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Function and regulation of *homothorax* in the wing imaginal disc of *Drosophila*

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SUMMARY

The gene *homothorax* (*hth*) is originally expressed uniformly in the wing imaginal disc but, during development, its activity is restricted to the cells that form the thorax and the hinge, where the wing blade attaches to the thorax, and eliminated in the wing pouch, which forms the wing blade. We show that *hth* repression in the wing pouch is a prerequisite for wing development; forcing *hth* expression prevents growth of the wing blade. Both the Dpp and the Wg pathways are involved in *hth* repression. Cells unable to process the Dpp (lacking *thick veins* or *Mothers against Dpp* activity) or the Wg (lacking *dishevelled* function) signal express *hth* in the wing pouch. We have identified *vestigial* (*vg*) as a Wg and Dpp response factor that is involved in *hth* control. In contrast to its repressing role in the wing pouch, *wg* upregulates *hth* expression in the hinge. We have also identified the gene *teashirt* (*tsh*) as a positive regulator of *hth* in the hinge. *tsh* plays a role specifying hinge structures, possibly in co-operation with *hth*.

Key words: Wing disc, *homothorax*, *wingless*, *decapentaplegic*, *vestigial*, *Drosophila*

INTRODUCTION

In the body of *Drosophila*, there is a clear distinction between the main body trunk and the cephalic and thoracic appendages. These are formed by the derivatives of the imaginal discs that also contribute to the body trunk (see Morata and Sanchez-Herrero, 1999, for a review). Some developmental genes appear to play a role in establishing the differences between trunk and appendage. The case best characterised so far is that of *extradenticle* (*exd*) and *homothorax* (*hth*), two related homeobox genes. *exd* acts as a cofactor of Hox function (Peifer and Wieschaus, 1990; see review by Mann, 1995), but it also has other developmental roles (Gonzalez-Crespo and Morata, 1995; Rauskolb et al., 1995). A characteristic feature of *exd* is that it is regulated at the subcellular level; in the imaginal discs, *exd* is transcribed uniformly (Flegel et al., 1993; Rauskolb et al., 1995), but the Exd protein accumulates only in the nuclei of cells that give rise to the proximal region of the discs (Mann and Abu-Shaar, 1996; Aspland and White, 1997). The nuclear transport of Exd depends on the activity of *hth*, which thus acts as a positive regulator of exd (Rieckhof et al., 1997). It is not yet clear whether *hth* has an independent role of its own. In this work, we assume *exd* and *hth* encode equivalent developmental functions.

In the leg disc, the distinction between body trunk and appendage is based genetically on the activity of the *exd/hth* genes in the proximal region and of the Hh/Wg/Dpp signalling pathway in the distal part. The growth of the appendage requires full activity of the Hh/Wg/Dpp pathway (Diaz-Benjumea et al., 1994; Gonzalez-Crespo and Morata, 1996; Gonzalez-Crespo et al., 1998), which activates the response genes *Distal-less* (*Dll*), *dachshund* (*dac*) (Lecuit and Cohen, 1997) and possibly others, which determine the characteristic development of the appendage. *Dll* in particular is a principal determinant of ventral appendage development: in absence of its function ventral appendages do not form (Cohen et al., 1989) and ectopic *Dll* expression induces leg or antennal development, depending on the local genetic context (Gorfinkel et al., 1997). The functions of *exd/hth* and of the Hh/Wg/Dpp pathway are mutually antagonistic (Abu-Shaar and Mann, 1998; Gonzalez-Crespo et al., 1998; Wu and Cohen, 1999): *exd* activity in the proximal region blocks the response of Hh/Wg/Dpp target genes such *optomotor-blind* (*omb*) and *dac*, whereas high levels of Wg and Dpp signalling in the distal region eliminate *exd* function by repressing *hth*. This repression is mediated by the activity of *Dll* and *dac* (Abu-Shaar and Mann, 1998).

From the above it can be concluded that one prerequisite for appendage development is the inactivation of the *exd/hth* genes. This allows full activity of the Hh signalling pathway, which, in turn, induces the necessary proliferation in the cells fated to form the appendage. When *exd* or *hth* function is forced in the domain of Hh signalling in the leg (Gonzalez-Crespo and Morata, 1996; Mercader et al., 1999), the appendage does not develop and only the proximal structures differentiate.

The interactions between *exd/hth* and the signalling pathways in the wing disc have not been studied. This disc forms a part of the trunk, the mesothorax and an appendage, the wing blade, which attaches to the mesothorax through a

proximal region, called the wing hinge. Functional and expression studies indicate that *exd* is expressed in the nucleus and required in the mesothorax, but is largely dispensable in the wing, except in the hinge region (Gonzalez-Crespo and Morata, 1995; Rauskolb et al., 1995). The various signalling pathways are very well characterised (Affolter et al., 1994; Neumann and Cohen, 1996) and are similar to those in the leg disc. A significant difference is that, in the wing, the Dpp and Wg signals emanate from the anteroposterior (AP) and dorsoventral (DV) compartment borders, respectively, whereas they both originate in the AP border in the leg disc (Affolter et al., 1994; Diaz-Benjumea et al., 1994; Campbell and Tomlinson, 1995). This indicates that, in the wing disc, the two signals are established independently. Other differences concern the response genes; in the wing disc, *spalt* (*sal*), *omb* and *vestigial* (*vg*) are activated by appropriate concentrations of Dpp (Lecuit et al., 1996; Nellen et al., 1996; Kim et al., 1997) and, of these, only *omb* is expressed in a thin dorsal stripe in the leg disc (Gonzalez-Crespo and Morata, 1996). Conversely, *Dll* and *dac* are activated by the Wg and Dpp signals in the leg but, in the wing, *dac* is not expressed and *Dll* is activated only by *wg* in late development.

In this report, we describe the regulatory interactions between the Dpp and Wg pathways and *hth* in the wing disc and also explore the developmental function of *hth* in the wing hinge. We show that the two signalling pathways repress *hth* activity in the wing pouch. This results in the functional elimination of the Exd protein, which accumulates in the cytoplasm. We have identified *vg*, a target gene common to both pathways (Kim et al., 1996, 1997), as one of the effectors involved in the control mechanism. In the wing hinge, Wg signalling and the gene *teashirt* (*tsh*) upregulate *hth* expression.

MATERIALS AND METHODS

Drosophila mutations

The following mutant strains were used in this work: vg^{83b27R} (Williams et al., 1991), dsh^{V26} (Perrimon and Mahowald, 1987), Mad^{B1} (Wiersdorff et al., 1996), tkv^{a12} (Nellen et al., 1996), $tsh^{12.5}$ (Fasano et al., 1991) and *Df(2L)305*, which lacks the *tsh* gene (Roder et al., 1992).

Generation of mutant clones

The clones were generated by the FRT/FLP method (Chou and Perrimon, 1992). The following stocks were used that had the FRT sequences in the appropriate locations: *FRT42* vg^{83b27R}, *FRT40* Mad^{B1}, *FRT40* tkv^{a12} and *FRT101* dsh^{V26}. Recombination was induced in larvae carrying two copies of the FRT insertion and a marker for discs (either *arm-lacZ* or Myc) in *trans* to the chromosome carrying the mutation. The FLP recombinase was provided by a 1 hour heat-shock treatment at different developmental stages depending on the experiment. The clones were visualized in discs by staining with either anti-β-galactosidase or anti-Myc antibodies. For staining of discs with anti-Myc antibody a 1 hour heat shock was given to the larvae to induce the Myc expression, followed by 1 hour recovery at 25°C, before the larvae were dissected.

Gal4/UAS experiments

The Gal4/UAS system (Brand and Perrimon, 1993) was used to induce ectopic function of the different gene products. The following Gal4 lines were used: *ap-Gal4*, *omb-Gal4* and *nub-Gal4* (Calleja et al., 1996; M. C. and G. M., unpublished data) confer the expression corresponding to the *apterous*, *optomotor-blind* and *nubbin* genes, respectively. The line *MS1096* has been described (Guillen et al., 1995); it induces expression mainly restricted to the dorsal wing compartment. The *MD735* line found in our laboratory confers expression in most of the prospective wing region. The *UAS-hth* stock was obtained from A. Salzberg (Pai et al., 1998), the *UAS-vg* is described (Kim et al., 1996) and the *UAS-tsh* was constructed in our laboratory by Sergio Gonzalez-Crespo and will be published elsewhere.

To generate marked clones of cells containing ectopic expression of any of the UAS chromosomes, we used the stock *ywFLP*f^{36}*; UAS-y abx>*f^{+}*>Gal4-lacZ*. The *abx* promoter is an *Ubx* regulatory element that confers high expression levels in the thoracic discs. Clones were heat-shock induced at 37°C at convenient times during larval development. To examine clones in the discs, these were dissected from late third instar larvae, fixed and stained as described below. The clones of cells arising from FLP-induced recombination in the FRT sequences gain Gal4 and *lacZ* activity and lose the wild-type allele of *forked*, a bristle and trichoma marker. In the discs, the clones can be scored with anti-β-gal or with antibodies that recognize the protein encoded by the corresponding *UAS (hth, vg, tsh)* gene. In the adult cuticle, the clones can be scored as they are mutant for the f^{36} allele and are also y^{+} because the Gal4 activity triggers the *UAS-y* construct present in the chromosome.

Immunostaining of discs

Wing discs were dissected in PBS and fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. They were blocked in PBS, 1% BSA, 0.3% Triton for 1 hour, incubated with the primary antibody (anti-β-gal diluted 1:2000, anti-Hth 1:2000, anti-Wg 1:10, anti-Tsh 1:200, anti-Myc 1:1000) overnight at 4°C, washed 4× in blocking buffer and incubated with the appropriate fluorescent secondary antibody for 1 hour at room temperature in the dark. They were then washed and mounted in Vectashield.

Antibodies were kindly provided by A. Salzberg and H. Sun (anti-Hth), S. Kerridge (anti-Tsh), Sean Carroll (anti-Vg) and the Hybridoma Center (anti-Wg). β-Gal antibody (rabbit) was purchased from Cappel and Myc antibody (mouse) from BAbCO. Images were taken in a laser scan Zeiss microscope and subsequently processed using Adobe Photoshop.

Preparation of adult cuticle

The adult flies were dissected in water and cut into pieces. They were subsequently treated with 10% KOH at 95°C for 3-5 minutes to digest the internal tissues, washed with water, rinsed in ethanol and mounted in Euparal. The preparations were photographed on a Nikon AFK-II microscope using Fujichrome 64T film.

RESULTS

Wild-type *hth* expression in the wing imaginal disc

We have analysed in detail *hth* expression in the wing disc using a polyclonal antibody (Pai et al., 1998). Protein distribution in second and early third instar discs is uniform in most or all the disc (Fig. 1A,B). In late third instar, *hth* expression is modulated in the thoracic as well as in the appendage part. There is no expression in the central part of the disc, which corresponds to the wing appendage, but the Hth protein accumulates in two concentric peripheral rings, in the region that corresponds to the wing hinge (Fig. 1C).

The domain of *hth* expression has been mapped by double-label experiments in relation to the domains of *wg*, *nubbin* (*nub*) and *vg* (Fig. 1D-F). *hth* and *wg* are co-expressed in the two rings, whereas *hth* and *vg* are expressed in near

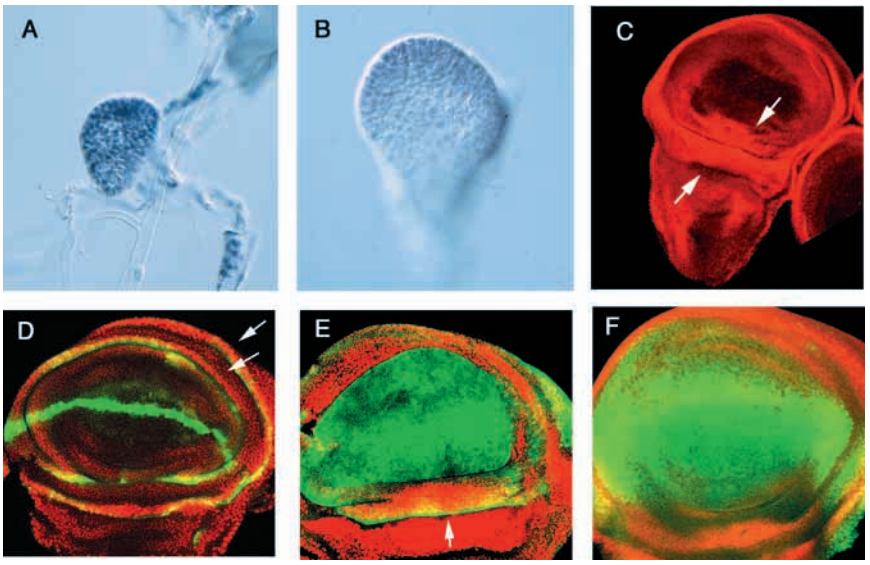

Fig. 1. Domains of expression of *homothorax*. (A) Second instar wing imaginal disc stained with anti-Hth antibody showing a general and uniform *hth* expression. (B) Early third instar wing disc stained as the one in A beginning to show loss of expression in the central region of the disc. (C) Late third instar disc. In the region corresponding to the wing blade, *hth* is expressed in two ring domains (arrows). The central part of the disc contains no expression. (D) Double staining for *hth* (red) and *wg* (green) in the region of the disc corresponding to the wing blade. Note that the two genes are co-expressed in the two concentric rings, the prospective wing hinge region (arrows). (E) Double staining for *hth* (red) and *nub* (green). *nub* is expressed in the wing pouch and extends to the inner ring of *hth* expression (arrow). (F) Double staining for *hth* (red) and *vg* (green) in a late third instar disc. The expression domains are largely complementary but overlap a little in the sides of the disc.

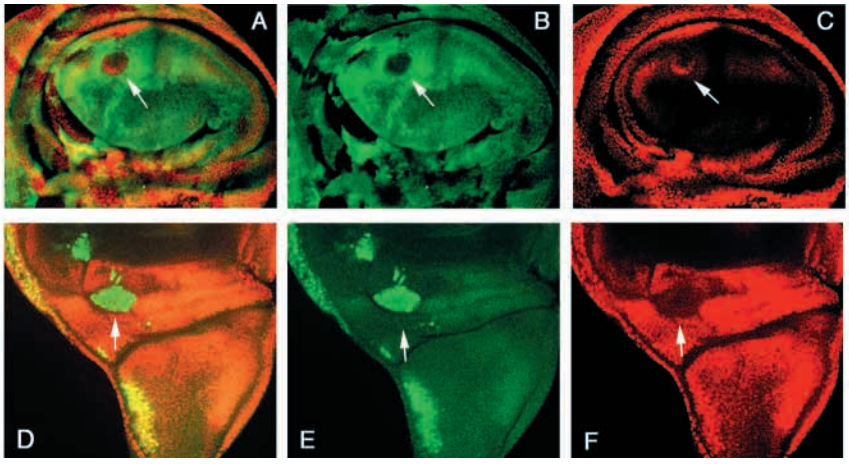

Fig. 2. *hth* repression by *vg*. (A-C) A *vg* mutant clone (arrows) in the wing pouch showing *hth* (red) expression in some of the cells. The clone is marked by the absence of arm-lacZ expression (green). (D-F) A clone (arrows) of *vg*-expressing cells (green) represses *hth* (red) expression in the wing hinge.

complementary domains that only overlap in the anterior and posterior sides of the disc. The expression of *nub* covers the wing pouch but also extends to the inner ring of *hth* and *wg* expression. The rings of *wg* and *hth* expression may be used to discriminate between wing hinge and wing pouch; hereafter we will refer to prospective wing pouch as the region that does not express *hth*, and prospective hinge as the region with *hth* (and *wg*) expression.

Negative regulation of *hth* in the wing pouch

(1) *vg* represses *hth* expression

The *vg* gene is a key element in the development of the wing pouch (Williams et al., 1991). Mutant flies lacking *vg* activity show loss of most of the wing, but the hinge region remains (Williams et al., 1991; Lindsley and Zimm, 1992). It is specifically expressed in the wing pouch cells (Williams et al., 1993) (Fig. 1F) and its ectopic expression in other imaginal discs induces wing tissue (Kim et al., 1996). Because of the near reciprocity of the *vg* and *hth/exd* domains and the parallel with *Dll* function in the leg (Abu-Shaar and Mann, 1998; Gonzalez-Crespo et al., 1998), we tested the possibility that *vg* is involved in the repression of *hth* in the wing pouch.

We induced clones of cells mutant for the vg^{83b27R} (see Methods for details) and examined *hth* expression. In the wing pouch, these clones are infrequent and round, suggesting that they sort out from the surrounding wild-type cells. However, those that can be detected show ectopic *hth* expression (Fig. 2A-C). We note that, although all the clones examined show some ectopic *hth* activity, it does not affect all the cells. This observation suggests that, although *vg* is involved in *hth* repression in the wing pouch, there probably are other genes implicated.

To analyse further the ability of *vg* to repress *hth*, we generated clones of *vg*-expressing cells. As expected, these clones have no effect in the wing pouch but, in the hinge, they repress *hth* activity (Fig. 2D-F). In contrast to the loss-of-function clones, all the cells expressing *vg* show repression of *hth*.

(2) The activities of the Dpp and Wg signalling pathways repress *hth*

The preceding experiment indicates that *vg* is part of the mechanism controlling *hth* expression in the wing. The transcription of *vg* in the wing is under the control of two

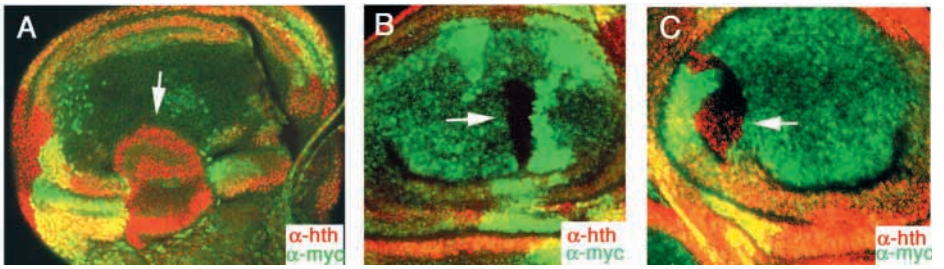

Fig. 3. *hth* repression by the Dpp and Wg signalling systems. (A) Late third instar wing disc with a *tkv* mutant clone (arrow) in the wing pouch showing ectopic *hth* (red) expression. The clone is marked by the absence of Myc expression (green). (B) A *dsh* mutant clone (arrow) located close to the AP border, marked by the absence of Myc expression. Ectopic *hth* (red) expression is not detected in the clone. (C) A *dsh* mutant clone (arrow) located far from the AP border. The clone is marked by the absence of Myc expression (green) and shows ectopic *hth* expression (red) in some cells.

different enhancers, the boundary and the quadrant enhancers (Kim et al., 1996). The activity of the boundary enhancer depends on Notch signalling arising from the DV axis (Kim et al., 1996; Klein and Martinez-Arias, 1999); Notch induces *wg*, whose product diffuses from the border and activates *Dll* and *vg* in third instar wing discs (Diaz-Benjumea and Cohen, 1995; Kim et al., 1996). The quadrant enhancer is responsible for *vg* expression in the wing pouch away from the DV border and is activated by Dpp (Lecuit et al., 1996; Kim et al., 1997). Thus both the Dpp and the Wg signals activate *vg* and are likely to be involved in the control of *hth* expression in the wing pouch.

The role of Dpp was tested by inducing clones of cells that were either mutant for *thick veins* (*tkv*) or for *Mothers against Dpp* (*Mad*). In the first case, the cells are unable to respond to the Dpp gradient and, in the second, do not transduce the Dpp signal (Newfeld et al., 1996; Wiersdorff et al., 1996). Both experiments gave the same result: either *tkv* or *Mad* mutant cells showed ectopic *hth* expression in the wing pouch (Fig. 3). A significant difference with *vg* mutant clones is that, in *tkv* and *Mad* clones, all the cells show *hth* derepression, whereas *vg* mutant clones only activate *hth* in some cells of the clone. We searched for *tkv* mutant clones in the adult cuticle as they were marked with *yellow*, but the great majority of them fail to develop in the distal and medial wing blade. Sometimes round vesicles of invaginated tissue, which are possibly formed by mutant cells, are present. In contrast, *tkv* clones are readily found in the Costa and alula region, where their differentiation is normal (data not shown; Burke and Basler, 1996), suggesting that the Dpp gradient does not pattern this region.

The involvement of Wg signalling was tested by inducing clones of cells mutant for *dishevelled* (*dsh*), one of the intracellular transducers of the pathway. The loss of *dsh* activity affects *hth* expression differently, depending on their position with respect to the AP axis. As observed in Fig. 3C, *hth* is ectopically activated only in dsh^- clones localised far from the AP border, but inside the prospective wing pouch region. dsh^- cells at the AP axis, or close to it, do not show *hth* expression (Fig. 3B).

Taken together, these observations suggest that the Dpp and Wg signalling pathways repress *hth* in the prospective wing pouch, probably by activating *vg* and maybe other response gene(s).

Positive regulation of *hth* in the wing hinge

As described above (Fig. 1), *hth* expression is uniform in the early wing disc, and it is also known (Rieckhof et al., 1997) that it is uniformly expressed at high levels in the thoracic segments of the late embryo. Thus it is likely that *hth* expression in the hinge is not activated de novo but inherited from embryonic cells. Nevertheless, we have studied the role of some of the genes co-expressed with *hth* in the maintenance and regulation of the high expression levels observed in late third instar discs. In particular, we considered *wg* and *tsh*. The expression of *wg* in the wing hinge may be regulated differently from the expression in the DV border and is likely to perform a different developmental role. The zinc-finger gene *tsh* is co-expressed with *hth* in the outer ring (Fig. 4A-C) and it is also known that, in the leg disc, *tsh* is entirely coincident with nuclear *exd* expression (Gonzalez-Crespo and Morata, 1996), suggesting it may have some functional interaction with *hth*.

The role of *wg* was studied as in the previous experiments by inducing clones of cells mutant for dsh^{v26} allele. The mutant clones generated in the prospective hinge region show reduction, but not elimination, of *hth* expression (Fig. 4D-F). This observation suggests that *wg* is necessary for the high levels of *hth* expression in the hinge, but is not the only factor necessary for *hth* activity.

We have also examined the possible role of *tsh* in regulating *hth*. The effect of the loss of *tsh* function was not studied because the necessary *FRT* tsh^- recombinant chromosome could not be constructed (due to the very proximal location of *tsh*). The effect of ectopic expression was studied using the Gal4/UAS method (Brand and Perrimon, 1993). Driving *tsh* activity in the wing disc with the *ap-Gal4*, *MS1096*, *omb-Gal4* and *nub-Gal4* lines (Calleja et al., 1996; Guillen et al., 1995) results in ectopic *hth* expression in the pouch (Fig. 4J-L), and a reinforcement of its expression in the wing hinge (Fig. 4J-L, and data not shown). We also induced marked clones of *tsh*-expressing cells. These clones tend to be round suggesting that they sort out from surrounding cells; those in the wing hinge are of normal size considering the time of induction and show increased levels of *hth* expression (Fig. 4G-I). In the wing pouch, they are more heterogenous; those near the DV border are small and show no *hth* activity, whereas those closer to the hinge are larger and have ectopic *hth* expression.

Developmental effects of ectopic expression of *hth* and of *tsh*

Both *hth* and *tsh* are strongly expressed at high levels in the wing hinge region but their respective developmental roles are not clarified. There is good evidence for the requirement for *hth/exd* function in the hinge; clones of *hth or exd* mutant cells develop aberrant structures in the hinge and produce outgrowths (Gonzalez-Crespo and Morata, 1995; Casares and Mann, 2000). In contrast, *tsh* mutant phenotype in the wing is

not known, although its strong expression in the hinge suggests a function. To study the developmental roles of *hth* and *tsh*, we have induced their ectopic activity in the wing pouch. A number of Gal4 lines were used to force expression in various regions of the wing (Brand and Perrimon, 1993). In other experiments, marked clones of *hth*-expressing and of *tsh*-expressing cells were induced (see Methods).

The principal result concerning ectopic *hth* expression is that it prevents growth; in lines conferring high and overall *hth* activity (*nub-Gal4/UAS-hth*, *1096/UAS-hth* or *C765/UAS-hth* genotypes), the wing pouch does not develop, but proximal structures like sclerites or costa are normal (Fig. 5). In lines inducing locally restricted expression, the effect on growth is mainly limited to the region containing Gal4 activity. For example, the line *omb-Gal4*, confers Gal4 activity only to the central region of the wing pouch. In *omb-Gal4/UAS-hth* flies, the central region of the wing pouch grows very little and differentiates only proximal pattern elements (Fig. 5D). In contrast, the anteriormost and posteriormost wing regions are much less affected.

In addition, some of these genotypes present an excess of proximal pattern elements (also reported in Mercader et al., 1999), probably at the expense of the more distal ones, suggesting that the presence of the Hth product is influencing the cellular response to the proximodistal signals. For example, in the genotype *MS1096/UAS-hth* the number of alula (a proximal posterior wing structure) bristles is 29.5 (n=14), which is higher than in the wild type (23.5, n=10). We have studied this "proximalizing" effect in some detail using the Gal4 line C735, that confers a general expression in most or all the wing pouch and whose activity responds readily to temperature changes between 18°C and 29°C. The phenotype of *C735/UAS-hth* wings shows a distal-proximal gradation depending on the temperature; weak *hth* activity (at low temperature) only affects the distal wing, but stronger activity affects the distal and medial regions (Fig. 5F-H). In the strongest phenotypes only proximal wing elements remain.

Few clones of *hth*-expressing cells can be recovered in the differentiated cuticle, the majority of them probably sort out and are eliminated. Many of those that can be scored are in the proximal wing. In the margin they are associated with loss of marginal elements (Fig. 5I,J), while those located closer to the AP border are associated with the loss of large portions of the middle of wing blade (Fig. 5K), suggesting an effect on growth in the proximodistal axis. A few cases were found near the medial DV wing margin; they only differentiate wing trichomes, but appear to induce local duplications of wing margin elements (Fig. 5L, see below).

Since both the effects on growth and on the proximodistal pattern may be due to an interference of the Hth product with the production or diffusion of the morphogenetic signals Wg and Dpp, we examined *wg* and *dpp* expressions in the genotypes above. The overall result is that *wg* expression is altered but that *dpp* expression is not. As shown in Fig. 6A-C, *ap-Gal4/UAS-hth* wing discs show loss of *wg* expression along the DV border. This is accompanied by an enhancement of *wg* expression in the inner ring in the hinge. This distinct effect of Hth on *wg* expression in the hinge and in the wing

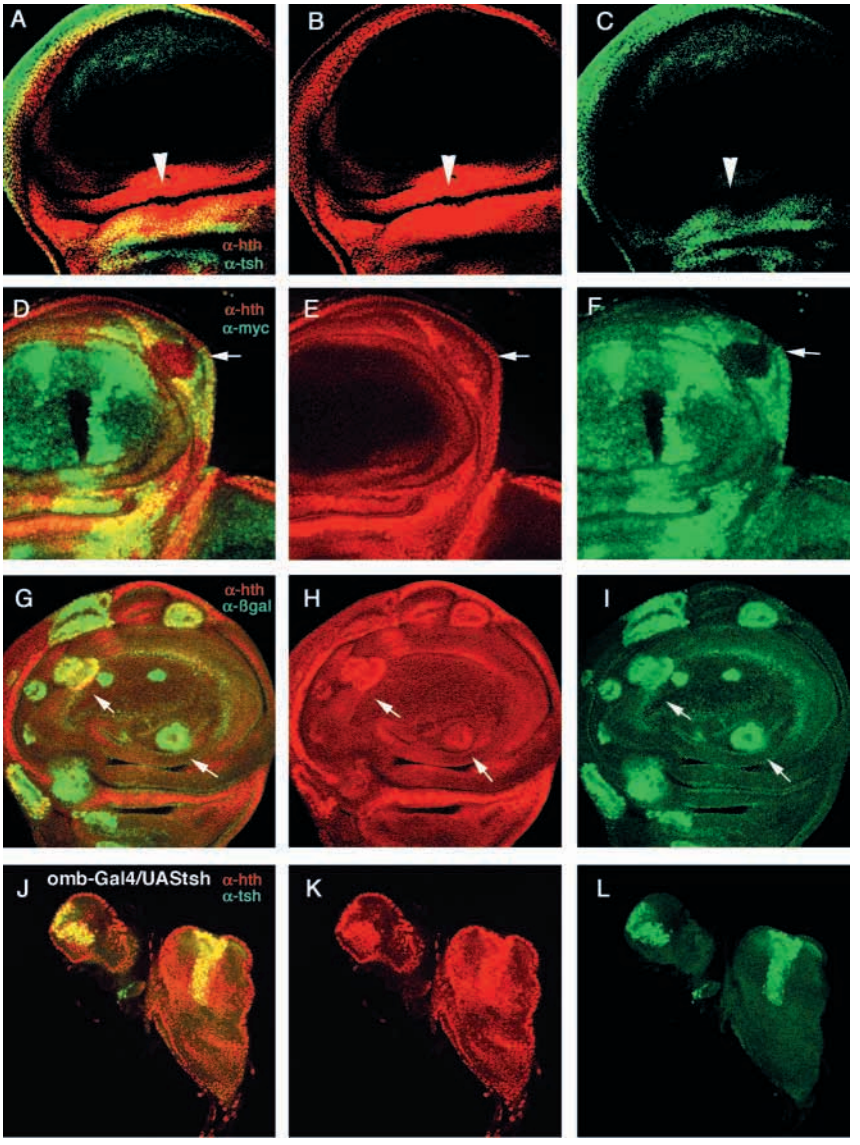

Fig. 4. *tsh* and the Wg signalling positively regulate *hth* in the wing hinge. (A-C) Wild-type third instar wing disc showing co-expression of *tsh* (green) and *hth* (red) in the outer ring (appears as yellow in the merged A). (D-F) *dsh* mutant clone (arrow) in the wing hinge showing decreased levels of *hth* expression (red). The clone is marked by the absence of Myc expression (green). (G-I) Clones of *tsh*-expressing cells (arrow) in the wing disc marked by the presence of β-gal expression (green). Increased levels of *hth* expression (red) in the wing hinge. Clones in the wing pouch near the DV border show no *hth* expression (red), whereas those close to the hinge ectopically activate *hth*. (J-L). Third instar haltere and wing discs of genotype *omb-Gal4/UAS-tsh*, stained for *tsh* (green) and *hth* (red). Driving *tsh* expression in the omb domain of the wing pouch ectopically activates *hth* in the same domain. Note that both discs are reduced in size.

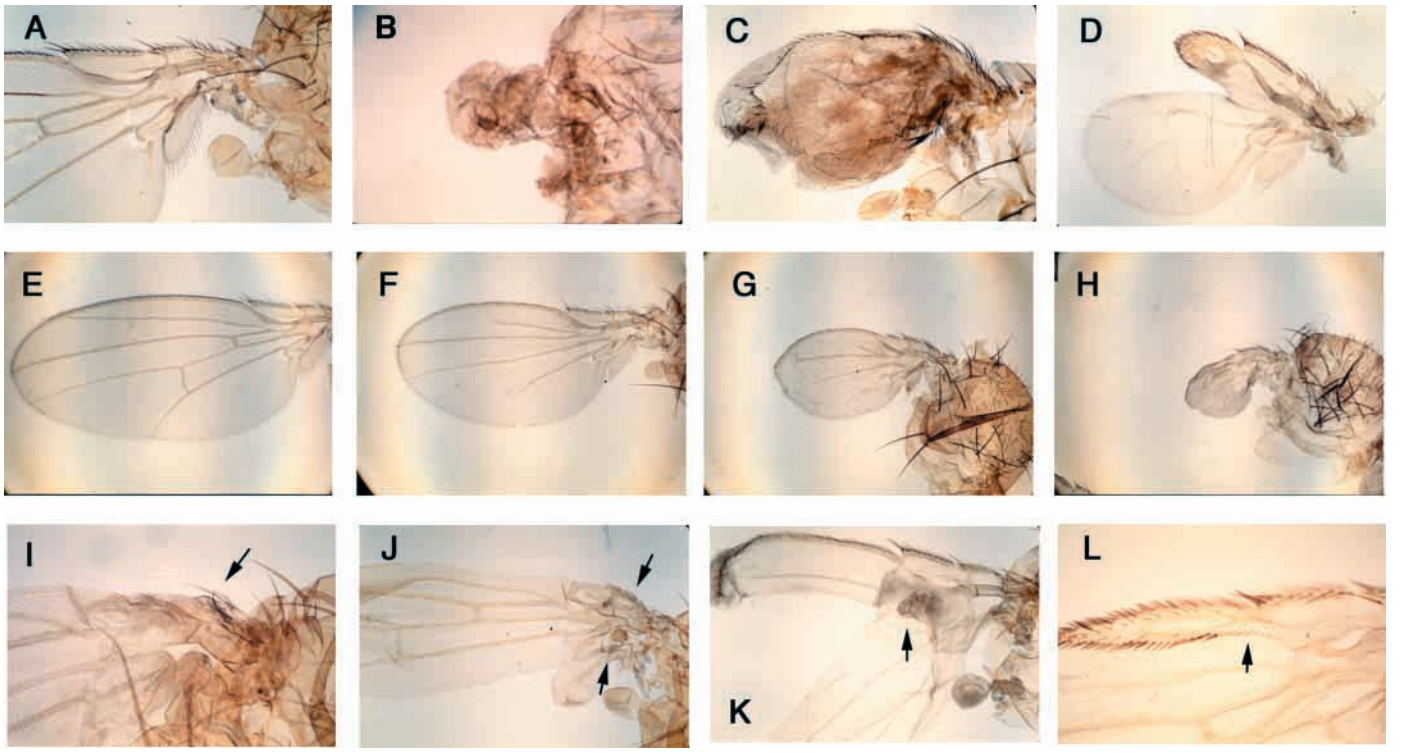

Fig. 5. Effects of *hth* expression in the adult wing. (A) Wild-type wing showing the normal costa and hinge structures. (B) Wing rudiment of an *ap-Gal4/UAS-hth* fly. The high and uniform expression of *ap* in the dorsal wing compartment prevents growth of the wing. (C) Wing of the genotype *MS1096/UAS-hth*. The wing is reduced in size predominantly in the distal region, but no reduction is seen in the proximal region. (D) Wing of genotype *omb-Gal4/UAS-hth* showing lack of the distal medial region. The deleted part corresponds largely to the area of expression of *omb*, which includes the central and distal part of the wing blade. (E-H) A series of wings of genotype *C765/UAS-hth* grown at 18° (E), 22° (F) 25° (G) and 29°C (H) to illustrate the response to different levels of *hth* activity. Note the gradual "proximalization" of the wing; the effect of *hth* is stronger in the distal than in the proximal structures. (I-K) Three cases of *hth*-expressing clones (marked with y^+ and f^{36a}). The clones are localized (arrows) in the proximal regions but appear to reduce or eliminate growth of the distal part of the wing. (L) A small *hth*-expressing clone that appears to induce the formation of a duplication of the wing margin in the vicinity.

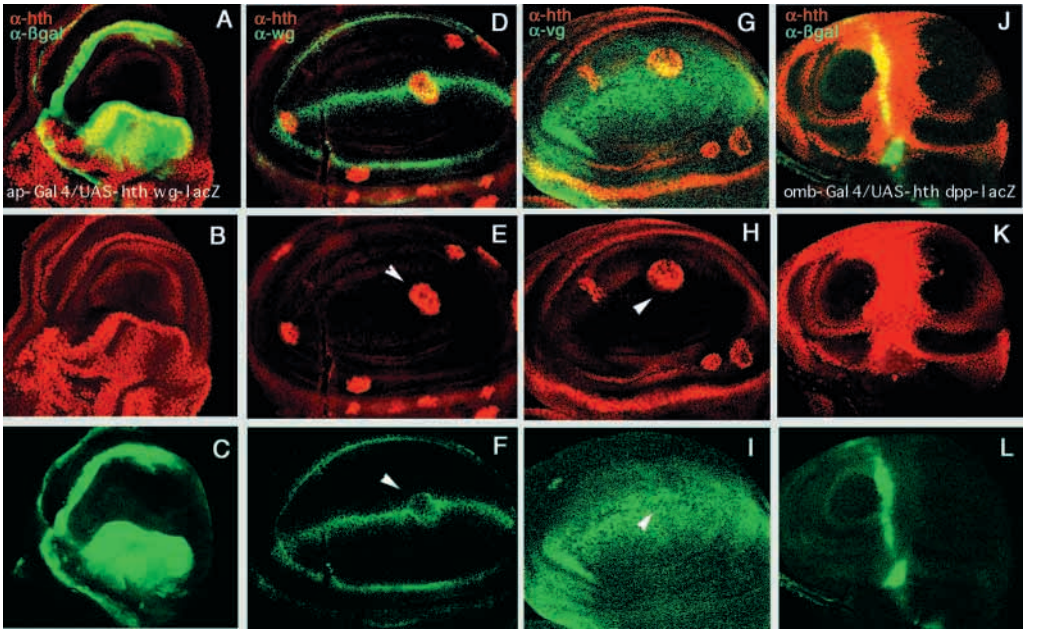

Fig. 6. Effect of ectopic expression of *hth* on *wg* and *dpp*. (A-C) Third instar wing disc of the genotype *ap-Gal4/UAS-hth wg-lacZ* stained for *hth* (red) and β-gal (green). Ectopic expression of *hth* in the wing pouch represses *wg* expression in the DV border. In the hinge, gain-of-function *hth* reinforces *wg* expression in the inner ring, but not in the outer one. (D-F) A clone (arrowhead) of *hth*-expressing cells (red) in the wing pouch represses local *wg* expression (green) and induces ectopic *wg* activity adjacent cells. (G,H) The arrowhead points to a clone of *hth*-expressing cells (red) in the wing pouch. This clone exhibits normal *vg* expression (green). (J-L) Third instar wing disc of the genotype *omb-Gal4/UAS-hth; dpp-lacZ* stained for *hth* (red) and β-gal (green). Ectopic expression of *hth* in the omb domain does not alter *dpp* expression in the AP border.

pouch illustrates the different regulation of *wg* in the two domains.

Clones of *hth*-expressing cells (see Methods) also have a similar effect on *wg* activity. As shown in Fig. 6D-F, *hth*-expressing clones in the DV border repress local *wg* expression, but, surprisingly, they cause ectopic *wg* activity in cells located in the vicinity. This is consistent with the observation above (Fig. 5L) that *hth*-expressing cells are associated with wing margin duplications. Despite the effect on *wg*, *hth*-expressing clones do not repress *vg* (Fig. 6G-I).

The effect on *dpp* expression was studied in the Gal4 lines described above and is illustrated in the genotype *omb-Gal4/UAS-hth* (Fig. 6J-L). Wing discs of this genotype show normal *dpp* expression. This result was anticipated as, in *omb-Gal4/UAS-hth* flies, the anterior and posterior patterns are near normal (Fig. 5D) and this requires normal *dpp* activity. The lack of effect on *dpp* provides an explanation for the lack of effect of ectopic *hth* on *vg* expression, as the latter is also activated by Dpp signalling (Kim et al., 1997).

Ectopic expression of *tsh* strongly modifies the adult wing pattern. In genotypes *ap-Gal4/UAS-tsh* or *1096/UAS-tsh*, the dorsal wing compartment differentiates hinge structures (Fig. 7A,B, data not shown), especially sclerites and proximal costal elements, that derive from a region of the disc with high levels of *hth* and *tsh*. In wing discs of these genotypes, the expression of *wg* in the DV border disappears, although it is likely due to the activation of *hth* described above (data not shown). The behaviour of clones of *tsh*-expressing cells also point to a similar conclusion; although most of the clones fail to differentiate properly, we frequently observe vesicles of differentiated tissue bearing the genetic markers of the clone. In favorable cases (Fig. 7C), the pattern differentiated by these clones can be identified as proximal hinge structures.

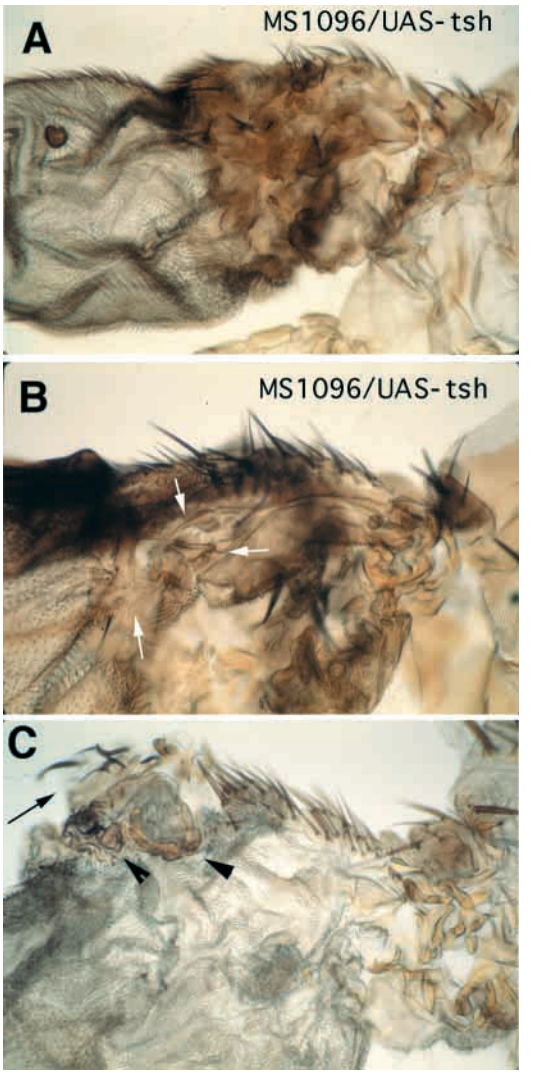

Fig. 7. Effects of the ectopic expression of *tsh* in the wing disc. (A) Wing of the genotype *MS1096/UAS-tsh.* The dorsal wing compartment differentiates a large number of hinge structures. (B) Higher magnification of a wing of the same genotype as in A. The arrows point toward sclerites (typical hinge structures) and some proximal pattern elements that appear in the dorsal compartment of the wing. (C) Wing with a y^+ f^{36a} clone (arrow) of *tsh*-expressing cells. The arrowheads indicate the presence of sclerites associated with the clone.

DISCUSSION

Negative regulation of *homothorax* in the wing pouch

In the second instar wing disc, the Hth product accumulates uniformly in the thoracic and appendage regions of the disc (Fig. 1A), but throughout the third larval period *hth* expression is downregulated and, by the late third instar, Hth only appears in the presumptive regions of the thorax and the wing hinge. The central part of the disc, which gives rise to the wing pouch, shows no *hth* expression (Fig. 1C). The repression of *hth* function is important for wing development since, if *hth* activity is forced in the wing pouch, the wing does not form (Fig. 5B). A similar observation has been made in the leg disc; *hth* or *exd* expression in the distal part results in truncated appendage in which all the distal components are missing (Gonzalez-Crespo and Morata, 1996). In the leg, the subdivision between distal and proximal regions results from the antagonism between Hh signalling and *exd/hth* function (Gonzalez-Crespo et al., 1998). Hh response genes such as *Dll* and *dac* are instrumental in repressing *hth* (Abu-Shaar and Mann, 1998; Wu and Cohen, 1999).

Our results indicate that the downregulation of *hth* in the wing pouch is a consequence of the activity of the Dpp and the Wg signaling pathways. Cells in which the response to the Dpp signal is prevented, as in *tkv* or *Mad* mutant cells, *hth* is expressed at high levels (Fig. 3A). Similarly, *dsh*$^-$ cells, in which the transduction of Wg is blocked, show ectopic *hth* activity and consequently nuclear *exd* expression. These results also indicate that *hth* is latently active in the wing cells and has to be repressed by the continuous activity of the Dpp and Wg signals. The inability of cell clones, in which the Dpp or the Wg pathways have been totally eliminated, to proliferate may be due to high levels of *hth* expression. Our results also indicate that the Dpp and Wg pathways repress *hth* expression independently. This is illustrated by the experiments inducing *dsh* mutant clones: ectopic *hth* expression is only observed in clones located away from the AP border. Our interpretation is that the high levels of Dpp expression near the AP border are sufficient to impede *hth* expression despite the removal of the control by Wg.

We have identified *vg* as one of the factors involved in the downregulation of *hth:* the elimination of *vg* activity in the wing pouch (Fig. 2) results in *hth* activation and its ectopic expression in the hinge region represses the normal activity of *hth*. As a principal role of *vg* is to specify wing development (Williams et al., 1991), it appears that a component of this function is to eliminate *hth* activity and therefore *exd* function. As *vg* is a target gene of both the Hh and the Wg pathways in the wing, it seems that the downregulation of *hth* by both pathways is mediated by *vg*. One question that is not fully understood about the role of *vg* is that, although it is able to repress *hth*, there is some *vg* activity normally in the wing hinge that coincides with that of *hth*. The levels of the Vg protein appear to be similar in the pouch and the hinge regions so that different levels of product do not seem to be a likely reason. We believe that there may be other factors in the hinge; *tsh* is a likely candidate that counteracts the repression by Vg.

Although *vg* clearly has a regulatory role, we note that, in contrast with the observed in *tkv* and *Mad* clones, not all the cells lacking *vg* activity in the wing pouch express *hth*, suggesting that there probably are other response genes involved in the repression of *hth*. We have not checked other Dpp response genes, such as *omb* or *spalt* (*sal*) (Nellen et al., 1996; Lecuit et a., 1996) that may mediate this control. *Dll* is a Wg target gene in the wing blade but, unlike in the leg disc (Abu-Shaar and Mann, 1998), *Dll* mutant clones do not affect *hth* expression (R. S. Mann, personal communication).

Maintenance of *hth* expression in the wing disc

The negative regulation of *hth* discussed above is a modification of the original uniform expression found in the early wing disc. As the wing disc derives from the second thoracic embryonic segment, which shows high and uniform levels of *hth* expression (Rieckhof et al., 1997), the initial levels of *hth* in the thoracic region and the wing hinge are likely to be inherited from the progenitor cells. The mechanism of *hth* activation during embryogenesis is not known, although its expression is modulated by the activity of the BX-C genes (Azpiazu and Morata, 1998).

Our results suggest that, at least during the larval period, *hth* expression in the wing disc is positively regulated by *tsh*. This is based on two findings. (1) An increase of Tsh levels in the hth domain results in increased levels of Hth product; possibly, one of the normal functions of *tsh* in the wing hinge is to maintain high *hth* levels. (2) Ectopic *tsh* expression in the wing pouch causes ectopic *hth* activity, at least in the clones located close to the hinge (or far from the DV border) and in the Gal4 lines. The fact that clones located close to the DV border are not able to induce *hth* activity, whereas the Gal4 lines do, could be due to a timing effect. However, our results also indicate that *tsh* is not the only factor involved: *hth* and *tsh* are normally co-expressed only in the proximal ring of *hth* expression, therefore there should be other factor(s) maintaining *hth* expression in the distal ring. Since *dsh* mutant clones show a reduction in *hth* expression in the hinge, a *wg* response gene is likely to be involved. In addition, the fact that *tsh* cannot activate *hth* near the DV border in the wing pouch may suggest that other factors are required.

The developmental role of *hth* in the wing disc

The main role of *hth* is to regulate *exd* function (Riekhof et al., 1997). The loss of *hth* activity during adult patterning results in changes in segmental identity and morphogenetic alterations that appear to be similar or identical to those produced by eliminating *exd* (Gonzalez-Crespo and Morata, 1995; Rauskolb et al., 1995). Thus *hth* and *exd* can be considered to perform the same developmental function.

In the wing disc, *hth* and *exd* are only required in the wing hinge region and, in their absence, the cells proliferate but form aberrant patterns (Gonzalez-Crespo and Morata, 1995; Casares and Mann, 2000) indicating that *hth/exd* function is involved in specifying the wing hinge region. The experiments inducing ectopic *hth* expression suggest that it has a role in controlling growth, for *hth* is able to prevent the formation of the wing pouch. It is also consistent with the observation (Casares and Mann, 2000) that *hth* mutant clones in the hinge may reach very large size. The finding that *hth* suppresses *wg* activity in the DV border may be related with the repression of growth, a process with which *wg* has been shown to be involved (Zecca et al., 1996). The lack of effect of *hth* on *dpp* expression emphasizes the independence of the AP wing axis from *hth/exd* function.

One aspect that we do not fully understand is the effect of *hth* on the proximodistal pattern, which has also been observed on the leg disc and on the chicken limb (Mercader et al., 1999). In our experiments, the presence of the Hth product influences the reading of proximodistal signals by the cells towards differentiating more proximal patterns. We do not know which factors are responsible for the proximodistal pattern in the wing, but as *hth* prevents *wg* response to Notch, it is possible that a Wg response element or some other Notch response gene may be involved in patterning.

It is also not clear what is the role of *hth* in the specification of the wing hinge, where it is expressed at high levels. Its ectopic expression in the pouch does not produce any specific transformation towards hinge structures, but rather a general proximalization of the whole pattern. This is in contrast with the effect of ectopic *tsh* that induces sclerites and very proximal hinge structures. Since *tsh* activates *hth* in the hinge (Fig. 4G-I), it suggests that the formation of proximal hinge requires the activity of the two gene products.

Altogether, the results presented here suggest the subdivision of the non-thoracic part of the wing disc into two major domains: the wing hinge, where *hth* is expressed and Exd is functional (nuclear), and the wing pouch where *hth* is not expressed, Exd is cytoplasmic and therefore inactive. By homology with the leg disc (Gonzalez-Crespo and Morata, 1996; Gonzalez-Crespo et al., 1998), the latter would be the genuine appendage part of the disc. These two regions are formed by two antagonistic genetic systems: in the hinge, the high levels of *hth*, inherited from the embryo and probably maintained by *wg*, *tsh* and may be other regulators, prevent *wg* response to Notch signalling, which is necessary for the development of the wing pouch. In the wing pouch, the activities of the Wg and Dpp pathways suppress *hth* so that Notch may induce *wg* activity and the appendage is formed (see also Casares and Mann, 2000).

In addition to its role in preventing excessive proliferation, *hth* may also contribute, together with *tsh*, *wg* and *nub*, to the partition of the wing hinge into two regions that correspond to the outer and inner rings of *hth* expression. The outer ring domain expresses *tsh*, *wg* and *hth*, has nuclear Exd and does not express *vg* and *nub*. The inner domain expresses *wg*, *nub*

and *hth*, has nuclear Exd and does not express *tsh*. The individual role of these genes is not yet established, but it is possible that they function in some combinatorial manner.

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