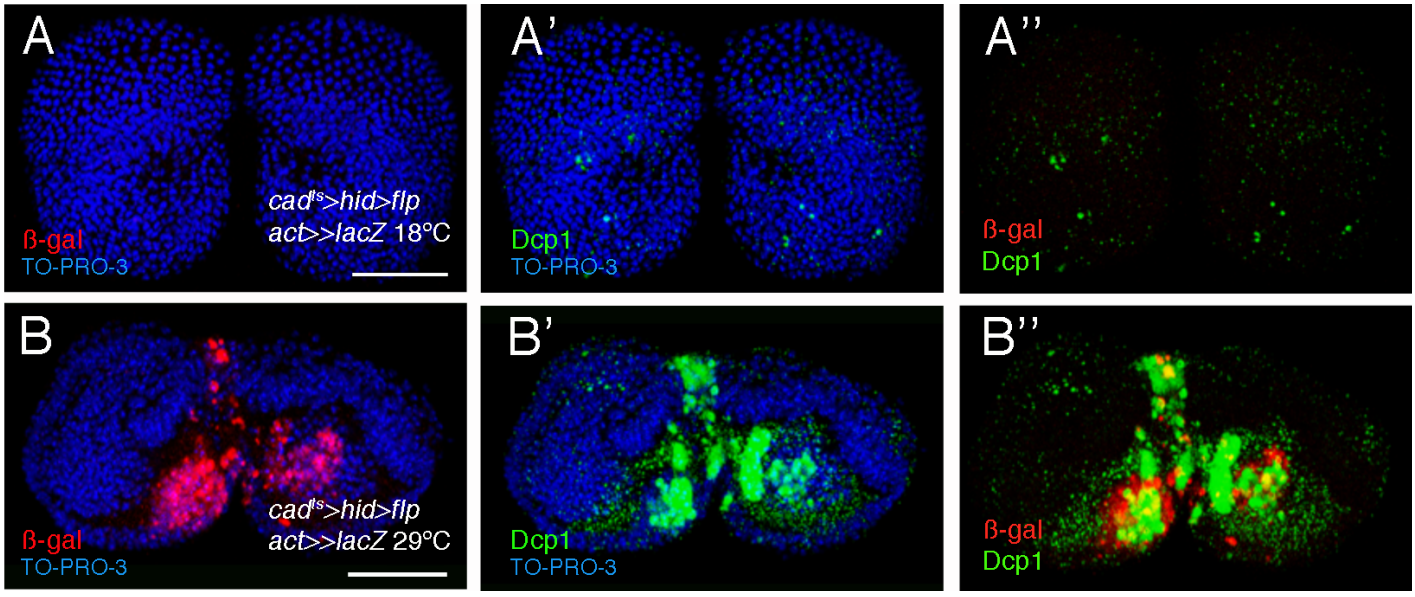


Fig. 3. Caspase activation after induction of cell death in the genital disc. (A-B'') Cleaved Dcp1 staining (in green) and ß-galactosidase signal (in red) in the A10 (distorted in B-B'') of third instar larvae male genital discs of genotype *cad*-Gal4/UAS-*hid tub*-Gal80^{ts}; UAS-*flp act*>stop>*lacZ*/+, in which *cad* lineage expression and apoptosis are either not induced (control discs, A-A'') because the larvae are grown at 18ºC, or induced (B-B''), when they are shifted to 30ºC. TO-PRO-3 is in blue. The >> symbol stands for FRT-stop-FRT. Scale bars are 50 µm.

We turned then to the study of the adult morphology. In contrast to the overlapping expression observed in third-instar genital discs, *Abd-B* and *cad* expression patterns become mutually exclusive during the pupal stage (McQueen *et al.,* 2025). This is consistent with the discovery that clones induced early in development are confined to either genitalia or analia (Dübendorfer and Nöthiger, 1982). Also consistently, the *cad*-Gal4 driver directs *yellow* expression in the analia of both sexes, but not in the adjacent genital structures (Fig. 4A, B).

To analyze if cells change segment identity during regeneration, we induced cell death and regeneration in the analia primordium while marking the *cad* lineage by the loss of the *yellow* (*y*) cuticular marker, present in the cassette *actin*-FRT-*y*$^+$-FRT-Gal4 (abbreviated as *act*>*y*+>Gal4), in a *y* mutant background. Adults of the genotype *y w*; *cad*-Gal4 *tub*-Gal80^{ts}/*act*>*y*+>Gal4; UAS-*flp*/+ kept at 18ºC do not have any *y* bristle in the analia (Fig. 4C), but this organ is full of *y* bristles when the animals were raised at 30ºC (Fig. 4D). Following the regeneration protocol, in 55,5% of control males (*cad*-Gal4/*tub*-Gal80^{ts} UAS-*GFP*; UAS-*flp*/*act*>*y*+>Gal4) (n=18) the analia are completely *y*, while the rest of males have a small number of *y*$^+$ bristles, with an average of 2,9 *y*$^+$ bristles per analia (Fig. 4E, G). By contrast, in the experimental males (*cad*-Gal4/*tub*-Gal80^{ts} UAS-*hid*; UAS-*flp*/*act*>*y*+>Gal4) (n=20), only 25% of them have all the analia bristles *y*, and the average number of *y*$^+$ bristles is significantly higher, 6,6 (Fig. 4F, G). Although in some of the analia cells there might have been no removal of the cassette, accounting for some *y*$^+$ bristles in control and experimental groups, the significant increase of *y*$^+$ bristles in the experimental set is probably due to cells coming from the adjacent genitalia primordium, that expressed *Abd-B* before regeneration and lose this expression to gain *cad* and develop analia.

Cells can gain *Ultrabithorax* expression in regenerating *bithorax* haltere discs

To further investigate the relationship between *Hox* gene expression and tissue regeneration, we examined regeneration in haltere discs with compartment-specific Hox expression. The haltere disc is homologous to the wing disc but expresses *Ubx* (White and Wilcox, 1984; 1985; Cabrera *et al.,* 1985; Beachy *et al.,* 1985). *bithorax* (*bx*) or *postbithorax* (*pbx*) mutations strongly reduce *Ubx* expression in the anterior or posterior compartments of the haltere disc, respectively, resulting in a homeotic transformation towards a wing fate (Lewis, 1963; Cabrera *et al.,* 1985; White and Wilcox, 1985). In these mutants the A/P boundary separates compartments of distinct fate, haltere or wing, with or without *Ubx* expression. We investigated whether, during regeneration of the posterior compartment of *bx* or *pbx* haltere discs, cells from the anterior compartment could gain (in *bx*) or lose (in *pbx*) *Ubx* expression as they rebuild the damaged compartment. The *hh*-Gal4 strain was used as a posterior specific Gal4 driver for these experiments.

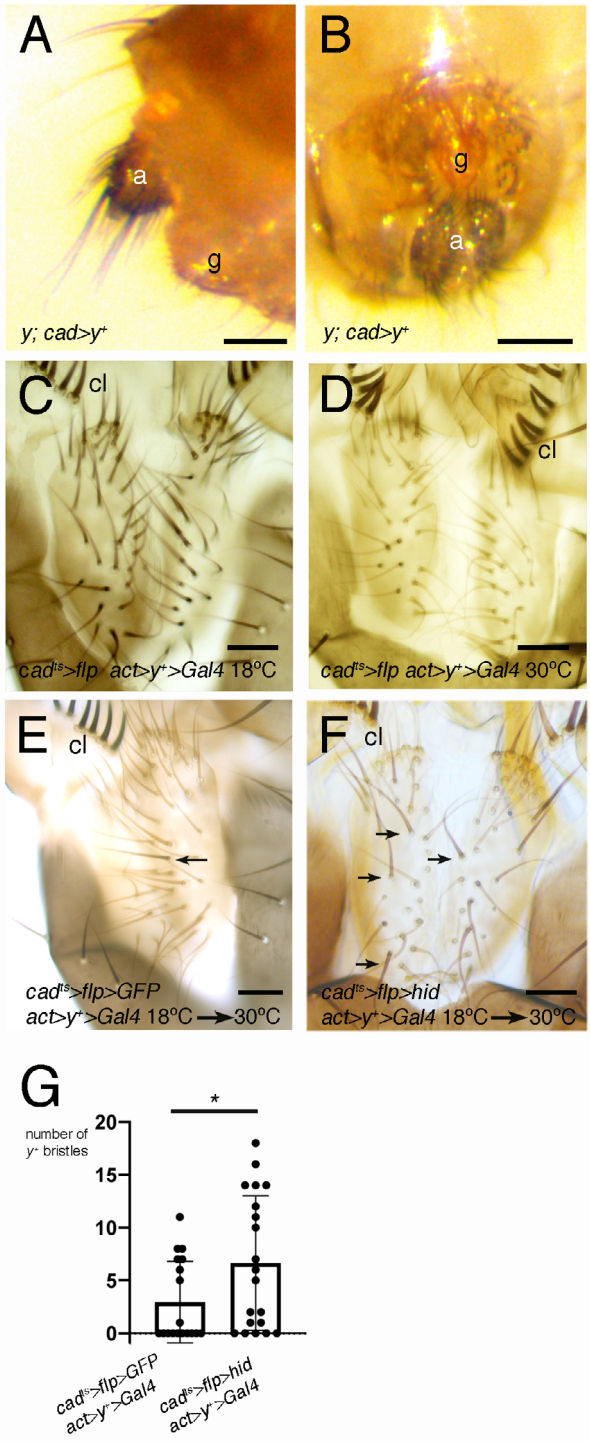


Fig. 4. Increase of *yellow* mosaicism in the analia after cell death and regeneration. (A, B) Analia of females (A) and males (B) in adults of the genotype *y*; *cad*-Gal4/UAS-y^+, showing the restriction of the dark tissue (y^+) to the analia (a); g, genitalia. (C) Analia of a male of the genotype *y w*; *cad*-Gal4 *tub*-Gal80^{ts}/ *act*>*y+*>Gal4; UAS-*flp*/+ grown at 18ºC, showing the bristles are of brown color (no recombination of the cassette that liberates the y^+ gene; n=6). The FRT sequences are represented by the symbol > (which also indicates Gal4/UAS). In this and following panels cl means clasper teeth, structures of the genitalia. (D) Analia of a male of the same genotype as in C but grown at 30ºC: in this case the Gal4 activates the flipase in the analia, and this leads to recombination of the cassette and losing of the y^+ gene, so that the whole analia is of yellow color (n=6). (E) Only one y^+ bristle (arrow) is observed in a male analia of the control genotype, *cad*-Gal4/*tub*-Gal80^{ts} UAS-*GFP*; UAS-*flp*/actin>y^+>Gal4 (n=18), after the regeneration treatment. (F) In the analia of males of the genotype *cad*-Gal4/UAS-*hid tub*-Gal80^{ts}; UAS-*flp*/act>y^+>Gal4, several y^+ bristles are observed (arrows) (n=20) with the same regeneration treatment. Quantification of y^+ bristles in the indicated genotypes. Scale bars are 100 µm in A and B, and 25 µm in C-F.

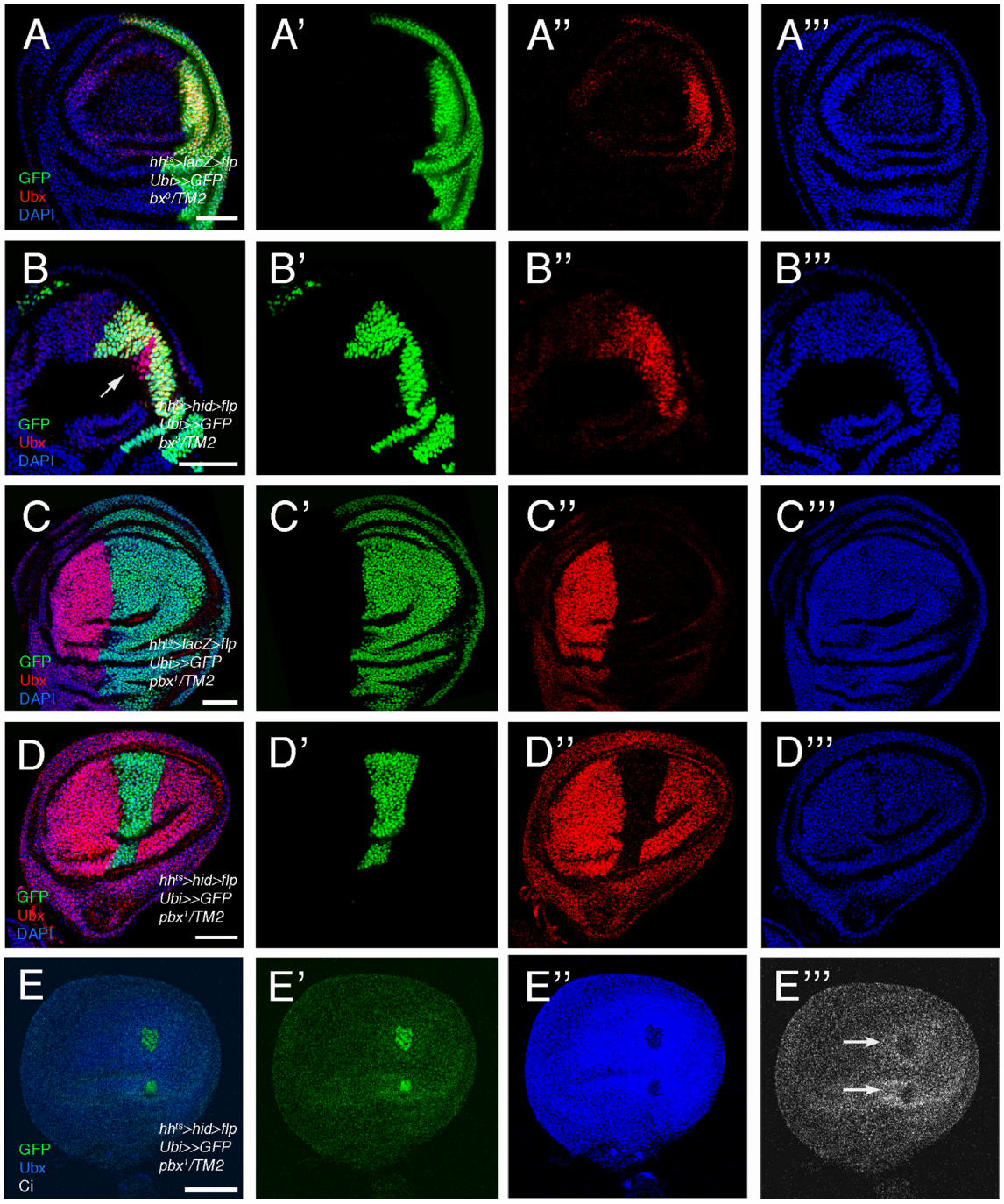


Fig. 5. Regeneration of haltere disc tissue after cell death and recovery in the posterior compartment. (A-A''') Third instar haltere disc mutant for *bx* in which *lacZ* is expressed in the posterior compartment and the cell lineage in the same compartment is marked by GFP expression (genotype *ubi*>stop>*GFP* UAS-*flp*/UAS-*lacZ tub*-Gal80^{ts}; *bx^3 hh*-Gal4/*TM2*). Ubx is almost completely absent from the anterior compartment and its expression coincides with that of GFP. In this and following Figures the double arrowhead (>>) stands for FRT-stop-FRT. (B-B''') Third instar haltere disc of the genotype *ubi*>stop>*GFP* UAS-*flp*/UAS-*hid tub*-Gal80^{ts}; *bx^3 hh*-Gal4/*TM2* showing some anterior cells (not marked with the GFP lineage tracer) integrated in the posterior compartment and showing Ubx antibody staining (arrow). (C-C''') Third instar haltere disc mutant for *postbithorax* in which *lacZ* is expressed in the posterior compartment and the cell lineage in the same compartment is marked by GFP expression (genotype *ubi*>stop>*GFP* UAS-*flp*/UAS-*lacZ tub*-Gal80^{ts}; *pbx^1 hh*-Gal4/*TM2*). Ubx antibody signal is observed just in the anterior compartment, complementary to that of GFP. (D-E''). Two examples of haltere discs in which "duplications" or anterior compartment extensions are observed. The genotype of the discs is *ubi*>stop>*GFP* UAS-*flp*/UAS-*hid tub*-Gal80^{ts}; *pbx^1 hh*-Gal4/*TM2*. The cell lineage of the posterior compartment is marked by GFP (D', E'), and is surrounded by anterior cells marked by Ubx (D'', E'') or Ci (E'''). The reduction of the posterior compartment is extreme in the haltere disc in E-E'''. Note the increased size of the pouch A compartment in D and E, and the reduced notum region, what gives globular shape to the haltere disc in E. Scale bars are 50 μm.

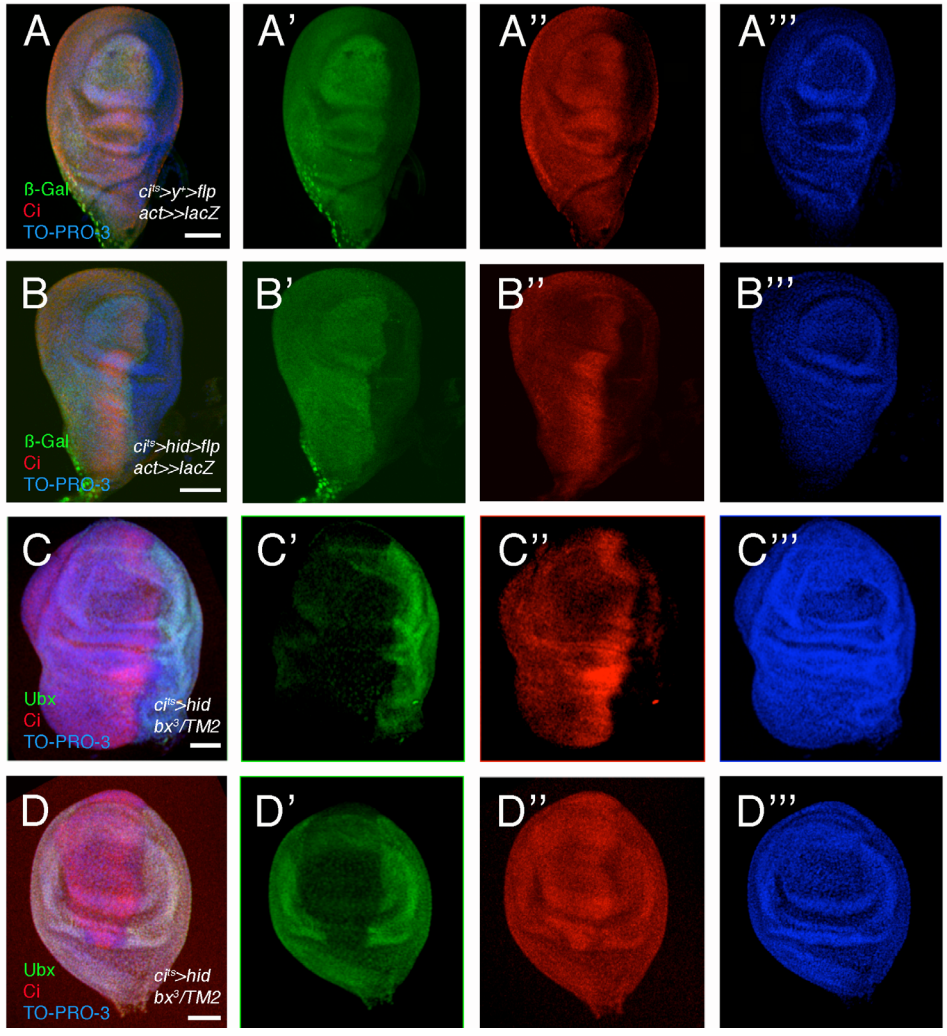


Fig. 6. Development of *bithorax* mutant haltere discs after cell death and recovery in the anterior compartment. (A-A''') Control haltere discs of genotype *tub*-Gal80^{ts}/UAS-*y*$^{+}$; *ci*-Gal4/UAS-*flp act*>stop>*lacZ*. The anti-ß-galactosidase staining in the anterior compartment, marking the cell lineage, coincides with Ci signal (n=15). (B-B'') The coincidence of cell lineage and Ci staining (UAS-*hid tub*-Gal80^{ts}/+; *ci*-Gal4/UAS-*flp act*>stop>*lacZ)* is also observed in haltere discs in which there is cell death and recovery in the anterior compartment (n=18). (C-C''') Haltere disc mutant for *bx* in which cell death and recovery were induced in the anterior compartment (genotype UAS-*hid tub*-Gal80^{ts}/+; *ci*-Gal4 *bx*^{3}/*TM2*. There is normal *Ubx* and *ci* expression. This occurs in 21/22 discs observed. (D-D''') Haltere disc of the same genotype as that in C but which shows duplication in the disc: posterior cells, marked by Ubx staining, surround anterior cells expressing *ci* (there is high background in the Ci staining, but high levels at the A/P interphase could still be distinguished). Scale bars are 50 µm.

Before analyzing results in *bx* or *pbx* mutants, we first examined lineage tracing in wildtype wing and haltere discs. In larvae of the genotype UAS-*y*$^{+}$/+; UAS-*flp act>stop>lacZ*/*hh*-Gal4 *tub*-Gal80^{ts}, shifted during the second larval instar from 18ºC to 30ºC for 30h, we observed, consistent with their established development patterns, that cells marking the posterior lineage abut the anterior ones, marked by Cubitus interruptus (Ci) (Eaton and Kornberg, 1990), in both wing and haltere discs (Fig. S4A-B''') (Table 2).

To investigate the plasticity of *Ubx* during regeneration, we analyzed haltere discs mutant for *bx*^{3} (*bx*^{3}/*TM2*), which exhibit a strong reduction of *Ubx* in the anterior compartment (Fig. S5B, B', the wildtype in A). After apoptosis induction via *hid* expression, detected by massive Dcp-1 signal in the P compartment coinciding with the *hh* lineage (Fig. S5E, E'), we analyzed *Ubx* expression in the regenerating tissue. In the control discs (expressing *lacZ* instead of *hid*), the *hh* lineage consistently coincided with the *Ubx* expression in the posterior compartment (Fig. 5A-A''') (Table 2). In most of the experimental larvae (16/18; 89%), we observed the posterior compartment regenerated exclusively with cells expressing both *Ubx* and the *hh* lineage. However, in 2 haltere discs (2/18; 11%) we observed a group of cells apparently integrated in the posterior compartment and close to the A/P boundary that expressed *Ubx* but lacked GFP signal, that is, they were not part of the *hh* lineage (Fig. 5B-B'') (Table 2). These cells are likely to be anterior cells that penetrated into the posterior compartment and activated *Ubx* expression during the regeneration process.

TABLE 2

NUMBER OF WING (W) AND HALTERE (H) DISCS UNDERGOING REGENERATION, SHOWING PARTIAL DUPLICATIONS OR EXTENSION OF ANTERIOR COMPARTMENT, OR ABNORMAL DEVELOPMENT IN THE DIFFERENT GENOTYPES INDICATED. *TWO OF THE HALTERE DISCS SHOW CROSSING OF ANTERIOR CELLS TO THE POSTERIOR COMPARTMENT; **IN 12 DISCS THERE ARE A FEW POSTERIOR CELLS IN THE ANTERIOR COMPARTMENT. ***THERE ARE CASES OF REDUCED POSTERIOR COMPARTMENT AND ABNORMAL COMPARTMENT BOUNDARY (MORE EASILY OBSERVED IN THE WING DISC)

Genotype	Total	Normal regeneration	"Duplications" / compartment extensions	Abnormal development
UAS-*y*$^{+}$/+; UAS-*flp act*>*stop*>*lacZ*/*hh*-Gal4 *tub*-Gal80^{ts}-w	24	24	0	0
UAS-*y*$^{+}$/+; UAS-*flp act*>*stop*>*lacZ*/*hh*-Gal4 *tub*-Gal80^{ts}-h	21	21	0	0
ubi>stop>*GFP* UAS-*flp*/UAS-*lacZ tub*-Gal80^{ts}; *bx*^{3} *hh*-Gal4/*TM2*-h	11	11	0	0
ubi>stop>*GFP* UAS-*flp*/UAS-*hid tub*-Gal80^{ts}; *bx*^{3} *hh*-Gal4/*TM2*-h	18	18*	0	0
ubi>stop>*GFP* UAS-*flp*/UAS-*lacZ tub*-Gal80^{ts}; *pbx*^{1} *hh*-Gal4/*TM2*-h	25	25**	0	0
ubi>stop>*GFP* UAS-*flp*/UAS-*hid tub*-Gal80^{ts}; *pbx*^{1} *hh*-Gal4/*TM2*-h	21	13	8	0
tub-Gal80^{ts}/UAS-*y*$^{+}$; *ci*-Gal4/UAS-*flp act*>stop>*lacZ*-h	15	15	0	0
UAS-*hid tub*-Gal80^{ts}/ +; *ci*-Gal4/UAS-*flp act*>stop>*lacZ*-h	18	18	0	0
UAS-*hid tub*-Gal80^{ts}/+; *ci*-Gal4 *bx*^{3}/*TM2*-h	22	21	1	0
UAS-*hid tub*-Gal80^{ts}/+; *pbx*^{1}*hh*-Gal4 /*TM2*-w	27	8***	14	5
UAS-*hid tub*-Gal80^{ts}/+; *pbx*^{1}*hh*-Gal4 /*TM2*-h	14	7***	4	3
UAS-*hid tub*-Gal80^{ts}/; *hh*-Gal4/ UAS-*flp act*> *stop*>*lacZ*-w	18	17***	1	0
UAS-*hid tub*-Gal80^{ts}/; *hh*-Gal4/ UAS-*flp act*> *stop*>*lacZ*-h	15	15***	0	0

Duplications and increased anterior compartment size in haltere discs after ablation of posterior cells

In the complementary experiment, in which we used the same experimental setup but in haltere discs of *pbx*^{1}/*TM2* larvae, which lack *Ubx* expression in the posterior compartment (Fig. S5C, C', the control in A), we obtained a different result. We also observed strong anti-Dcp-1 signal in the posterior compartment, coinciding with the *hh* lineage (Fig. S5F, F'; the control in D). Whereas in more than half of the control discs the absence of *Ubx* in the posterior compartment strictly coincided with the *hh* lineage (Fig. 5C-C'"), in 48% of them we observed the GFP signal appeared in a subset of cells near the A/P boundary that are also expressing *Ubx* (Fig. S6A-A'") (Table 2). This is likely due to the occasional early expression of the *hh*-Gal4 driver in the anterior compartment in this mutant combination, a phenomenon previously reported in the wildtype wing disc (Bosch *et al.,* 2016).

In the experimental discs (genotype *ubi*>stop>*GFP* UAS-*flp*/ UAS-*hid tub*-Gal80^{ts}; *pbx*^{1} *hh*-Gal4/*TM2*), most of the discs (65%) regenerated the posterior compartment with cells failing to express *Ubx*, as in controls. However, in 38% of the discs we observed a quite different phenotype: these discs showed variable reduction of the posterior compartment, restricted to the most central region of the disc, with the most posterior pouch cells lacking the *hh* lineage and expressing *Ubx*; that is, the posterior compartment seemed to be surrounded by anterior cells. In a few extreme cases, the reorganization of the haltere disc led to a complete A-P-A disposition (what we call compartment "duplications") (Fig. 5D-D'"), while in most cases we observed just the expansion of anterior cells (Table 2). To confirm the anterior identity of the cells surrounding the posterior compartment, we stained with an anti-Ci antibody, which marks in the wildtype the anterior compartment, with higher levels in cells abutting the A/P boundary (Motzny and Holmgreen, 1995; Aza-Blanc *et al.,* 1997). In the regenerated discs we observed this increased signal surrounding the remaining posterior cells, which in some cases were reduced to small clusters (Fig. 5E-E'"; Fig. S6B-B'"). The expanding anterior cells are likely to derive from anterior pouch cells or from the hinge. Collectively, the data suggest that absence or reduction of trans-compartment regeneration led these discs to form a partial mirror image duplication or to the extension of the anterior compartment surrounding posterior cells.

To determine whether the unexpected duplications were specific to the posterior compartment, we tested if similar ef-

fects occurred following cell death induction in the mutant anterior compartment. In control (*ci*-Gal4 *tub*-Gal80^{ts} UAS-*y*$^{+}$ UAS-*flp act>stop>lacZ*) or experimental (*ci*-Gal4 *tub*-Gal80^{ts} UAS-*hid* UAS-*flp act*>stop>*lacZ*) larvae which underwent the same experimental treatment as the *hh*-Gal4 larvae, we observed strict coincidence of Ci and ß-galactosidase (the anterior cell lineage) expression in haltere discs (Fig. 6A-A'''; 6B-B''') (Table 2). We next induced cell death and recovery but in a *bx* mutant background. Although in these experiments we did not label the anterior cell lineage, by staining with anti-Ubx and anti-Ci antibodies we observed that, in all but one disc, there was the expected normal regeneration of the anterior compartment (Fig. 6C-C'''). In this single exception, however, we observed a mirror image duplication (P-A-P) with *Ubx* signal at the lateral sides of the disc, resembling the duplications we have described above for the *pbx* haltere discs after cell death induction in the posterior compartment (Fig. 6D-D''') (Table 2). Taken together, the experiments suggest there are instances, particularly when inducing cell death in the posterior compartment of *pbx* haltere discs, where regeneration fails. In these cases, the ablated compartment is reduced, becoming surrounded by the cells of the adjacent one.

Lack of complete regeneration after cell death in one compartment of the wing and haltere discs

To provide further experimental evidence, we induced regeneration in larvae of the genotype UAS-*hid tub*-Gal80^{ts}/+; *pbx*^{1} *hh*-Gal4/*TM2*. Although clonal lineage tracing was not employed, analysis of these discs revealed frequent expansions of the anterior compartment and "duplications". Among the 14 *pbx* haltere discs analyzed, we observed 4 with "duplications" or extended anterior compartments (Fig. 7A-A") and 3 with abnormal morphologies (Fig. S7A-A'"). Surprisingly, we also found similar effects in the wing disc. Out of 27 discs analyzed, we found 14 with some kind of reduction of the posterior compartment and "duplication" or expansion of the anterior domain (Fig. 7B-B") and 5 with abnormal development (Fig. S7B-B") (Table 2).

Given these results, we conducted a control experiment, analyzing the regenerative capacity of wing discs with wild-type *Ubx* function and the same experimental conditions as in previous experiments. The genotype of the 18 discs analyzed was UAS-*hid tub*-Gal80^{ts}/+; *hh*-Gal4/UAS-*flp act*>stop>*lacZ*. We found normal regeneration in most discs, and consistent with previous reports (Herrera and Morata, 2014), some control discs that displayed overlap of posterior lineage cells (ß-galactosidase-expressing cells) and Ci-positive anterior cells, or showed an irregular A/P boundary (Fig. S7C). We also found discs which a reduced P compartment (Fig. S6D-D") and, surprisingly, we identified a disc with a severely reduced P compartment surrounded by anterior cells, resembling the phenotype observed in *pbx* mutant wing or haltere discs (Fig. 7C-C") (Table 2). These findings suggest that in larvae wildtype for *Ubx* function there could be, though very infrequently, defects including partial extension of the anterior compartment and limited regeneration in the posterior one.

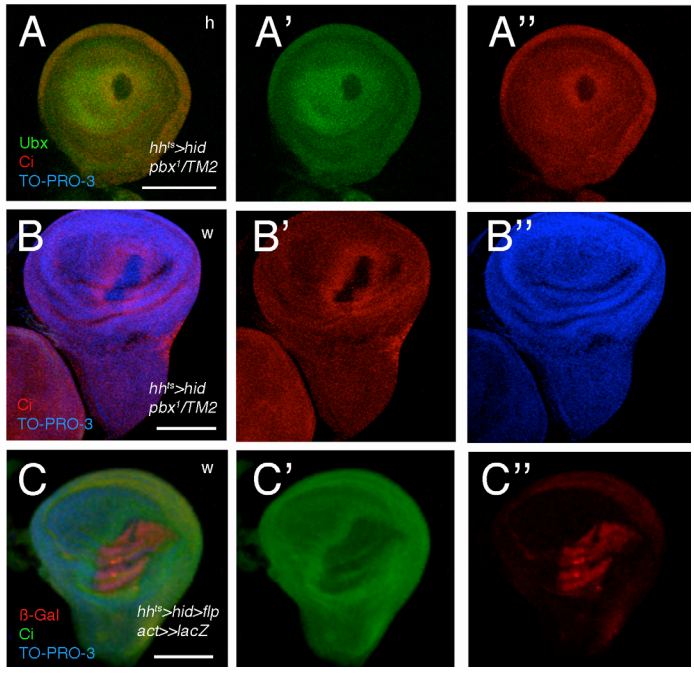


Fig. 7. Partial duplication and extension of anterior tissue in the wing and haltere disc after cell death induction in the posterior compartment. (A-A") *pbx* haltere disc (h) in which cell death was induced in the posterior compartment (genotype UAS-*hid tub*-Gal80^{ts}/+; *pbx*^{1} *hh*-Gal4/*TM2*). *Ubx* and *ci* expression occupy most of the disc, with a small group of posterior cells in the middle of the pouch. (B-B") Wing disc of the same genotype as in A, which similarly shows anterior cells (expressing *ci*) surrounding posterior cells in the middle of the pouch. (C-C") Wing disc (w) in which cell death was induced in the posterior compartment and cell lineage of posterior cells are marked by ß-galactosidase signal (genotype UAS-*hid tub*-Gal80^{ts}/+; *hh*-Gal4/ UAS-*flp act*>stop>*lacZ*). Similarly to the disc in B, the posterior compartment is reduced to a group of cells in the middle of the pouch and hinge, surrounded by anterior cells, expressing *ci*. Scale bars are 100 µm.

Discussion

A central issue in regeneration studies is identifying the origin and genetic status of the cells that recover the damaged tissue. In complex metazoans, epithelial cell plasticity can be triggered by stress and directed by diverse genetic and epigenetic mechanisms that modify the genetic profile of contributing cells (Nagai and Nakajima, 2026). Experiments of regeneration in amputated salamander limbs have demonstrated the formation of a blastema, wherein dedifferentiated cells give rise to tissues with genetic signatures distinct from those of progenitor cells (Otsuki and Tanaka, 2022). Similarly, work in *Drosophila* has shown that the damaged tissue is reconstructed by cells that originally have a different genetic constitution (Smith-Bolton *et al.,* 2009; Bergantiños *et al.,* 2010; Repiso *et al.,* 2013; Herrera *et al.,* 2013; Verghese and Su, 2016; Verghese and Su, 2017). Our experiments have shown that the differential activity of Hox

genes does not represent an absolute barrier for regeneration across segments or compartments, and that the Hox genetic status can change in this process, although such events are rare or even absent in some specific contexts.

The existence of developmental compartments in Drosophila suggested they act as restrictions to the regeneration process: the compartment boundary was not affected or rapidly re-established following injury (Smith-Bolton *et al.,* 2009), with regeneration driven exclusively by cells originated within the damaged compartment (Bosch *et al.,* 2008; Bergantiños *et al.,* 2010). However, experiments in which isolated imaginal discs were cut and let regenerate showed that cells from a compartment could cross the compartment boundary to restore disc integrity (reviewed in Worley *et al.,* 2012). Furthermore, studies using *Minute* technique clones, which provide a competitive growth advantage, showed that cells crossed the A/P boundary during regeneration (Szabad *et al.,* 1979). More recent experiments, in which the posterior compartment was precisely targeted to death, demonstrated that cells from the anterior compartment migrated and contributed to the reconstruction of the damaged posterior territory (Herrera and Morata, 2014).

Based on these latter experiments, we tested whether differential *Hox* expression imposes an absolute restriction on compartment or segment regeneration. Such differences are known to be sufficient in some cases for the segregation of cells at *Drosophila* compartment boundaries or vertebrate rhombomeres (Curt *et al.,* 2013; Prin *et al.,* 20014), processes typically governed by the *engrailed/hedgehog/cubitus interruptus* system in Drosophila (Wang and Dahmann, 2020), or genes like *Kros20* in vertebrate rhombomeres (Addison and Wilkinson, 2016). Our analyses of genital and haltere discs (in a *bx* mutant background) suggest that distinct Hox gene expression does not result in an absolute impediment to cells crossing boundaries to rebuild an organ, even though the canonical signaling systems restricting this movement are most likely still active. However, these crossing events in the *bx* haltere disc are rare (2 out of 18 cases), and in regeneration of the analia, although individual cases could not be unambiguously identified as segment crosses, analysis of the data suggest these trans-compartmental transgressions are infrequent. These examples of rare compartment/segment crossing might be due to the harsh cell death conditions imposed in our experiments: a milder cell death induction regime might result in a compartment-restricted plasticity.

The segment/compartment transgressions observed in this study indicate that the cellular reprogramming during regeneration must overcome two distinct barriers: the first one is established by the *engrailed/hedgehog/cubitus interruptus* system, which separates anterior and posterior compartments in the haltere disc, and genitalia and analia primordia in the genital disc (Wang and Dahmann, 2020; Freeland and Kuhn, 1996; Casares *et al.,* 1997; Chen and Baker, 1997), and the second one is determined by the segment/compartment-specific Hox gene expression. We note that the frequency of cases crossing the A/P boundary during regeneration in the *bx* haltere disc (2/18) is significantly lower than that previously reported for the homologous, wildtype, wing disc (22/31; Herrera and Morata, 2014). This diminished frequency suggests that distinct Hox gene expression profiles (e.g., *Ultrabithorax* in the haltere disc) in our experiments act as a strong secondary barrier, hindering cellular trespassing. Interestingly, studies on axolotl forelimb (Carl-son *et al.,* 2001), mouse bone (Gersch *et al.,* 2005) and gecko tail (Nurhidayat *et al.,* 2025) have shown reactivation of Hox gene expression during regeneration, indicating these genetic changes can perhaps occur more freely without segmental/compartmental limits.

Such transgressions were not observed in a *pbx* mutant background. Instead, a substantial proportion of the discs dis-played a significantly reduced posterior compartment (some-times dramatically), with anterior cells surrounding the injured tissue. These observations suggest that anterior cells contrib-ute minimally, if at all, to the regeneration of the posterior com-partment; differential regeneration from surviving posterior cells probably account for the variable compartment size. The ex-treme examples where the haltere disc posterior compartment is reduced to a few cells (see Fig. 5E), suggest that the additive effects of the two barriers described above may impose a com-plete restriction to compartment crossing, leading to significant reduction of posterior compartment and expansion of the an-terior cells. We argue against a model of superior competitive-ness of anterior cells accounting for this phenotype: *Ubx* mutant clones grow similarly to *Ubx/+* twins (de Navas *et al.,* 2006), and classical cell competition does not operate across compart-ment boundaries (Simpson, 1976; Simpson and Mo rata, 1981).

Surprisingly, the effects present in the *pbx* haltere discs are also observed in the wing disc, where *Ubx* is not expressed in the proper disc epithelium, suggesting the phenotype observed in the *pbx* mutant combination is something unrelated to *Ubx*. Furthermore, we observed occasional specific compartmental interactions, where cells from one compartment surround a re-duced adjacent one, in different genetic backgrounds. This was observed not only in a *bx* mutant background, where anterior cells are undergoing apoptosis, but also, remarkably, in a wing disc from a larva with no *Ubx* mutation. We also noticed several examples of wing discs with reduced posterior compartment in Ubx^{+} larvae, which we interpret as evidence of limited regener-ation, likely stemming from a reduced number of anterior cells crossing the anterior-posterior boundary.

Finally, our data implicate a non-autonomous mechanism to regulate size after regeneration. Compartments of the wing disc grow independently of one another when they have differ-ent growth rates (Martin and Morata, 2006) or when cell division is arrested in one compartment (Weigmann *et al.,* 1997; Neufeld *et al.,* 1998), scenarios where final compartment size is gener-ally maintained. However, perturbations in the size of the posterior wing disc compartment can non-autonomously influence the size of the anterior compartment (Mesquita *et al.,* 2010). Furthermore, *bx* or *pbx* mutants induce homeotic transforma-tions that significantly enlarge one compartment of the haltere disc, and these transformations induce subtle size adjustments in the corresponding, untransformed compartment (González-Gaitán *et al.,* 1990). Our analysis of regenerating tissues in-dicates that a similar non-autonomous, inter-compartmental mechanism likely operates during restoration of injured discs. Although we did not conduct a detailed study of compartmental growth, we observed that in regenerating discs where the poste-rior compartment was significantly reduced, the size of the an-

terior one was frequently larger than that of wildtype controls. This suggests a compensatory, non-autonomous growth response mechanism to maintained disc size during regeneration.

Collectively, our findings demonstrate that regeneration is occasionally compartment-restricted, likely due to limited boundary crossing by cells from the adjacent compartment, resulting in a reduced size of the damaged one. Differences in Hox gene expression may contribute to this restriction, although such differences are not an absolute barrier to regeneration. Strangely, the *pbx* mutant combination significantly increases the frequency of this phenomenon in both haltere and wing discs. This suggests the *pbx* mutant combination includes an unspecific element which hampers normal regeneration.

Material and Methods

Drosophila Stocks

The *Drosophila melanogaster* wildtype strain used is Vallecas. *bx*^{3}*, pbx*^{1} and *TM2, Ubx*^{130} (Lewis, 1963) are mutations in the *Ubx* gene; TRE-DsRed is a reporter of JNK activity (Chatterjee and Bohmann, 2012) and *BRV-B-GFP* is an enhancer of *wingless* that also responds to JNK activity (Harris *et al.,* 2016); the Gal4/UAS system (Brand and Perrimon, 1993) was used with the following Gal4 strains: *cad*-Gal4^{459} (Calleja *et al.,* 1996)*, hh*-Gal4 (Tanimoto *et al.,* 2000), *ci*-Gal4 (Croker *et al.,* 2006), *tsh*-Gal4 (Calleja *et al.,* 1996), *sd*-Gal4 (Calleja *et al.,* 1996), and the following UAS strains: UAS-*GFP* (Ito *et al.,* 1997) (BDSC 6874), UAS-*lacZ* (Brand and Perrimon, 1993), UAS-*hid* (Igaki *et al.,* 2000), UAS-*rpr* (Igaki *et al.,* 2000), UAS-*y*$^{+}$ (Calleja *et al.,* 1996) and UAS-*flp* (BDSC 4539 and BDSC 4540). The *CyO, Tb* balancer has the *Tubby* (*Tb*) dominant marker inserted on the second chromosome (Lattao *et al.,* 2011), and was used, as the *TM6B, Tb* balancer on the third chromosome, to identify mutant larvae for disc dissection. Temporal control of gene activity in the Gal4/UAS system was achieved with *tub*-Gal80^{ts} strains (McGuire *et al.,* 2003) (BDSC 7017 and BDSC 7019). Larvae were normally shifted from 18ºC to 30ºC during second or early third larval instar.

Flip-out clones

Cell lineage clones were generated using the flip-out technique and the following stocks: *act>stop>lacZ* (Struhl and Basler, 1993), *ubi*>stop>*GFP*^{nls} (BDSC32250, BDSC32251) and *act>y*$^{+}$>Gal4 (Ito *et al.,* 1997) (BDSC 3953). Clones were inducing by setting the larvae in a water bath 10 min at 37ºC after 4-5 days of development at 18ºC.

Analysis of adult cuticles

Flies were dissected and macerated in a 10% KOH solution at 100ºC to remove the internal structures. The cuticles were mounted in glycerol, adding a small amount of Tween-20.

Regeneration experiments

For regeneration studies in the analia we followed this protocol: after 24h egg laying at 18ºC, larvae were left growing for 5 or 6 days at 18ºC, then shifted to 30ºC for 30h or 40h (for experiments expressing *hid*) or 4h 30min (for experiments expressing *rpr*) and shifted back to 18ºC for recovery during 2-4 days for disc dissection, or until adult eclosion. For studies in haltere and wing discs, females were laying eggs for 24 h at 18ºC, larvae were left growing for 6 days at 18ºC, then shifted to 30ºC for 30h and shifted back to 18ºC for 2-4 days for disc dissection.

Dissection and antibody staining

Larvae were dissected in cold PBS and fixation was carried out in 4% PFA for 30 min. Dissection, fixation and staining of imaginal discs were done according to standard protocols (Sullivan *et al.,* 2000). The antibodies used were mouse anti-Ubx 1:10 (White and Wilcox, 1984), rabbit anti-ßgalactosidase 1:2000 (Cappel), mouse anti-ßgalactosidase 1:100 (Sigma), mouse anti-Abd-B 1:125 (DSHB) (Celniker *et al.,* 1989), rat anti-Ci 1:200 (DSHB 2A1) (Motzny and Holmgreen, 1995), rabbit anti-Cad 1:200 (McDonald and Struhl, 1986), guinea-pig anti-Cad 1:200 (Kosman *et al.,* 1988) and rabbit anti-Dcp1 1:200 (Cell Signaling, antibody #9578). Secondary antibodies were fluorescently labeled secondary antibodies (Jackson Immunoresearch) used at 1:200 dilution. DAPI (MERCK) and TO-PRO-3 (Invitrogen) were used in a 1:1000 dilution to label nuclei.

Image acquisition

Photographs of adult flies were taken with a Leica MZ12 stereomicroscope and a Leica DFC5000 camera, and images were acquired using Leica LAS software (3.7). Images from mounted adults were taken with a Zeiss Axiophot microscope. Confocal images were taken in the following microscopes: Zeiss LSM710, Zeiss LSM810, Zeiss LSM710 and Nikon A1R+ Eclipse Ti-E. Image treatment and analysis were performed using Image J and Adobe Photoshop and Powerpoint softwares.

Statistical analysis

To compare bristle number between *cad*-Gal4 *tub*-Gal80^{ts} UAS-*flp* UAS-*GFP act>y*$^{+}$>Gal4 and *cad*-Gal4 *tub*-Gal80^{ts} UAS-*flp* UAS-*hid act>y*$^{+}$>Gal4 males, the two-tailed non-parametric Student's *t*-test test was used, using the GraphPad Prism software. Symbol to indicate significance was *0.01<*p<0.05*.

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