Differing strategies for the establishment and maintenance of 
teshirt and homothorax repression in the Drosophila wing

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Summary

Secreted signaling molecules such as Wingless (Wg) and Decapentaplegic (Dpp) organize positional information along the proximodistal (PD) axis of the Drosophila wing imaginal disc. Responding cells activate different downstream targets depending on the combination and level of these signals and other factors present at the time of signal transduction. Two such factors, teshirt (tsh) and homothorax (hth), are initially co-expressed throughout the entire wing disc, but are later repressed in distal cells, permitting the subsequent elaboration of distal fates. Control of tsh and hth repression is, therefore, crucial for wing development, and plays a role in shaping and sizing the adult appendage. Although both Wg and Dpp participate in this control, their specific contributions remain unclear. In this report, we analyze tsh and hth regulation in the wing disc, and show that Wg and Dpp act independently as the primary signals for the repression of tsh and hth, respectively. In cells that receive low levels of Dpp, hth repression also requires Vestigial (Vg). Furthermore, although Dpp is required continuously for hth repression throughout development, Wg is only required for the initiation of tsh repression. Instead, the maintenance of tsh repression requires Polycomb group (PcG) mediated gene silencing, which is dispensable for hth repression. Thus, despite their overall similar expression patterns, tsh and hth repression in the wing disc is controlled by two very different mechanisms.

Key words: Drosophila, Wing, Teashirt, Homothorax, Wingless, Decapentaplegic, Polycomb

Introduction

In the Drosophila wing imaginal disc, different domains along the proximodistal (PD) axis are present as concentric regions, each with a unique genetic address. Accordingly, distal wing blade, medial hinge and proximal notum map to discrete territories in the mature wing disc (Fig. 1). The initial subdivision of the wing disc into prospective wing and notum territories depends on the activities of the Wingless (Wg) and the Epidermal Growth Factor Receptor (Egfr) signaling pathways, respectively (reviewed by Klein, 2001). wg expression in the ventro-anterior region of the second instar wing disc represses the expression of the Egfr ligand Vein, which is necessary for notum specification (Wang et al., 2000; Zecca and Struhl, 2002a; Zecca and Struhl, 2002b). Consequently, loss of wg in ventral cells results in a complete loss of wing tissue and the duplication of proximal structures (Couso et al., 1993; Morata and Lawrence, 1977; Ng et al., 1996; Sharma and Chopra, 1976). Decapentaplegic (Dpp), secreted from cells next to the anteroposterior (AP) compartment boundary, also plays a role in PD subdivision, in part by restricting expression of the Iroquois complex (Iro-C) genes to the prospective notum (Cavodeassi et al., 2002). Although both Wg and Dpp are necessary to restrict proximal fates, it is not known if these pathways function synergistically or independently. Here, we address this question and provide novel insights into how these signals initiate and maintain unique identities along the PD axis of the wing disc.

The formation of the PD axis has been studied extensively in the leg, where Wg and Dpp act combinatorially to distalize cells in a concentration-dependent manner (Campbell et al., 1993; Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997; Wilder and Perriman, 1995). One of the earliest manifestations of this process is the restriction of the Meis-family homeobox gene homothorax (hth), and the zinc-finger transcription factor teashirt (tsh), to the most proximal regions of the leg disc (Abu-Shaar and Mann, 1998; Wu and Cohen, 1999; Wu and Cohen, 2000). In the wing disc, both hth and tsh are initially expressed in every cell, but, as in the leg disc, are later repressed in distal cells (Azpiazu and Morata, 2000; Casares and Mann, 2000; Wu and Cohen, 2002). Moreover, ectopic expression experiments demonstrate that hth and tsh activities are incompatible with wing blade development (Azpiazu and Morata, 2000; Casares and Mann, 2000). Thus, given these similarities, it is plausible that in the wing disc, as in the leg, Dpp and Wg act combinatorially to repress hth and tsh in distal cells, allowing for the growth and specification of the appendage.

A number of observations support the idea that both wg and dpp act together to induce distal fates in the wing disc. wg mutant discs fail to repress tsh distally, whereas ectopic activation of the wg pathway represses tsh proximally (Azpiazu
and Morata, 2000; Wu and Cohen, 2002). Wg and Dpp signal transduction also appear to be necessary for complete hth repression in the pouch (Azpiazu and Morata, 2000). However, the relationship between Dpp signaling and tsh regulation remains unclear. Ectopic activation of the Dpp pathway in early-induced clones only represses tsh in the most lateral regions of the disc (Wu and Cohen, 2002), and hypomorphic dpp mutant larvae still show some tsh repression in the distal wing disc (Cavodeassi et al., 2002). The combinatorial model of Wg and Dpp function in the wing is also weakened by the observation that wg activity is only required for hth repression in a subset of the wing pouch (Azpiazu and Morata, 2000).

Differences in the timing of tsh and hth repression also indicate that the two genes are regulated by distinct mechanisms in the wing disc. tsh is repressed in distal wing disc cells in the early second larval instar, coincident with the ventro-anterior wedge of wg expression (Wu and Cohen, 2002). By contrast, hth repression begins shortly after tsh repression. Furthermore, Notch-dependent activation of wg at the DV compartment boundary (Diaz-Benjumea and Cohen, 1995; Neumann and Cohen, 1996) is not required for tsh repression (Wu and Cohen, 2002), raising the question of what maintains tsh repression as wing development progresses. Notch also activates the gene vestigial (vg), an essential factor for wing blade growth and patterning (Halder et al., 1998; Kim et al., 1996; Simmonds et al., 1998; Williams et al., 1991; Williams et al., 1994). However, vg is neither necessary nor sufficient for tsh repression in the wing disc (Wu and Cohen, 2002). By contrast, the loss of vg activity causes hth de-repression in a subset of distal clones. Because vg is activated by Dpp and Wg (Kim et al., 1997; Kim et al., 1996; Neumann and Cohen, 1997; Zecca et al., 1996), these results have been interpreted to suggest that vg may be responsible for Dpp- and Wg-mediated repression of hth (Azpiazu and Morata, 2000; Wu and Cohen, 2002). However, the endogenous vg and hth expression domains overlap considerably outside of the wing pouch, suggesting a more complex relationship between these two factors.

To better understand how PD domains in the wing disc are established and maintained, we executed a genetic screen to identify genes involved in these processes. Two of the genes identified by this screen, which we focus on in this report, were Medea (Med), a downstream component of the Dpp pathway, and Sut(z)12, a member of the Polycomb group (PcG) of genes. The identification of these genes prompted us to examine the role of the Dpp, Wg and PcG pathways in the formation of the PD axis of the wing disc. We demonstrate that Dpp signal transduction is necessary for hth, but not tsh, repression in the distal cells of the wing disc throughout larval development. Wg signal transduction is also dispensable for the maintenance of tsh repression. We also elucidate the distinct temporal and spatial requirements of vg as a mediator of hth repression. Finally, we show that PcG-mediated gene silencing maintains the separation between wing and body through tsh, but not hth, repression, accounting for the inability of distal cells to express tsh, even when both the Dpp and Wg signaling pathways are compromised.

Materials and methods
Mutagenesis/mapping
Isogenized yw males with third chromosome FRTs were fed 10 mM ethyl methanesulfonate (EMS) and mated to yw; vgBE-Gal4 UAS-flp; FRT y+ virgin females. The F1 progeny were scored for defects in wing morphology and backcrossed to test for the heritability of the phenotypes. Stable mutant lines were established using balancer stocks. Mutations were mapped by meiotic recombination and complementation tests. The aerodrome (adro) and daedalusian (daed) alleles were named after pre-Wright Brothers attempts at heavier-than-air flight.

Medadro is homozygous larval lethal, and lethal in trans to the strong hypomorph Medf3 (Hudson et al., 1998) and to Df(3R)il-e. The transheterozygous phenotype is slightly more severe than the homozygous phenotype, indicating that Medadro is not a null allele. In clones, Medadro and Medf3 phenotypes are indistinguishable.

Sut(z)12med was mapped by its failure to complement Df(3L)Keto2 and the strong Sut(z)12 allele (Kehle et al., 1998). Hox gene de-repression in Sut(z)12med clones closely resembles the effect reported for strong Sut(z)12 hypomorphic alleles (Birve et al., 2001).

Flies stocks
Mutant alleles: arr2 (Tearle and Nusslein-Volhard, 1987) (see FlyBase); brk8H (Campbell and Tomlinson, 1999); Madk (Wiersdorff et al., 1996); Peg109 (Franke et al., 1995); vg8B27R (Williams et al., 1991); k8o (Tearle and Nusslein-Volhard, 1987) (see FlyBase).

Gal4 drivers: vgBE-Gal4 (Simmonds et al., 1995); act>hs-CD2>Gal4 (Pignoni and Zipursky, 1997).

UAS lines: UAS-GFP; UAS-brk (Lammel et al., 2000); UAS-DTCfDN (van de Wetering et al., 1997).

Mutant clones
vgBE-Gal4, UAS-flp (vgBE::flp) clones:

yw; vgBE-Gal4, UAS-flp; FRT82B Medadro/FRT82B hs-CD2 y+ (FRT82B ubiquitinGFP and FRT82B hs-CD2 y+ M(3)w24 chromosomes were substituted as noted in the results section); yw; vgBE-Gal4, UAS-flp; Sut(z)12med FRT80>hs-GFP y+ FRT80; and

yw; vgBE-Gal4, UAS-flp; P-X109 FRT2A/ubiquitinGFP y+ FRT2A.

Heat shock clones:
yw hs-flp; FRT82B Medadro/FRT82B hs-CD2 y+ M(3)w24, yw hs-flp; P-X109 FRT2A/ubiquitinGFP y+ FRT2A; yw hs-flp; FRT42 vg8B27R/FRT42 arm-lacZ M(2)IK; vgQE-lacZ, yw hs-flp; FRT42 arr2/FRT42 hs-GFP y+; and

brk8H FRT101/hs-GFP, hs-flp FRT101.

Larvae were heat shocked for 45 minutes to an hour at 37°C. The developmental stage of clone induction for each experiment is indicated in the results section.

Misexpression
yw hs-flp; act>hs-CD2>Gal4, UAS-GFP was used to drive expression of UAS-DTCfDN in clones. Prior to heat shock, larvae were grown at 22°C. Second instar larvae were heat shocked for 30 minutes at 35°C, then transferred to 25°C until dissection.

yw hs-flp; act>hs-CD2>Gal4, UAS-GFP was used to drive expression of UAS-brk in clones. Prior to heat shock, larvae were grown at 22°C. Late first instar larvae were heat shocked for 30 minutes at 37°C, then transferred to 25°C until dissection.

Larvae of the genotype yw hs-flp tub>Gal4, UAS-GFP; UAS-DTCfDN; FRT82B Med1/FRT82B tub>Gal80 hs-CD2 y+ M(3)w24 were heat shocked for 45 minutes to an hour at 37°C during the second instar. Perdurance of Gal80 prevented strong expression of UAS-GFP until 12 to 24 hours after clone induction.

Larvae of the genotype yw hs-flp tub>Gal4, UAS-GFP; UAS-Nr1 wg; P-X109 FRT2A tub>Gal80 hs-CD2 FRT2A were heat shocked for one hour at 37°C during the early second instar.

Immunostaining
Antibodies: mouse anti-β-galactosidase (Promega); mouse anti-rat CD2 (Serotec); mouse anti-Dll (Cohen et al., 1993); guinea pig anti-
Repression of proximal fates by Wg, Dpp and Pc

Results

By the late third larval instar stage, the wing imaginal disc is subdivided into a series of concentric domains, corresponding to distinct fates along the PD axis, wing pouch, hinge and notum (Fig. 1A,B). For reasons that will become clear below, we distinguish between the distal hinge (DH), which is continuous with the blade, and the proximal hinge (PH), which is required for wing flapping (Fig. 1B). In the imaginal disc, both hinge regions express a ring of \( w_g \), separated by a deep epithelial fold. We also make a distinction between the lateral hinge, which is far from the source of Dpp, and hinge cells that receive high levels of Dpp input because they are close to the AP compartment boundary (Fig. 1A,B). Initially, all cells of the wing disc express both \( t_s_h \) and \( h_t_h \). By the third instar, \( h_t_h \) is repressed in the pouch, but remains expressed in the DH and PH, where it is activated by Wg (Azpiazu and Morata, 2000; Casares and Mann, 2000; Rodriguez, 2004) (Fig. 1C,D). \( t_s_h \) is absent in the DH, and in the subset of PH cells expressing high levels of \( w_g \) and \( h_t_h \), but remains expressed in the more proximal cells of the PH (Casares and Mann, 2000; Wu and Cohen, 2002) (Fig. 1C,E). Thus, by the third instar, both \( t_s_h \) and \( h_t_h \) are completely absent from the wing pouch, but the distal limit of their expression domains are distinct (Fig. 1C-E).

To identify factors necessary for the establishment and maintenance of these PD domains, we performed an F1 genetic screen (see Materials and methods). This screen used a wing-specific source of Gal4 to drive expression of the FLP recombinase, generating clones by the FRT-FLP system (Golic, 1991; Xu and Rubin, 1993). We selected the vestigial boundary enhancer-Gal4, UAS-flp (vgBE::flp) combination for its robust wing expression and its ability to drive clones along the entire PD axis. Although primarily active at the dorsoventral (DV) margin under the control of the Notch pathway (Kim et al., 1996), the \( vgBE \) is transiently active throughout the wing disc (Vegh and Basler, 2003). This property allowed us to screen adult wings for PD defects caused by large mutant clones.

One mutation identified by this screen, dubbed aerodrome (adro), profoundly affected the growth and patterning of the wing, and mapped to the Medea (Med) gene (see Materials and methods). Medea, a homolog of vertebrate Smad4, functions downstream of Dpp as a DNA-binding partner for Mothers against Dpp (Mad) (Hudson et al., 1998; Wisotzkey et al., 1998). Med<sup>adro</sup> clones driven by \( vgBE::flp \) caused a non-autonomous reduction of wing size and growth along the PD axis (Fig. 2A), evident in both the blade and the hinge. Wing blade clones tended to sort out from the surrounding wild-type cells, forming vesicles (Fig. 2B). Clones located close to the DV boundary sometimes induced non-autonomous duplications of margin structures (Fig. 2C). We also observed cell autonomous differentiation of lateral hinge elements in Med<sup>adro</sup> clones near the AP boundary of the hinge (Fig. 2D).

Dpp is necessary for repression of \( h_t_h \) but not \( t_s_h \)

Med<sup>adro</sup> (Fig. 2A-D) and Med<sup>13</sup> (a strong hypomorphic Medea allele; data not shown) clones exhibit adult phenotypes similar to those produced by ectopic \( h_t_h \) expression (Azpiazu and Morata, 2000; Casares and Mann, 2000). Because other Dpp pathway members have been implicated in \( h_t_h \) repression (Azpiazu and Morata, 2000), we induced Med<sup>adro</sup> clones in a Minute background and stained for both Hth and Tsh in wing discs. Large Med<sup>adro</sup> clones in the wing pouch induced by \( vgBE::flp \) or heat-shock-flp (HS:flp) showed strong autonomous \( h_t_h \) expression, but no \( t_s_h \) expression (Fig. 2E-H).

To discriminate between a requirement for Med in the initiation versus the maintenance of \( h_t_h \) repression, we examined HS:flp-induced clones generated in the mid-third instar, after \( h_t_h \) repression in the pouch has occurred (Casares and Mann, 2000; Wu and Cohen, 2002). Med<sup>adro</sup> clones induced during the mid-third instar also de-repressed \( h_t_h \) throughout the wing pouch (Fig. 2I-K). Like Med<sup>adro</sup> clones, clones of two other Dpp pathway components, thickveins (tkv), which encodes a type I Dpp receptor (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Ruberte et al., 1995), and Mad, also de-repressed \( h_t_h \), but not \( t_s_h \) (data not shown). Thus, Dpp signaling is necessary to maintain \( h_t_h \), but not \( t_s_h \), repression, at least until mid-third instar stage.

Although the above results demonstrate that, by the late second instar, Dpp signaling is not required to repress \( t_s_h \) in the pouch, early-induced clones expressing a constitutively active form of Tkv (Tkv*) have been reported to repress \( t_s_h \) in some contexts (Wu and Cohen, 1996), the \( t_s_h \) gene (from Michalis Averof); rabbit anti-Tsh (from SK Chan); rabbit anti-Vg (Williams et al., 1991); mouse anti-Wg (4D4; (Neumann and Cohen, 1997) (Iowa University Hybridoma bank). Fluorescent secondary antibodies (FITC, Cy3, Texas Red and Cy5) were from Jackson Laboratories. All imaginal discs were analyzed with a Bio-Rad 1024 confocal system.

Fig. 1. Subdivision of the proximodistal (PD) axis in the wing disc. (A) Cartoon of a third instar wing imaginal disc, showing wing pouch (P), distal hinge (DH), proximal hinge (PH), lateral hinge (L) and notum (N). Anterior is up and dorsal is to the left in all figures. (B) Adult wild-type wing showing PD subdivisions. Labels are the same as in A, except for the wing blade (WB), which fates maps to the pouch. (C–E) Late third instar wing disc stained for Tsh (red) and Hth (blue). Neither is expressed in the pouch. Hth extends throughout the entire hinge, while Tsh is restricted to the PH. A deep fold in the epithelium (yellow arrowhead in C) marks the separation between DH and PH.
2002). It was therefore possible that Dpp might play an early role during the establishment of tsh repression in the second instar. However, according to another report, a strong dpp hypomorphic combination is not sufficient to de-repress tsh in the distal wing disc (Cavodeassi et al., 2002). We addressed the requirement for Dpp signal transduction during the establishment of tsh repression by compromising the Dpp pathway in the early wing disc. First, we induced flip-out clones expressing brinker (brk) in late first instar larvae. brk encodes a repressor of Dpp target genes and is normally expressed in the lateral regions of the wing imaginal disc, as its transcription is itself negatively regulated by Dpp (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Jazwinska et al., 1999b; Minami et al., 1999). Most ectopic Brk-expressing cells in the pouch are eliminated from the epithelium soon after clone induction. However, we obtained a small number of Brk-expressing clones in the distal regions of early and mid-third instar wing discs. Such clones de-repressed hth, but had no effect on the expression of tsh or the distal marker, nubbin (nub) (Ng et al., 1995) (Fig. 2L,M). In addition, ectopic expression of brk using the Dpp-Gal4 driver line, which is active before tsh is initially repressed, also results in hth, but not tsh, de-repression (data not shown).

As a second test for an early role of Dpp in tsh repression, we induced Medadro clones by heat shock during the first larval instar in a Minute background. Even in these early-induced clones, tsh expression was absent from the distal-most portion of these almost entirely mutant discs (Fig. 2P-W). By contrast, hth was expressed in the mutant cells (Fig. 2P-R). As expected, these discs are much smaller than wild type, and resemble those obtained from dpp and dpp larvae, which also maintain some tsh repression in distal cells (Cavodeassi et al., 2002).

Together, these experiments strongly suggest that the Dpp pathway is not required for the establishment or maintenance of tsh repression. By contrast, Dpp signaling is required to both establish and maintain hth repression throughout wing disc development.

**Temporal and spatial requirements for vg repression of hth**

As a downstream target of Dpp, vg is a good candidate to mediate the Dpp-dependent repression of hth. Two previous results suggest that vg plays a role in hth repression in the pouch: (1) some vg loss-of-function clones in the pouch ectopically express hth; and (2) ectopic vg expression in the hinge represses hth (Azpiazu and Morata, 2000). However, the latter observation is complicated by the fact that vg and hth are normally co-expressed in the wild-type lateral hinge (Fig. 3A-C). To better assess the requirement for vg in hth repression, we used the Minute technique to recover multiple vg mutant clones, which tend to survive poorly. Unlike Med mutant clones, which de-repress hth at all positions along the AP axis of the pouch, vg mutant clones, induced in early third instar larvae, only de-repressed hth in pouch cells far from the AP compartment boundary (Fig. 3D-F). The failure of vg mutant clones to de-repress hth near the AP boundary – the source of

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Fig. 2. Repression of hth, but not tsh, requires Dpp signaling. (A-D) Adult wings containing multiple Medadro clones induced in a Minute background by vgBE::flp. Mutant clones are marked by y- tissue, and denoted by black arrows. (A) Medadro clones result in reduced growth of the wing blade and hinge along the PD axis. (B) A mutant Medadro clone sorts out from surrounding wild-type tissue in the wing blade. (C) A mutant Medadro clone differentiates wing margin bristles and non-autonomously induces wing margin elements in adjacent wild-type cells. (D) Medadro clones located near the anteroposterior (AP) compartment boundary of the hinge differentiate bristles characteristic of the lateral hinge. (E-U) Immunostains of wing discs; Hth staining is in blue, Tsh is in red, and clones are marked by the absence of CD2 in green. (E-H) Late third instar wing disc containing Medadro M+ clones induced by vgBE::flp. (F-H) Magnification of the region denoted by the white box in E. A large mutant clone in the wing pouch ectopically expresses hth, but not tsh. (I-K) Late third instar wing disc containing Medadro M+ clones induced by heat shock in the mid-third instar. (J,K) Magnification of the region denoted by the white box in I. The yellow arrowheads point to ectopic hth expression in small wing pouch clones. (L) Mid third instar wing disc containing flip-out clones of brk marked by GFP, and stained for Hth and Tsh. The magnification shows a clone that fails to repress hth but has no effect on tsh expression. (M) Mid third instar wing disc containing flip-out clones of brk marked by GFP, and stained for Nubbin (Nub) and Tsh. The magnification shows a clone that has no effect on either tsh or nub expression. (N-Q) Late third instar wing disc containing Medadro M+ clones induced by heat shock in the first instar. tsh repression is maintained in distal cells, which strongly express hth. (R-U) Late third instar wing disc containing Medadro M+ clones induced by heat shock in the first instar. The distal limit of tsh repression coincides with the proximal limit of Dil (violet) expression.
secreted Dpp — suggests that vg is not necessary for hth repression in cells that receive high levels of this signal.

The limited requirement of vg for hth repression only applies to clones induced in third instar larvae. Clones induced earlier, in second instar larvae, caused a dramatic reorganization of cell fate in the distal wing, including the ectopic expression of hth and the induction of epithelial folds that are reminiscent of the hinge (Fig. 3G-I). Thus, although vg is largely dispensable for hth repression in the third instar wing pouch, earlier in development it is required for the distinction between pouch (hth-non-expressing) and hinge (hth-expressing) fates.

The absence of Dpp signaling permits co-expression of vg and hth

To further characterize the relationship between Dpp, vg and hth, we examined their expression in Medadro clones. In Medadro clones near the DV boundary of the pouch, we observed strong hth de-repression but no effect on vg expression, leading to the co-expression of these two transcription factors (Fig. 4A-D). The inability of Vg to repress hth in the absence of Dpp signaling might provide an explanation for the co-expression of these two factors in the wild-type lateral hinge. In this region, low Dpp signaling leads to high levels of brk, which represses Dpp target genes. We tested whether brk expression is required for hth expression in the lateral hinge by inducing brk loss-of-function clones in second instar larvae. Lateral distal hinge (DH) brk– clones expressed vg but not hth, and tended to grow larger than their wild-type twin spots (Fig. 4E-H). This result complements our observation of vg and hth co-expression in Medadro clones (Fig. 4A-D), and suggests that Brk represses an as yet unidentified repressor of hth (see Discussion).

Altogether, our results indicate a complex relationship between Dpp signaling and the expression of hth and vg. Where Dpp activity is absent or very low (as in the lateral hinge or in Med mutant clones), vg and hth are co-expressed, suggesting that in the absence of Dpp signaling Vg is not able to repress hth. In cells where Dpp activity is moderate (as in the lateral wing pouch), Vg is required for hth repression. However, Vg is dispensable for hth repression near the AP boundary of the pouch, where Dpp activity is highest. We note, however, that this formulation does not apply to cells of the proximal hinge (PH), where reducing Dpp signaling results in vg expression (data not shown). As tsh is expressed in the PH, but not the DH or pouch, it may in part be responsible for the distinct responses to Dpp signaling in these regions.

Wg is not required for the maintenance of tsh repression

Previous results suggested that Wg signaling is necessary for both hth repression and the early establishment of tsh repression in the wing pouch. Clones mutant for the Wg signal transducer dishevelled (dsh) ectopically express hth when located far from the AP compartment boundary (Azpiazu and Morata, 2000), and transheterozygotes of a disc-specific regulatory allele and a null allele of wg fail to repress tsh in the wing pouch (Wu and Cohen, 2002). The DV stripe of wg expression, however, is dispensable for tsh repression (Wu and Cohen, 2002). To demonstrate directly that Wg signal

Fig. 3. Repression of hth near the AP boundary does not require vg. (A-C) Wild-type third instar wing imaginal disc, stained for Hth (blue) and Vg (red). (A) White arrows point to the lateral hinge, where hth and vg are co-expressed at high levels. (D-I) Clones are labeled by the loss of Vg protein (red), and the loss of vgOE-lacZ and arm-lacZ expression (green). (D-F) Late third instar wing disc containing multiple vg– M+ clones, induced in the early third instar. Hth (blue) is not de-repressed in wing pouch clones near the AP boundary (a subset are outlined in white), but clones in the lateral wing pouch express Hth (yellow arrowheads). (G-I) Late third instar wing disc containing a large vg– M+ clone (white outline) induced in the second instar. The clone expresses two rings of Hth (blue), separated by a large ectopic fold.

Fig. 4. Antagonism between Vg and Hth requires Dpp signaling. In all panels, Hth staining is in blue, Vg is in red, and clones are marked by the absence of GFP or CD2 in green. (A-D) vgBE::fpr-induced Medadro M+ clone in the wing pouch. (B-D) Magnification of the region denoted by the white box in A. hth is de-repressed even though vg is still highly expressed in the clone (white outline). (E-H) brk– clones, induced by heat shock during the second instar. The yellow arrowhead points to a lateral DH clone, which loses hth expression and maintains vg expression. The white dotted line marks the epithelial fold between the DH and PH.
transduction is not necessary for the maintenance of tsh repression in the pouch, we eliminated the ability of pouch cells to respond to Wg, by generating clones of cells mutant for the Wg co-receptor arrow (arr) (Wehrli et al., 2000). arr mutant clones located in the pouch far from the AP boundary, expressed hth, but never expressed tsh (Fig. 5A-C). Similarly, pouch clones expressing dTCF<sup>DN</sup>, a dominant-negative form of the Wg pathway transcription factor (van de Wetering et al., 1997), also expressed hth, but not tsh (Fig. 5D-F). Thus, although Wg signal transduction is required for the initiation of tsh repression, it is not required to maintain tsh repression in the wing pouch. We also note that the subset of pouch cells that requires Wg signaling to maintain hth repression is the same subset that requires Vg for hth repression. Because Vg is a target of Wg, these observations suggest that the requirement for Wg to maintain hth repression in the lateral wing pouch may be mediated through Vg.

In the second instar, hth and tsh expression patterns are coincident in the hinge (Casares and Mann, 2000; Wu and Cohen, 2002). tsh is subsequently repressed in the DH and the Wg-expressing cells of the PH, whereas hth is upregulated by Wg in these same domains (Casares and Mann, 2000; Rodriguez, 2004; Wu and Cohen, 2002). In contrast to clones in the wing pouch, we observed ectopic tsh expression in both arr and dTCF<sup>DN</sup> clones that were located in the hinge (Fig. 5A-C,D,F). These clones were induced in the second instar, prior to the repression of tsh in this domain, indicating that Wg signaling is required for tsh repression in the hinge that normally occurs during the third instar.

**Dpp and Wg are not redundantly required to repress tsh**

Based on the Dpp and Wg pathway loss-of-function experiments described above, as well as the previous analysis of Wg mutant discs (Wu and Cohen, 2002), we suggest that Wg signaling establishes tsh repression in the second instar wing pouch, but that neither signaling pathway is necessary to maintain this repression. One possibility not addressed by our previous experiments is that Dpp and Wg are able to redundantly repress tsh expression. We tested this question by making clones that are compromised for both signaling pathways. We used the MARCM system (Lee and Luo, 2001) to ectopically express dTCF<sup>DN</sup> in Med<sub>13</sub> clones. The experiment was performed in a Minute background to ameliorate the severe growth disadvantage of these clones. We included a UAS-GFP transgene to mark these clones and also monitored Dll (a target of Wg signaling) expression to confirm that Wg signaling was compromised (Neumann and Cohen, 1996; Zecca et al., 1996). By the late third instar, tub>Gal4; UAS-dTCF<sup>DN</sup>; Med<sub>13</sub> clones strongly expressed GFP and hth, but showed no tsh expression (Fig. G-I). We also observed the loss of Dll expression in these clones (Fig. 5J). Because the morphology of the disc is severely disrupted by these doubly mutant clones, we were concerned that the mutant cells could be derived from the peripodial membrane. To rule this out, we examined hth and tsh in mutant peripodial cells, and found no change in the endogenous expression levels of both genes (Fig. 5K). Thus, tsh remains repressed in the main epithelium even when the activities of both long-range signaling pathways of the wing pouch are simultaneously compromised.

**PcG genes are required to maintain repression of tsh in the wing pouch**

If neither Dpp nor Wg are required for the maintenance of tsh repression, what, then, performs this essential function? In the course of our F1 screen, we identified a mutation, dubbed daedalian (daed), that caused non-autonomous reduction of wing blade and hinge size along the PD axis (Fig. 6A), and mapped to the Suppressor of zest 12 (Su(z)12) gene. Su(z)12 is a PcG member, required to maintain the heritable silencing of Hox genes throughout development (Birve et al., 2001). Su(z)12<sup>daed</sup> clones tended to sort out from the surrounding wild-type tissue, frequently forming vesicles (Fig. 6B). Some wing blade clones cell autonomously differentiated bristles characteristic of proximal hinge and notum (Fig. 6C). When examined in the wing imaginal disc, we found that Su(z)12<sup>daed</sup>
clones induced by vgBE::flp weakly expressed hth in some of the mutant pouch cells (Fig. 6D,E). However, the same clones ectopically expressed high levels of tsh throughout the mutant tissue (Fig. 6D,F). Thus, in contrast to Wg and Dpp pathway mutations, Su(z)12 daed clones de-repressed tsh more readily than hth.

More than a dozen PcG genes have been characterized in Drosophila (reviewed by Brock and van Lohuizen, 2001; Mahmoudi and Verrijzer, 2001; Pirrotta, 1997). To determine whether the effect on tsh expression is specific to Su(z)12 or is a more general characteristic of PcG members, we examined clones mutant for a null allele of Polycomb (Pc). Like Su(z)12 daed, Pc mutant clones induced by vgBE::flp strongly de-repressed tsh, but not hth (Fig. 6G-I). The Su(z)12 daed phenotypes in the adult wing and the ectopic tsh expression in the wing pouch suggest that the mutant tissue has some characteristics typical of more proximal fates. Consistent with this, we found that Su(z)12 daed clones downregulated the expression of the distal wing markers Dll (Fig. 6J,K) and nub (Fig. 6L,M).

**Temporal and spatial regulation of tsh in PcG mutants**

The extent and timing of loss of PcG-mediated silencing depends on both the PcG member and the particular target gene being examined (Beuchle et al., 2001). To confirm that PcG mutant clones are defective in the maintenance of tsh repression, and to better assess the kinetics of tsh de-repression, we induced Pc– clones by heat shock at different time-points during larval development (Fig. 7A).

When larvae were heat shocked 84-96 hours after egg laying (AEL) and dissected in the late third instar, Tsh was rarely observed in Pc– pouch clones, but was ectopically expressed in some DH clones (Fig. 7B,C). However, ectopic tsh expression was widespread, in Pc– clones induced 72-84 hours AEL. Although not at endogenous hinge levels, tsh was expressed throughout the resulting pouch clones (Fig. 7D,E). The levels of tsh expression peaked in Pc– clones induced 48-60 hours AEL (Fig. 7F,G). There was no discernable increase in Tsh levels in Pc– clones induced during the first instar (Fig. 7H,I). All of the clones induced 72 hours AEL or later were generated after the initiation of tsh repression in the second instar. We conclude that the presence of Tsh in Pc and Su(z)12 mutant clones results from a failure to maintain, rather than a failure to establish, tsh repression.

The spatial pattern of tsh de-repression in PcG mutant clones differs significantly from the pattern for other reported PcG targets. Ubx, and other Hox genes, are de-repressed first in the prospective wing pouch, and later in the hinge and notum (Beuchle et al., 2001). By contrast, late induced Pc– clones only expressed tsh along the periphery of the wing pouch (Fig. 7B,C). Earlier induced clones expressed tsh in more distal regions (Fig. 7D-I). However, even in large clones induced during the first or second instar, tsh expression was not observed close to the DV boundary or in most of the hinge (Fig. 7H,I). As both of these regions of the disc express Wg, this pattern points to the possibility that tsh expression remains sensitive to repression by Wg signaling even in the absence of PcG function. To test this, we used the MARCM system to express the membrane tethered, non-diffusible, Nrt-Wg (Zecca et al., 1996) in Pc– cells. We found that Nrt-Wg strongly repressed the ectopic tsh resulting from loss of Pc function (Fig. 7J-M), indicating that Wg retains the ability to repress tsh in the absence of PcG-mediated silencing.

**Discussion**

This report examined two aspects of Drosophila dorsal appendage formation: (1) the role of Wg and Dpp signaling in establishing PD domains; and (2) the maintenance of these
domains during disc development. Prior to this work, much of our understanding of PD axis formation in the fly stemmed from the analysis of the leg imaginal disc, where the combined activities of Dpp and Wg induce distal fates and repress proximal fates (Abu-Shaar and Mann, 1998; Campbell et al., 1993; Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997; Wilder and Perrimon, 1995; Wu and Cohen, 1999; Wu and Cohen, 2000). This model has, by analogy, been applied to the wing imaginal disc, where high levels of Dpp and Wg overlap in the prospective distal region, marked, as in the leg, by the absence of tsh and hth expression. However, we demonstrate by loss-of-function experiments that Dpp and Wg act independently to repress these genes. Furthermore, our observation that repression of tsh, but not hth, requires PcG gene activity has several implications for the maintenance of PD domains in the wing and other appendages.

**Dpp signaling during PD axis formation in the wing**

In the course of a screen for mutations affecting the PD axis of the wing, we isolated an allele of the Drosophila Smad4 homolog Med. Like other Dpp pathway mutations, Med<sup>shi</sup> clones located in the wing pouch cell autonomously de-repress hth. This is evident even in late-induced clones, demonstrating the continuous role of Dpp signaling in shaping the wing blade/hinge subdivision during larval development. By contrast, we did not detect any de-repression of tsh resulting from any of our manipulations of the Dpp pathway.

The ability of ectopic Dpp activity to repress tsh in early-induced proximal clones was interpreted to suggest that Wg and Dpp cooperate to repress tsh in the early pouch (Wu and Cohen, 2002). However, because Dpp is dispensable for tsh repression, this model must be an over-simplification. We conclude that Wg, not Dpp, must be considered the primary repressor of tsh in the wing. The lack of synergy between the two pathways is reminiscent of the regulation of Dll, which is activated in the leg by the combined activities of Wg and Dpp, but requires only Wg for its expression in the wing pouch (Neumann and Cohen, 1996; Zecca et al., 1996). In the absence of Dpp signaling, wing pouch cells co-express hth, nub and Dll, but not tsh. This combination of factors is normally only found in the distal hinge (DH), suggesting a transformation from pouch to DH when the Dpp pathway is compromised (Azpiazu and Morata, 2000; Casares and Mann, 2000). The expression of the Iro-C genes, normally restricted to the notum, extends to the distal limit of the tsh domain in dpp mutant discs, leading to the hypothesis that Dpp signaling is essential for the separation of wing and body wall (Cavodeassi et al., 2002). However, because loss of Dpp signaling transforms wing pouch to DH, we propose an alternative view, in which Dpp further divides an already extant appendage/trunk subdivision by repression of hth in the pouch and Iro-C in the proximal hinge (PH). According to this proposal, the distal limit of tsh expression, initiated by early Wg expression and maintained by PcG silencing, denotes the boundary between the appendage and the body.

**hth repression through multiple mechanisms**

Our results suggest that repression of hth in the wing disc only occurs in cells with a history of vg expression and continuous Dpp input. Consistent with this, ectopic vg expression in the medial DH (Azpiazu and Morata, 2000) and loss of brk in the lateral DH both result in hth repression. The requirement for vg can be separated into two distinct stages. The first stage occurs in the second instar, when vg expressed at the DV compartment boundary determines which cells are competent to repress hth in response to Dpp signaling (Fig. 8B). Thus, both vg or Dpp-pathway mutant clones induced at this early stage fail to repress hth.

In the third instar, vg expression is only required for hth repression at the lateral edges of the wing pouch, whereas Dpp signaling is required at all positions along the AP axis. Accordingly, the boundary between the lateral hinge and pouch
Repression of proximal fates by Wg, Dpp and Pc

is dictated by the threshold of Dpp activity that permits the Vg-dependent repression of \textit{hth}. Wg signal transduction is also required to repress \textit{hth} in pouch cells far from the AP boundary (Azpiazu and Morata, 2000) (our results). However, the requirement for Wg signaling in this part of the wing pouch could be due to its role in \textit{vg} activation (Kim et al., 1996; Neumann and Cohen, 1997; Zecca et al., 1996). Alternatively, it is possible that Wg and Vg are independently required to repress \textit{hth} in these cells.

A model that encompasses these observations is that Vg and Dpp activate another factor that directly represses \textit{hth} (Fig. 8C). This factor would be activated in Vg-positive cells by Dpp signaling beginning in the late second instar. By the third instar, high levels of Dpp signaling would be sufficient to maintain its activation, with additional input by Vg and Wg required only at the lateral regions of the pouch. Even further from the source of Dpp, in the lateral hinge, high levels of Brk would prevent expression of this factor, thus allowing \textit{hth} expression despite the presence of Vg. This model is consistent with the idea that Brk is a transcriptional repressor (Hasson et al., 2001; Kirkpatrick et al., 2001; Rushlow et al., 2001; Sivasankaran et al., 2000; Zhang et al., 2001) and Vg is a transcriptional activator (Halder and Carroll, 2001; Halder et al., 1998). There is also precedent for the idea that early \textit{vg} expression predisposes cells to a particular Dpp response, which was also proposed for the activation of the \textit{vg}\textsubscript{QE} (Klein and Arias, 1999).

We note that the above model does not apply to PH cells, which have a distinct response to Dpp signaling. For example, we found that \textit{Med}\textsuperscript{dro} clones located near the AP boundary of the PH ectopically expressed \textit{vg} (data not shown). \textit{tsh} is an attractive candidate for mediating this switch in response to Dpp signaling, as it is expressed in the PH but not the DH, and is reported to bind Brk in vitro (Saller et al., 2002). However, the absence of reagents to readily examine \textit{tsh} loss-of-function clones prevents us from testing this idea at this time.

\textbf{PcG genes and the maintenance of \textit{tsh} repression}

If \textit{tsh} repression marks a fundamental subdivision along the PD axis, then the maintenance of \textit{tsh} repression is crucial for the maintenance of this subdivision. Although Wg signaling is clearly required for the initiation of \textit{tsh} repression, it is dispensable by the time the DV margin is established (Wu and Cohen, 2002). The \textit{elbow-no ocelli (el-noc)} gene complex has been identified as a target of both Dpp and Wg that is necessary for \textit{tsh} repression in the wing (Weihe et al., 2004). However, \textit{tsh} de-repression is only observed in \textit{el-noc} loss-of-function clones induced in first or early second instar larvae. \textit{tsh} repression must therefore be maintained by a \textit{wg}- and \textit{el-noc}-independent mechanism. We ruled out the possibility of redundant Wg- and Dpp-mediated \textit{tsh} repression by making clones doubly mutant for both signaling pathways. Such clones upregulated \textit{hth}, and lost \textit{Dll} expression, but showed no ectopic \textit{tsh} expression. Thus, neither of the two major long-range signaling systems of the wing pouch is involved in the maintenance of \textit{tsh} repression.

Instead, our analysis of \textit{Su(z)12\textsuperscript{daed}} and \textit{Pc} mutant clones indicates that the maintenance of \textit{tsh} repression is mediated by a heritable silencing mechanism. By inducing \textit{Pc} mutant clones in third instar discs, we demonstrated that this ectopic \textit{tsh} expression represents a failure to maintain rather than a failure to establish repression. The weak \textit{hth} levels observed in some \textit{Pc} mutant clones may be due to the previously noted ability of \textit{tsh} to upregulate \textit{hth} (Azpiazu and Morata, 2000; Casares and Mann, 2000). This interpretation is supported by the fact that \textit{hth} expression is only seen in large \textit{Pc} mutant clones, and only in cells expressing the highest levels of \textit{tsh}. The general absence of \textit{hth} expression in \textit{Pc} mutant clones, together with the ectopic \textit{hth} expression resulting from late Dpp pathway disruption, points to the need for continuous signaling input to maintain \textit{hth} repression. By contrast, \textit{tsh} requires PcG gene activity, but not continuous Wg or Dpp input, to maintain its repression during the third instar.

We cannot at this stage rule out the possibility that the effects of \textit{Pc} mutant clones on \textit{tsh} repression described here are indirectly due to the de-repression of another factor. We suggest that this is unlikely, however, in part because the spatial distribution of \textit{tsh} de-repression in \textit{Pc} mutant clones differs significantly from reports of Hox gene de-repression (Beuchle et al., 2001). Additionally, the ectopic \textit{tsh} expression in \textit{Pc} mutant clones is repressible by \textit{Nrt-Wg}, indicating that \textit{tsh} is still subject to regulation by Wg signaling.

In the embryo, Hox genes are repressed in some segments by the transient presence of the gap genes (reviewed by Bienz and Muller, 1995). This initial repression is then maintained by
the PcG proteins through a heritable silencing mechanism. Our model of tsh repression follows this general outline, whereby Wg signaling is required transiently to establish the limits of the tsh expression domain (Fig. 8A). PeG proteins subsequently maintain the tsh silenced state, while the appendage is further subdivided along the PD axis (Fig. 8A-C). Similar mechanisms may be important for tsh regulation in other tissues, as was suggested by a recent report showing tsh de-repression in PeG mutant clones in the eye disc (Janody et al., 2004).

tsh and hth repression are distinct events during the development of the wing imaginal disc. The requirement for PeG activity in tsh, but not hth, repression points to the primacy of tsh repression in determining appendage versus trunk fate. PeG regulation ensures a strict and inflexible pattern of gene expression, ideal for defining the fundamental divisions of the disc. Within the specified appendage domain, Wg and Dpp signaling can then modify the shape and size of the hinge and wing blade through continuous input into transcription factors that control patterning and growth. In the absence of Tsh, Hth is an essential mediator of this process, as it promotes hinge development at the expense of wing pouch growth. The complexity of hth relative to tsh regulation may therefore, reflect the greater need for plasticity in the response of hth to the Wg and Dpp morphogen gradients.

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