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Temporally dynamic response to Wingless directs the sequential elaboration of the proximodistal axis of the *Drosophila* wing

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Abstract

The *Drosophila* wing imaginal disc gives rise to three main regions along the proximodistal axis of the dorsal mesothoracic segment: the notum, proximal wing, and wing blade. Development of the wing blade requires the *Notch* and *wingless* signalling pathways to activate *vestigial* at the dorsoventral boundary. However, in the proximal wing, Wingless activates a different subset of genes, e.g., *homothorax.* This raises the question of how the downstream response to Wingless signalling differentiates between proximal and distal fate specification. Here, we show that a temporally dynamic response to Wingless signalling sequentially elaborates the proximodistal axis. In the second instar, Wingless activates genes involved in proximal wing development; later in the third instar, Wingless acts to direct the differentiation of the distal wing blade. The expression of a novel marker for proximal wing fate, *zfh-2,* is initially activated by Wingless throughout the "wing primordium," but later is repressed by the activity of Vestigial and Nubbin, which together define a more distal domain. Thus, activation of a distal developmental program is antagonistic to previously established proximal fate. In addition, Wingless is required early to establish proximal fate, but later when Wingless activates distal differentiation, development of proximal fate becomes independent of Wingless signalling. Since P-element insertions in the *zfh-2* gene result in a revertable proximal wing deletion phenotype, it appears that *zfh-2* activity is required for correct proximal wing development. Our data are consistent with a model in which Wingless first establishes a proximal appendage fate over notum, then the downstream response changes to direct the differentiation of a more distal fate over proximal. Thus, the proximodistal domains are patterned in sequence and show a distal dominance.

Keywords: Proximodistal axis; Wingless; Zfh-2; *Drosophila*

Introduction

The adult *Drosophila* appendages develop from small groups of cells, the imaginal discs, which are allocated early in embryogenesis. The wing imaginal disc gives rise to most of the dorsal mesothorax and its associated appendage, the wing. The mesothoracic segment comprises the notum, or body wall, the hinge and proximal wing, and the wing blade. These structures define different regions along the proximodistal (P/D) axis. While much is known about the development of the wing blade and the patterning mechanisms that specify the anterioposterior (A/P) and dorsoventral (D/V) axes (for review, see Cohen, 1993), very little is known about either the elaboration of the P/D axis or the development of the proximal regions.

Correct patterning and development of the wing requires the activity of Wingless (Wg), a member of the Wnt family of secreted proteins, from the early second larval instar onwards. Reduction of *wg* function causes a complete loss of wing structures and the duplication of notum tissue (Morata and Lawrence, 1977; Sharma and Chopra, 1976). Between mid-first and mid-second instar, the EGF-receptor (EGFR) signalling pathway is required in the dorsal part of the wing disc to activate *apterous (ap),* a gene encoding a LIM-homeodomain protein (Wang et al., 2000). The activity of Ap directs dorsoventral (D/V) compartmentalization (Blair et al., 1994; Cohen et al., 1992; Milan and Cohen, 2000). Subsequently, EGFR signalling and *ap* expression

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are antagonized ventrally by the expression of *wg* (Wang et al., 2000; Williams et al., 1993), which initiates the differentiation of the wing primordium (Ng et al., 1996). *wg* expression initiates in a ventral–anterior wedge of the early second instar wing disc and is extremely dynamic throughout the larval stages (Couso et al. 1993; Ng et al.,1996; Phillips and Whittle, 1993;). During the second instar, the combined activities of the Wg and Notch (N) signalling pathways induce the expression of the nuclear protein Vestigial (Vg), which is essential for wing blade development, along the D/V boundary through activation of the second intron or boundary enhancer (vgBE;Couso et al., 1995; Kim et al., 1996; Klein and Martinez Arias, 1998, 1999; Neumann and Cohen, 1997; Williams et al., 1991, 1994). In the early third instar, *N*, *wg*, and *vg* act together to activate a second *vg* enhancer, the *vg* quadrant enhancer (*vg*QE), through which *vg* is expressed across the wing pouch (Klein and Martinez Arias, 1999). Later, *vg*QE is regulated in a dosage-dependent manner by the Wg and Dpp signalling pathways (Kim et al., 1996, 1997; Klein and Martinez Arias, 1999). In this way, it is postulated that by late third instar *vg* is expressed in a graded response to signals emanating from the D/V and A/P boundaries to interpret putative signal gradients as positional information across the wing pouch.

Wg is also required for the correct development of the proximal region of the wing (Neumann and Cohen, 1996a). In contrast to cells of the adjacent wing pouch, in the developing proximal wing, *vg* is not expressed; instead, Wg regulates the expression of the homeobox gene, *homothorax (hth)*. In the proximal wing, Hth plays a dual role, to limit the size of the developing distal wing, by negative gene regulation, and also to upregulate proximal *wg* expression (Azpiazu and Morata, 2000; Casares and Mann, 2000). However, it is not known whether Hth is necessary to specify proximal *fate*. Together, these studies show that Wg signalling is able to direct a number of distinct downstream responses that control the differentiation of distinct P/D developmental programs. However, the mechanism and timing by which Wg signalling response coordinates the subdivision of the wing disc, in particular how the proximal wing differentiates, and hence establishes the P/D axis, remain largely unknown.

In this study, we utilize a novel marker for proximal wing fate, the zinc-finger homeodomain containing gene *zfh-2* (Fortini et al., 1991), to show that the downstream response to Wg signalling directs the sequential distalisation of the wing appendage. Initially, in the second instar, Wg allocates cells to a "wing primordium" developmental program, at which point Wg establishes proximal wing fate through activation of *zfh-2*. Later, Wg initiates the differentiation of the wing pouch by cooperating in the activation of *vg*QE. Vg then acts along with the POU-domain protein Nubbin (Nub) to repress proximal fate. Finally, Wg acts to pattern the most distal element, the wing margin, by activation of members of the *Achaete Scute Complex* (Couso et al., 1994; Neumann and Cohen, 1997; Phillips and Whittle, 1993). Here, we also show that the response to Wg signalling is temporally dynamic, since Wg is required early to activate *zfh-2*, but during the third instar, continued *zfh-2* expression is independent of Wg activity. In addition, we provide evidence that suggests that *zfh-2* function is required for the correct development of proximal wing structures.

Materials and methods

Fly stocks

wg^{cx4} is a null allele (J. de Celis); $wg^{spd\text{-}fg}$ is a regulatory mutant (Neumann and Cohen, 1996a); *wg-lacZ* (Kassis et al., 1992); wg^{IL114} temperature-sensitive mutant (Nüsslein-Volhard et al., 1984); vg^{1}, strong hypomorph (Lindsley and Zimm, 1992); nub^{2}, strong hypomorph (Lindsley and Zimm, 1992); hth^{C1}, hypomorphic allele (Casares and Mann, 1998). *dpp*- GAL4 (Staehling-Hampton et al., 1994) is driven by an enhancer/promoter element that only expresses in the imaginal discs along the A/P boundary. $zfh\text{-}2^{MS209\text{-}GAL4}$ is described as MS209 (Capdevila and Guerrero, 1994) and inserted in the *zfh-2* transcription unit (our unpublished observations). M390.R and M707.R are independent insertions in the *zfh-2* gene (Sun et al., 2000). hs-*flp; abx/Ubx<f*$^{+}$*>GAL4-lacZ* (de Celis and Bray, 1997). UAS-*lacZ* and UAS-*wg* were provided by the Cambridge Stock Collection; UAS-*Nrt-flu-wg* (Zecca et al., 1996); UAS-vg^{73} (Kim et al., 1996); UAS-$wg^{\Delta c}$ (Klein and Martinez Arias, 1999).

Misexpression experiments

Directed misexpression of genes used the GAL4/UAS technique (Brand and Perrimon, 1993). Clones of cells expressing GAL4 marked with *lacZ* were generated by a brief heat-shock of the *Ubx<f*$^{+}$*>GAL4-lacZ* chromosome (de Celis and Bray, 1997). Clonal expression of membrane-bound Wg was achieved by heat shocking 112- to 120-h larvae of genotype *hs-flp; abx/Ubx<f*$^{+}$*>GAL4-lacZ/* UAS-*Nrt-flu-wg*. Clones expressing dominant negative Wg were induced by heat shocking 72- to 96-h larvae of genotype *hs-flp/+; abx/Ubx<f*$^{+}$*>GAL4-lacZ/+; UAS-wg*$^{\Delta c}$*+*.

Mitotic recombination

Mitotic recombination was induced by the *hs-flp*/FRT method (Xu and Rubin, 1993). hth^{Cl} clones were generated by heat shocking 48- to 72-h or 72- to 96-h larvae of genotype *hs- flp; FRT 82B* hth^{C1}*/FRT 82B arm-lacZ* wg^{-} clones were generated by heat shocking 72-to 96-h larvae of genotype *hs-flp;* wg^{cx4} *FRT 40A/arm-lacZ FRT40A*. Under both conditions, mutant cells were detected by the absence of β-gal immunostaining.

Temperature shift assay

Flies bearing wg^{IL114} were crossed to a *wg-lacZ* line and allowed to lay for 4 h. These progeny were maintained at 17°C until late first instar (92–96 h) when they were transferred to 25°C. Discs were analyzed at late second/early third instar.

Immunohistology

The antibodies used were: rabbit anti-β-gal (Cappel); chick anti-Hth (Casares and Mann, 2000); rabbit anti-Nub (Ng et al., 1996); rat anti-Tsh (Roder et al., 1992); rabbit anti-Vg (Kim et al., 1996); mouse anti-Wg (4D4; Developmental Studies Hybridoma Bank, Iowa City, IA); mouse anti-Zfh-2 was used at 1:250 (Lai et al., 1991); rat anti-Zfh-2, used at 1:250, was a kind gift from M. Lundell (unpublished data). Fluorescence-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories Ltd. Immunostaining of imaginal discs was according to the procedure of Halder et al. (1998). Confocal analysis was conducted by using a Biorad 1024 system.

Histochemistry

X-gal chromogenic staining to detect β-galactosidase activity was performed on imaginal discs by using a standard protocol (Ashburner, 1989), and newly eclosed adults were described by Hama et al. (1990).

Results

Zfh-2 is a specific marker for proximal wing fate

The *Drosophila* wing imaginal disc gives rise to the structures of the dorsal mesothoracic segment. This is subdivided into three main regions: the notum, the wing blade, and the proximal wing and hinge (Fig. 1A). The wing is attached to the thorax via a complex joint comprising a small portion of the appendage, the hinge, which consists of several interlocking sclerites and plates. The wing blade tapers toward the body, forming a short, narrow region that is attached at the hinge. This region shall be referred to as the proximal wing as it is morphologically and mechanically distinct from the hinge itself. Fate mapping of the late third instar imaginal disc has determined that the central portion, the wing pouch, develops as wing blade, a ring surrounding the wing pouch develops as proximal wing and hinge, and the large dorsal territory and a narrow ventral domain form the notum and ventral pleura (Fig. 1B; Bryant, 1978).

Previous studies have attempted to follow the development of the proximal part of the wing by analysis of genes that have some expression in the proximal region of the wing disc, e.g., *wg* or *nub* (see Fig. 1C), or by the exclusion of markers for notum and wing fates, e.g., *teashirt (tsh)* and *vg*, respectively (Fig. 1D; Klein and Martinez Arias, 1998; Ng et al., 1996). Here, we describe the identification and analysis of a novel marker for proximal wing fate that specifically demarcates the whole of the developing proximal wing tissue, the zinc-finger homeodomain gene *zfh-2* (Fortini et al., 1991). In third larval instar (L3) wing discs, Wg is expressed in a stripe along the D/V boundary, forming the wing margin, and in two concentric rings around the wing pouch (Fig. 1C and E). In the adult wing, expression of a *wg-lacZ* reporter indicates that the two rings of *wg* delimit the proximal wing. The inner (distal) ring runs from the medial costa, through the humeral crossvein to the alula, and the outer (proximal) ring runs from the proximal end of the proximal costa to the axillary cord (Fig. 1F). We have used a GAL4 insertion within the *zfh-2* transcription unit, MS209, ($zfh\text{-}2^{MS209}$; Capdevila and Guerrero, 1994; our unpublished observations) and antisera against Zfh-2 to monitor the expression of *zfh-2*. In both L3 wing discs (Fig. 1E) and adult wings (Fig. 1G), Zfh-2 is expressed in a domain that completely overlaps the rings of Wg expression. In L3 wing discs, Zfh-2 does not extend either proximally into the notum or distally into the wing pouch. These observations indicate that, in late stages, Zfh-2 is specifically expressed throughout the developing proximal wing and therefore may be used as a useful marker for proximal wing fate.

wg expression, monitored by a *lacZ* reporter, is initiated in the early second instar (L2) in an approximately anterior–ventral domain (Fig. 2A; Couso et al., 1993). A *lacZ* reporter driven by $zfh\text{-}2^{MS209}$ is expressed in a very similar pattern (Fig. 2B). To determine the extent of coexpression of Zfh-2 and Wg, we examined early L2 discs with anti-Zfh-2 and anti-Wg antisera. Zfh-2 is expressed at this stage in a pattern that directly overlaps with Wg (Fig. 2C). It is apparent that, although the *wg-lacZ* reporter gene shows *wg* expression induced in a narrow domain, the protein can be detected at some distance outside of this region (Fig. 2C, arrow; compare with Fig. 2A). This is a measure of the mobility of Wg protein. Consistent with this, Zfh-2 nuclear expression is at high levels in the wedge-like domain of *wg-lacZ*, but is also detectable away from this region at lower levels (Fig. 2C, arrowhead). As development proceeds, Zfh-2 quickly expands to cover the whole of the ventral portion of the wing disc, accompanying the expansion of the Wg domain (Fig. 2D). The expression of Wg at this stage is proposed to determine the differentiation of the presumptive "wing primordium" as opposed to notum (Ng et al., 1996). However, since Zfh-2 is also widely expressed at this time, it suggests that the "wing primordium" has not been further subdivided into proximal or distal domains.

At the onset of L3, Zfh-2 begins to decline in the center of the disc, suggesting differentiation of more distal fates here (arrow, Fig. 2E). At this time, Wg is still expressed throughout the "wing primordium" but becomes upregulated at the D/V boundary, where it plays a central role in

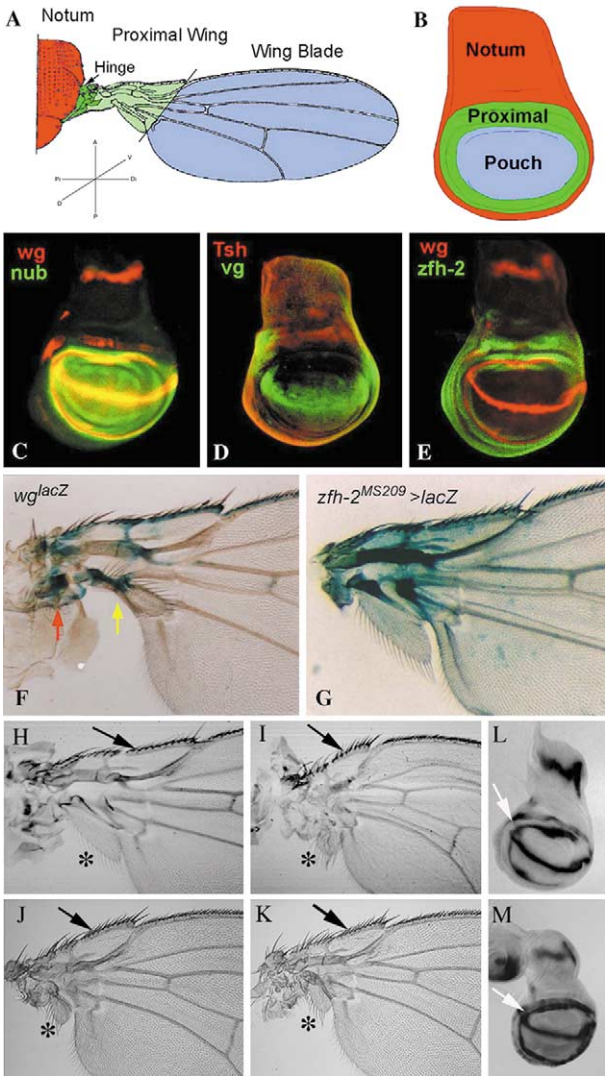


Fig. 1. Organization of the P/D axis of the *Drosophila* wing and the role of *zfh-2*. (A) A diagrammatic representation of an adult dorsal mesothorax highlighting the three main domains of the P/D axis: the notum, the proximal wing, and the wing blade. (B) Fate mapping indicates the regions of the wing disc that give rise to the wing; colors are coordinated to show the approximate origins of the adult epidermal structures. (C–E) Wild type gene expression in the imaginal disc. (C) Wg expression (red) includes two rings, which demarcate the proximal wing, and is partially overlapped by Nub (green). (D) Tsh (red) marks the notum tissue, and Vg (green) marks wing blade tissue. (E) Zfh-2 is expressed in a broad ring around the wing pouch (see text). (F) After eversion of the wing disc, the rings of Wg expression form two stripes bordering the proximal wing proximally (red arrow) and distally (yellow arrow). (G) *zfh-2* enhancer elements drive expression of a *lacZ* reporter gene throughout the adult proximal wing. (H, I) *zfh-2* mutations exhibit proximal wing deletions. (H) Wild type wing with the costa (black arrow) and the alula (asterisk) marked. (I) Homozygous $zfh\text{-}2^{MS209}$ wing demonstrating the deletion of proximal wing tissue. (J) $zfh\text{-}2^{MS209}/zfh\text{-}2^{M707.R}$ and (K) $zfh\text{-}2^{MS209}/zfh\text{-}2^{M390.R}$ show similar phenotypes. (L) Wild type and (M) $zfh\text{-}2^{MS209}/zfh\text{-}2^{MS209}$ third instar wing discs stained to reveal Wg. In the mutant discs, the tissue between the rings of Wg expression is missing (white arrows).

defining the wing margin. This is also the time when the *vg* quadrant enhancer (*vg*QE) is activated on either side of the D/V boundary, marking the establishment of the wing pouch (Klein and Martinez Arias, 1999). During mid-L3, the pattern of Wg expression is refined further, becoming upregulated at the periphery of the wing pouch and at the D/V boundary, the presumptive wing margin. Zfh-2 is also refined and is now only present in a ring around the wing pouch overlapping the rings of Wg (Fig. 2F and G).

Taken together, these observations suggest that, at the beginning of L2, the wing imaginal disc is divided into the presumptive notum and the appendage or "wing" primordium, and that the wing primordium is undifferentiated with respect to the proximodistal axis. This is supported by reports that *tsh* is also expressed throughout L2 wing discs, but is later restricted to the presumptive notum and hinge regions (Casares and Mann, 2000), and also that *vg*QE is not yet activated to differentiate the wing pouch (Klein and Martinez Arias, 1999). From the dynamic expression pattern of the proximal wing marker *zfh-2*, it appears that the elaboration of distal elements within the disc, marked by the disappearance of Zfh-2 and concomitant activation of the *vg*QE, is initiated at the start of L3 at the center of the wing disc where the A/P and D/V boundaries intersect. This suggests that the proximal wing and wing pouch differentiate sequentially, the distal wing pouch being induced later

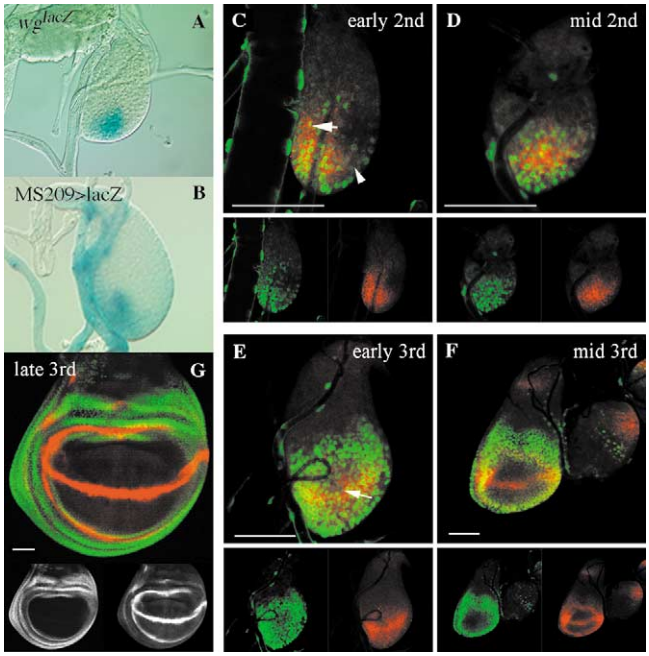


Fig. 2. Temporal dynamic progression of Zfh-2 expression. (A) A *wg-lacZ* reporter gene expression at early L2 shows a ventrally located wedge-like domain. (B) A similar pattern is observed with a reporter for $zfh\text{-}2^{MS209\text{-}GAL4}$ activity. (C) Early L2 shows colocalization of Zfh-2 with high levels of Wg. Wg can diffuse away from the site of expression, leading to low level induction of Zfh-2 at a distance (arrowhead). (D) Mid-L2, Wg expression expands to fill the "wing primordium" mirrored by Zfh-2. (E) Early L3, Zfh-2 starts to be repressed in the center of the disc (arrow), marking the differentiation of the "wing pouch". (F) Mid-L3, Wg and Zfh-2 domains are refined so that, by late L3 (G), Zfh-2 is completely restricted to the periphery of the wing pouch. (C–G) Anti-Wg is marked in red and anti-Zfh-2 in green. Scale bars of 50 μm are added for reference. Separate channels are shown below each panel.

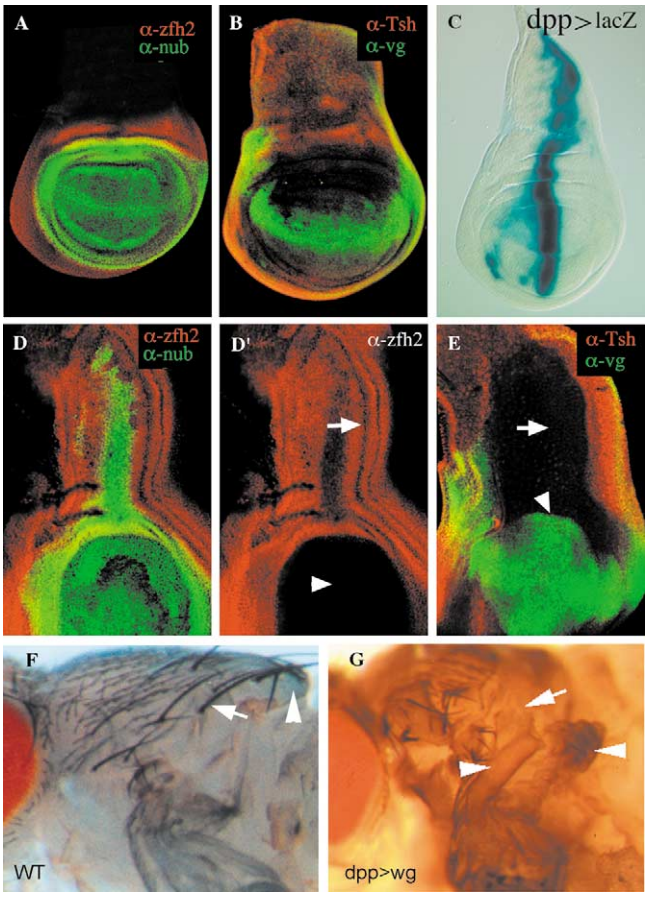


Fig. 3. Ectopic Wg can induce the differentiation of proximal wing fate. (A) Wild type expression of Zfh-2 (red) and Nub (green). (B) Wild type expression of wing blade marker Vg (green) and notum marker Tsh (red). (C) A stripe of *lacZ* induced by *dpp*-GAL4 along the A/P boundary. (D) Ectopic expression of Wg driven by *dpp*-GAL4 induces a broad domain of ectopic Zfh-2 expression (red) and a narrow domain of Nub (green). (D′) Zfh-2 is only ectopically expressed in the notum (arrow) and not in the wing pouch (arrowhead). (E) Ectopic Wg driven by *dpp*-GAL4 represses Tsh (red, arrow) and does not lead to expansion of Vg (green, arrowhead). Note the upregulation of Vg in the wing pouch. (F) Wild type pharate adult thorax displays characteristic macrochaete (arrow) and scutellum (arrowhead). (G) Pharate adult of genotype *dpp*-GAL4/UAS-*wg* shows loss of macrochaete and scutellum (arrow) and ectopic outgrowth and sclerites (arrowhead) characteristic of proximal wing structures.

than the already established proximal wing. The early expression of *zfh-2* indicates that it is a specific marker for proximal fate.

zfh-2 mutations have a proximal wing deletion phenotype

zfh-2^{MS209} homozygotes, while poorly viable, display a recessive proximal wing phenotype (Fig. 1I). The phenotype consists of deletion of both anterior and posterior wing structures, including the medial costa, parts of the radius, and the alula (compare with Fig. 1H). The P-element insertion in *zfh-2*^{MS209} was mapped to the first intron of the *zfh-2* transcription unit. Evidence that the insertion causes the wing phenotype is twofold; the phenotype can be reverted by loss of the P-element, and independently isolated P-element insertions in the same region of the *zfh-2* gene have similar phenotypes. M390.R and M707.R were isolated in a fourth chromosome P-element screen (Sun et al., 2000) and, like *zfh-2*^{MS209}, these insertions are poorly viable. Homozygous escapers and transheterozygotes with *zfh-2*^{MS209} display similar proximal wing phenotypes (Fig. 1J and K). When examined for *wg* expression, the L3 wing discs of *zfh-2*^{MS209} homozygotes show a loss of tissue between the rings of *wg* expression that demarcate the proximal wing; there are no effects on the expression of wing pouch markers, such as *nub* or *vg*, or the notum marker *tsh* (not shown). Although we have been unable to isolate null mutations in *zfh-2*, the fact that at least three independently isolated P-element insertions show similar phenotypes strongly suggests that, consistent with its expression pattern, *zfh-2* is required for the correct development of the proximal wing.

Wg can direct the differentiation of proximal wing

The overlap between Zfh-2 and Wg throughout the larval stages suggests that *zfh-2* may be activated by Wg signalling. In order to test this, we analyzed the effect of ectopic Wg expression on *zfh-2* expression. *dpp*-GAL4 was used to drive the expression of a UAS-*wg* construct along the A/P boundary in all domains along the P/D axis (Fig. 3C). Under these conditions, Zfh-2 shows a broad expansion into the presumptive notum region but no ectopic expression in the wing pouch (Fig. 3D and D′). This indicates that ectopic

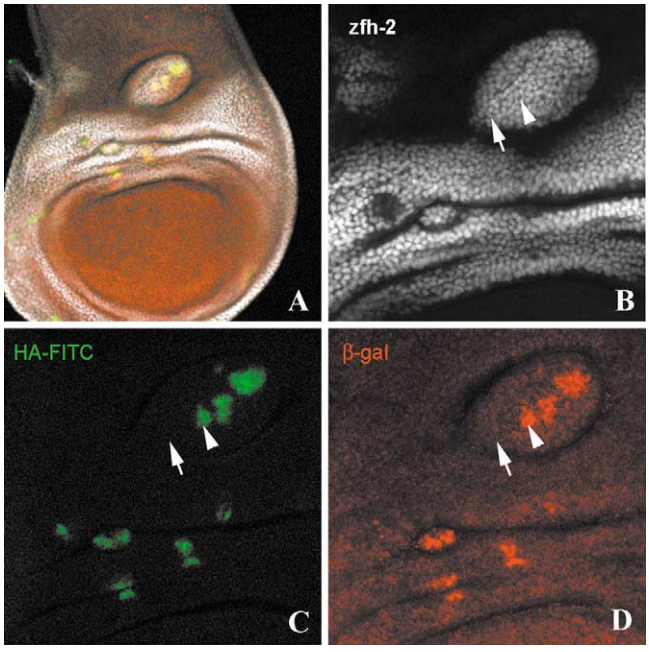


Fig. 4. Long-range induction of *zfh-2* by membrane-tethered Wg. (A, B) Wg can ectopically induce Zfh-2 expression in the notum. A small clone of cells expressing a membrane-bound form of Wg (Nrt-flu-wg) in the notum (arrowhead) marked with anti-HA-FITC (green, C) and anti-β-gal (red, D). Zfh-2 is ectopically induced many cell diameters away (arrow, B).

Wg can activate *zfh-2* at a distance from its site of expression. In the wild type proximal wing disc, Nub overlaps the Zfh-2 domain at the inner ring of Wg. This situation is recapitulated when ectopic Wg is driven by *dpp*-GAL4, since ectopic Nub is only detected in regions of high Wg expression (Fig. 3D). This indicates that ectopic Wg is inducing a response similar to the inner ring and suggests that the region expressing ectopic Zfh-2 is now differentiating as proximal wing.

To assess whether the cells expressing ectopic Zfh-2 have altered their fate, we analyzed the expression of the notum marker Tsh. Tsh is completely repressed throughout the region of ectopic Zfh-2 (Fig. 3E), indicating that cells are no longer fated as notum. Since Wg is an important factor in the development of wing blade, we also examined the expression of a wing blade marker, Vg. In agreement with previous results (Klein and Martinez Arias, 1998), Vg shows no expansion into more proximal regions and is still restricted to the wing pouch. These observations support the idea that Wg is able to direct the differentiation of proximal wing fate at the expense of notum. This can also be inferred from an examination of the phenotype of pharate adults of genotype *dpp*-GAL4/UAS-*wg* (Fig. 3G). We see an outgrowth of tissue with characteristic proximal wing sclerites and a concomitant loss of macrochaete and scutellum normally associated with the notum (compare Fig. 3G with Fig. 3F).

Long range induction of Zfh-2 by tethered Wg

The experiment to induce ectopic proximal wing was carried out in such a way that the ectopic Wg was expressed in a pattern that intersects the endogenous domain of Wg expression and resulted in a continuous region of Wg expression. The observed effects could therefore be interpreted as directed overgrowth of the endogenous proximal wing and not differentiation of proximal wing de novo. This is supported by observations that ectopic expression of Wg in the proximal wing anlagen causes disc overgrowth and consequently overgrows proximal wing tissue (Neumann and Cohen, 1996a; Russell, 2000). In view of this, we sought to reproduce the effects of ectopic Wg in a manner that was discontinuous with the endogenous *wg* domain. To achieve this, we induced clones of *wg*-expressing cells that were contained entirely within the notal region, outside of the endogenous proximal wing. To prevent diffusion of ectopic Wg, we used a construct (UAS-*Nrt-flu-wg*) that directs the expression of a *membrane-tethered* form of Wg marked with a Flu epitope tag (Zecca et al., 1996). Fig. 4 shows a small clone of cells expressing the Nrt-Wg bound to the cell surface (arrowhead), stained to reveal the GAL4-expressing cells and the Wg fusion protein. The colocalization of these two markers confirms two things; first, that the only cells expressing GAL4 induce expression of the UAS construct. Second, that the Flu epitope marker is not detectable beyond the site of expression, indicating that the Flu/Wg hybrid molecule is membrane-bound and not detectably diffusible. When stained to reveal Zfh-2 (white, Fig. 4B), we can clearly see that Zfh-2 is induced at a distance of several cell diameters from the site of Nrt-Flu-Wg expression (arrow), producing a large zone of Zfh-2-expressing cells surrounded by an epithelial fold. This observation is surprising considering that Wg protein is believed to be tethered to the cell membrane, although it is consistent with observations reported by Casares and Mann (2000). In the wing pouch, the same construct only elicits a Wg signal response in the expressing cell and its immediate neighbors (Zecca et al., 1996). Although the nature of the long-range induction cannot be explained at present, this does confirm our earlier indication that ectopically expressed Wg is able to induce the expression of Zfh-2 and therefore drive differentiation of proximal wing fate.

Taken together, these results show Wg is sufficient to direct the differentiation of proximal wing fate. Furthermore, Wg can only induce ectopic Zfh-2, and thereby proximal wing fate, in the more proximal notum tissue and not in the more distal wing pouch.

Temporal requirement of Wg to activate zfh-2

To determine whether Wg is required for *zfh-2* expression and how this changes through development, a number of methods were employed to remove Wg function at different developmental stages. The temperature-sensitive allele wg^{IL114} was used in *trans* to a *wg-lacZ* insertion line to create a conditional null mutant. When larvae were moved to the restrictive temperature just prior to L2, Zfh-2 expression was no longer detected in the wing primordium. Fig. 5A shows a late L2 wg^{IL114}/*wg-lacZ* wing disc stained for β-gal, to highlight *wg*-expressing cells, and for Zfh-2. No accumulation of Zfh-2 is observed in the wing primordium (Fig. 5B arrow; compare with Fig. 2D), although the tracheal nuclei remain immunoreactive (arrowhead). This indicates that Wg function is required at least for initiation of *zfh-2* expression in the L2 wing disc. Wg signal transduction can also be antagonised by the expression of a dominant negative TCF (DN-TCF), a component of the Wg signalling pathway. *dpp*-GAL4 was used to drive expression of DN-TCF along the A/P boundary from early larval stages (Fig. 5C). *zfh-2* fails to be activated in the presence of DN-TCF (arrow), even into L3. This further supports our findings that Wg signal transduction is absolutely required for initiation of *zfh-2* expression during L2.

However, when Wg signalling is removed later in L3, under all experimental conditions tested, we see no effect on *zfh-2* expression. Large *wg* null clones (Fig. 5D and G) or the expression of a dominant negative form of wg (Fig. 5E and H) during L3 shows no detectable reduction in Zfh-2 levels. Similarly, no loss of Zfh-2 is observed with clonal expression of DN-TCF (Fig. 5F and I). This shows that, after activation in the L2, Wg activity is no longer required during L3 for the maintenance of *zfh-2* expression.

Taken together, these results show that the regulation of

zfh-2 by Wg is temporally dynamic. Although Wg is required early to activate *zfh-2*, when they are extensively coexpressed, it appears not to be required later to maintain *zfh-2* expression. This raises the possibility that, once activated, *zfh-2* might regulate its own expression by an unknown mechanism. This interpretation would also mean that the downstream response to Wg signal is temporally dynamic, since it appears that one set of genes, e.g., those required to determine proximal wing fate, is activated early and later becomes independent of Wg, and then another set of genes is in turn activated, e.g., those delimiting the wing blade.

Proximal wing restricted by Vg and Nub

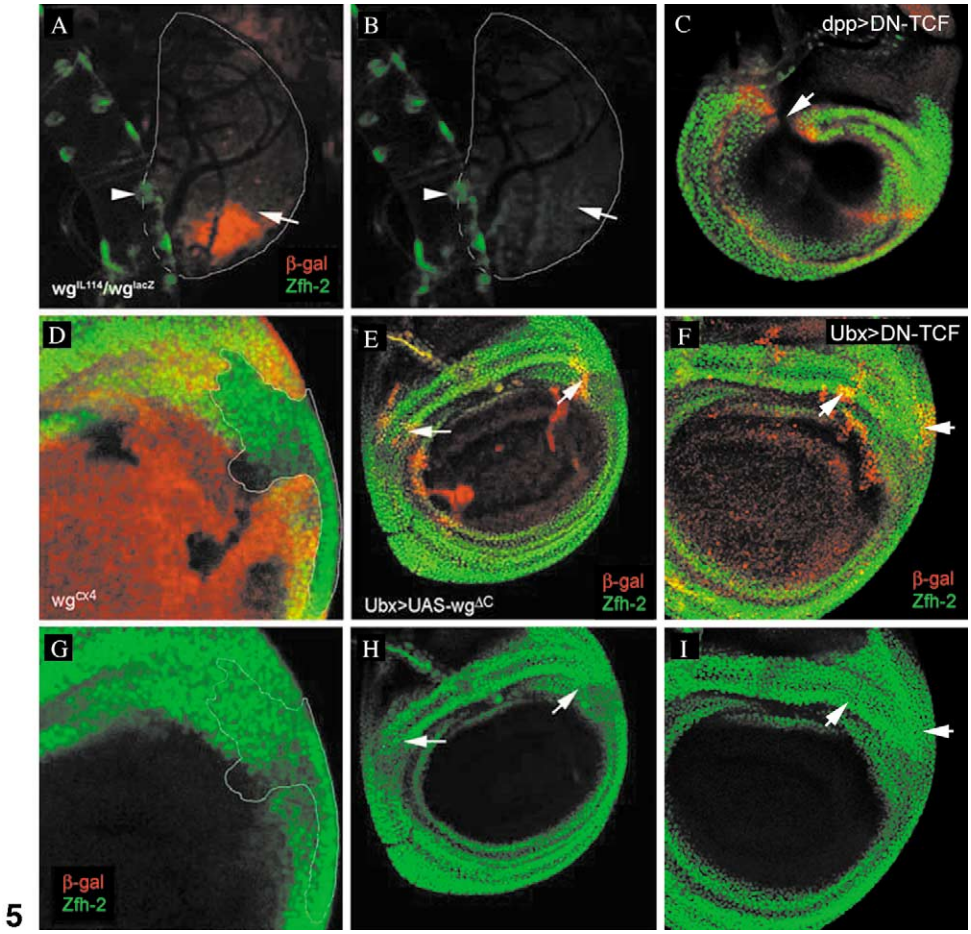
Ectopic expression of Wg can only induce *zfh-2* in regions outside of the wing pouch. This suggests that some factor has a repressive effect on *zfh-2* in the pouch that cannot be overcome by Wg activation. We considered genes fundamental to wing blade development that may be responsible for this repression. Since Vg expression is restricted to the presumptive wing blade and is required for wing blade development, we examined the effects of ectopic expression of *vg* on the proximal wing region (Fig. 6). Using *dpp*-GAL4 to direct expression of *vg* along the A/P boundary repressed *zfh-2* in the proximal wing region (Fig. 6E and E′, arrow). Endogenous *wg* expression, monitored with the *wg-lacZ* reporter, also showed complete repression at the point of intersection (arrow, Fig. 6C). Conversely, in vg^{1} mutant discs, the Zfh-2 expression domain is expanded into the remnant of the wing pouch and shows a greater overlap with Nub expression than in the wild type (Fig. 6F and F′). In vg^{1} discs, much of the wing pouch anlagen fails to develop, and this is accompanied by complete loss of Wg expression at the wing margin; however, the two rings of Wg delimiting the proximal wing are maintained (Fig. 6D). This suggests that derepression of the *zfh-2* domain into the pouch region is not caused by ectopic Wg activity. Recently, Azpiazu and Morata (2000) reported similar observations with derepression of proximally expressed Hth in vg^{-} clones.

Since the loss of *vg* does not result in complete derepression of *zfh-2*, it suggests that another repressor must be acting with *vg*. Nub is also required for wing blade development. Hypomorphic *nub* alleles display a severely reduced wing phenotype and a transformation of distal structures into proximal ones (Ng et al., 1995; Cifuentes and Garcia-Bellido, 1997). nub^{2} discs show a complete loss of the inner ring of Wg and an expansion of Wg expression at the wing margin (Fig. 7E, arrow). In nub^{2} mutant discs, Zfh-2 expression is expanded into the wing pouch, along the line of the wing margin (see Fig. 7D and F, arrowhead). This indicates two things; first, that Nub normally acts to repress *zfh-2* expression, and thus proximal wing fate, within the wing pouch; and second, that ectopic *zfh-2* is induced where Wg is expressed. Therefore, we would predict that, in an environment of reduced Nub, ectopic Wg would be able to induce ectopic Zfh-2. To test this, we expressed ectopic Wg in a *nub* mutant background. Fig. 7G shows an imaginal disc of genotype nub^{2}/nub^{2}, *dpp*-GAL4/UAS-*wg*, stained for Zfh-2 and Wg (Fig. 7H and I, respectively). As in the nub^{2} background, Zfh-2 is ectopically induced in the wing pouch along the wing margin (arrowhead, compare with Fig. 7F). In addition, Zfh-2 can now be detected in the wing pouch along the line of *dpp*-GAL4, where high levels of Wg are ectopically expressed (arrow). This demonstrates that, in an environment of reduced Nub, Zfh-2 expression can be induced wherever Wg is expressed and is no longer restricted from the pouch (compare with Fig. 7C). We note that, whereas Wg expression is expanded at the wing margin in *nub* discs, here, where ectopic Wg is induced in a *nub* background, endogenous Wg is expressed normally at the wing margin; however, the reason for this is unknown.

In *nub* discs, *vg* expression is unaffected (data not shown; Cifuentes and Garcia-Bellido, 1997), but *vg* is upregulated by high levels of ectopic Wg (Fig. 3E). Thus, in the experiment described above, it appears that the increased levels of Vg are not sufficient to repress Zfh-2 in the absence of Nub when Wg is present at high levels. However, further from the source of ectopic Wg, Zfh-2 is not induced in the *nub* background, and presumably here, Vg alone can repress Zfh-2. Taken together, these data suggest that *zfh-2* expression is regulated by a balance between activation by Wg and repression by a combination of Nub and Vg, acting together or independently. The loss of either Nub or Vg is enough to cause only a partial derepression of *zfh-2* in the wing pouch, indicating that alone neither Nub nor Vg is sufficient to completely repress proximal wing fate. However, their combined action, as is the case in the wild type, is able to completely repress *zfh-2* expression in the wing pouch. Thus, these factors act to restrict *zfh-2* expression to the periphery of the wing disc, thereby defining the distal limit of the proximal wing primordium.

Role of Hth in the proximal wing

Recent work has indicated that the homeobox gene *homothorax (hth)* is required for the correct development of the proximal wing by both upregulating Wg expression in the proximal wing and limiting the area of wing blade differentiation. Since loss of Hth function in the proximal wing leads to a dramatic reduction in the level of Wg expression (Fig. 8A and B; Casares and Mann, 2000), we sought to determine whether Hth is also required for regulation of Zfh-2 expression. In hth^{-} clones (Fig. 8), neither the expression pattern nor the level of Zfh-2 is altered compared with neighboring wild type tissue. This is consistent with the observations described above where late removal of *wg* does not affect the expression of *zfh-2*. Similarly, ectopic expression of Hth showed no effect on *zfh-2* expression (data not shown). These data suggest that



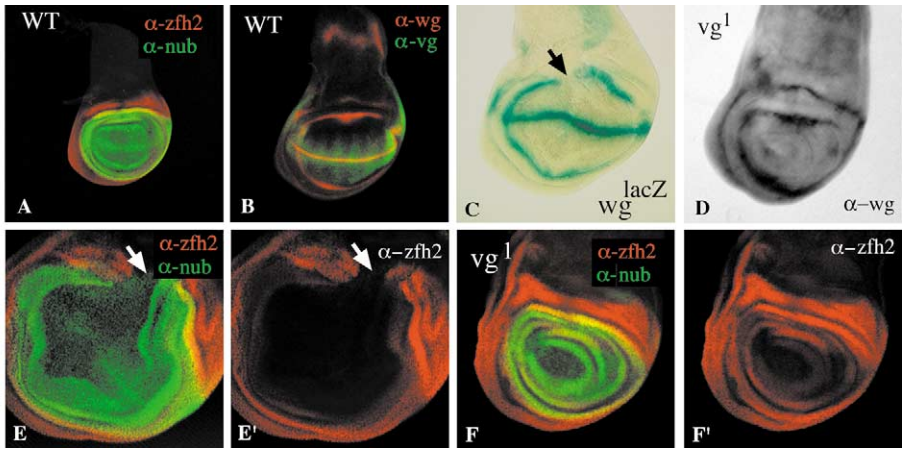


Fig. 5. *wg* is required to activate but not maintain *zfh-2* expression. (A, B) The wg^{IL114} temperature-sensitive allele in *trans* to a *wg-lacZ* shifted to the restrictive temperature by the second instar shows no initiation of Zfh-2 expression (arrow). Note that Zfh-2 expression is still observed in the trachea (arrowhead). (C) Early L3 disc showing *dpp*-GAL4-driven expression of dominant negative TCF, antagonising Wg signalling from early stages, shows Zfh-2 fails to be induced. (D, G) A large clone of wg^{cx4} cells induced at the start of L3, marked by absence of β-gal staining (red), shows no loss of Zfh-2. Late induced clones expressing a dominant negative Wg (E, H) or DN-TCF (F, I), marked in red (arrows), also show no loss of Zfh-2 expression.

Fig. 6. Ectopic Vg expression can repress the development of proximal fate. (A) Wild type expression of Zfh-2 (red) is in a proximal domain around the wing pouch partially overlapped by Nub (green). (B) Vg is expressed more distally (green). (C) Ectopic expression of Vg driven by *dpp*-GAL4 is sufficient to repress the expression of proximal Wg as measured by a *wg-lacZ* reporter (arrow). (D) Wg expression in vg^1 discs shows complete loss of marginal expression but retains the proximal rings. (E) Ectopic expression of Vg driven by *dpp*-GAL4 represses Zfh-2 (arrow). (F) Zfh-2 is derepressed in the wing pouch of a vg^1 mutant. This can also be inferred from the greater overlap with Nub, colored yellow (compare with A).

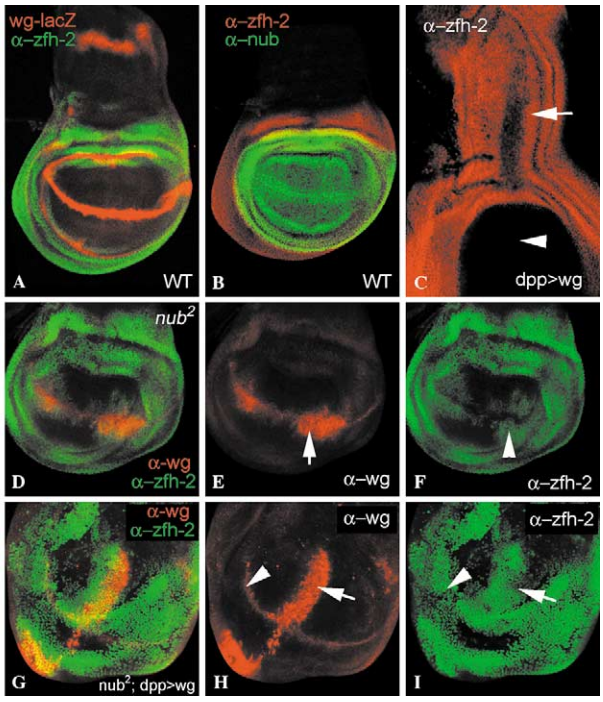


Fig. 7. Nub restricts activation of Zfh-2 by Wg to the proximal wing. (A) Wild type expression of Zfh-2 (green) and Wg (red) shows overlap in the proximal wing anlage. (B) Nub (green) domain also overlaps Zfh-2 (red), where Wg is highly expressed. (C) Zfh-2 is ectopically activated in the presumptive notum (arrow), but not the wing pouch (arrowhead), by ectopic Wg expression. (D–F) *nub*^{2} discs show an expansion of the Zfh-2 domain into the wing pouch along the wing margin (arrowhead in F), an area of high Wg. (E) Reduced Nub also causes an expansion of Wg at the wing margin (arrow). (G–I) A disc of genotype *nub*^{2}*; dpp-GAL4/UAS-wg* stained to show Zfh-2 (green) and Wg (red). In a reduced Nub background, ectopic Wg can now induce Zfh-2 in the wing pouch (arrow); compare with (C). Note that Wg is no longer expanded at the wing margin (arrowhead), and ectopic Zfh-2 β is still restricted to regions of high Wg.

Hth does not play a role in establishing or regulating the determination of proximal wing *fate*, since no change in the expression of Zfh-2 was observed. Thus, it appears that the prime functions of Hth in the proximal wing are to maintain Wg expression and define the limits of the wing pouch.

Discussion

The *Drosophila* imaginal disc gives rise to dorsal mesothorax and appendage, which can be subdivided into three main domains; the notum, the proximal wing and hinge, and the wing blade. While much is known about the development of the wing blade, and in particular the patterning of the A/P and D/V axes, we know little about the development of the proximal wing or the establishment of the P/D axis. We have identified the expression domain of *zfh-2* as a discrete marker for proximal wing tissue throughout larval development. The *zfh-2* gene is located on the fourth chromosome and encodes a large Zinc Finger Homeodomain protein. It is expressed in the CNS throughout embryonic and larval life (Lai et al., 1991; Lundell and Hirsh, 1992) and specifically in the wing imaginal disc. We have identified a set of P-elements inserted in the 5′ region of the gene; one of these, *zfh-2*^{MS209}, expresses GAL4 in the wing imaginal disc in a pattern indistinguishable from anti-Zfh-2 antisera. Significantly, *zfh-2*^{MS209} homozygotes and transheterozygotes between *zfh-2*^{MS209} and two other independently isolated P-elements (M390.R and M707.R) have a proximal wing deletion phenotype, suggesting that it is required for proximal wing development. Using *zfh-2* as a specific marker for proximal wing fate, we show that the P/D axis of the wing imaginal disc is sequentially elaborated from proximal notum to distal wing blade in a temporal sequence that is mediated by a set of differential responses to the signalling molecule Wg.

At the beginning of the second larval instar, the wing imaginal disc expresses markers of proximal fate, *hth* and *tsh*, in the entire anlage. During early L2, the expression of *wg* and *zfh-2* is initiated in an anterior–ventral wedge pattern. Our data indicate that Wg function is required to activate *zfh-2* expression at this stage, since early removal of Wg function leads to a simultaneous loss of *zfh-2* expression. As development proceeds, *wg* and *zfh-2* expression rapidly expands filling the whole of the ventral portion of the wing disc by the end of the second instar. Concomitant with the expansion of *wg* and *zfh-2*, both *hth* and *tsh* become repressed in the ventral portion of the disc (Casares and Mann, 2000; Ng et al.,1996). This transition appears to mark the first P-D differentiation of the wing disc into appendage and notum. However, since *zfh-2* is expressed in the entire wing anlage at this time, we believe that the

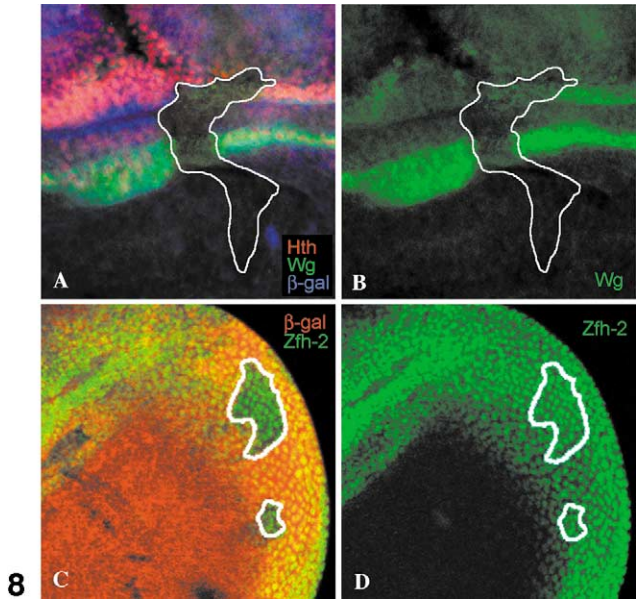


Fig. 8. Hth regulates Wg but not Zfh-2 expression in the proximal wing. Clones of *hth*$^{-}$ in the proximal wing lead to a dramatic reduction in level of Wg expression (green, A, B). However, expression of Zfh-2 remains unaffected in *hth*$^{-}$ clones compared with neighboring wild type cells (green, C, D).

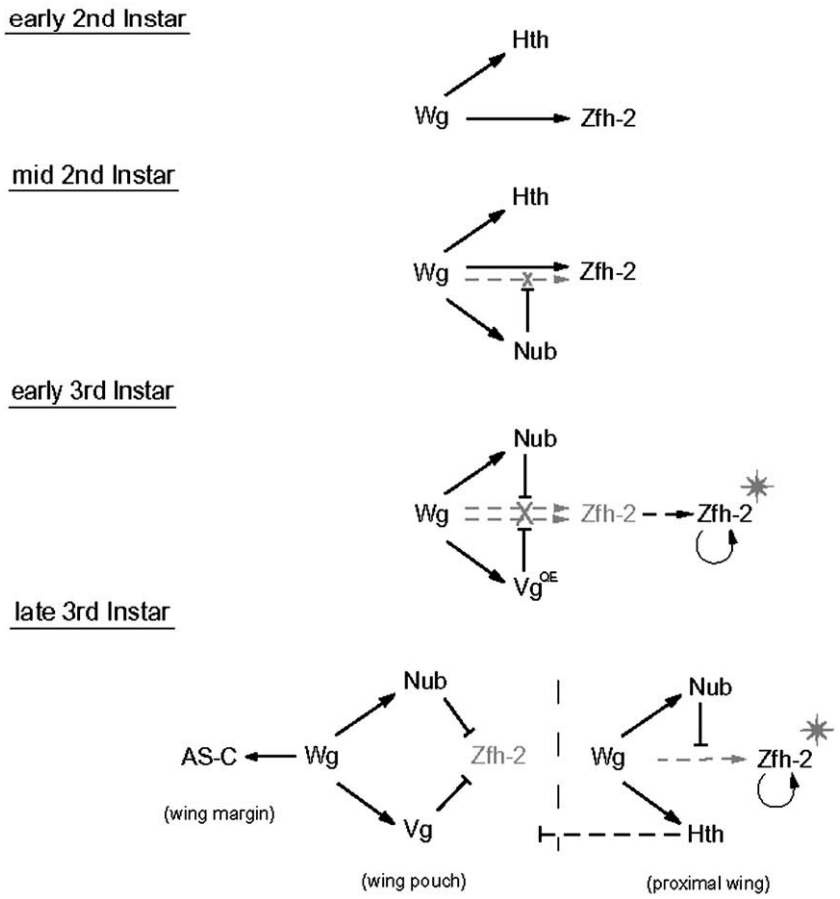


Fig. 9. Schematic representation of the dynamic response to Wg signalling in the establishment of distinct domains along the P/D axis. In early L2, Wg directs the differentiation of the "wing primordium." During L2, the proximal wing is established and Nub is activated. At the start of L3, Wg activates *vg*QE defining the wing pouch and Zfh-2 expression becomes independent of Wg. During L3, Vg acts together with Nub to repress proximal fate. Hth acts proximally to restrict the area of the wing pouch. Finally, by late L3, distal wing blade and proximal wing are differentiated, and Wg patterns the distal limit of the wing, the wing margin, through the AS-C.

appendage has not differentiated proximal wing and blade. Around the L2–L3 transition, the wing blade markers *nub* and *vg*QE are activated by the combined activity of the Wg and N signalling pathways (Klein and Martinez Arias, 1998; Ng et al., 1996). Nub and Vg, acting together or independently, repress *zfh-2* expression in the center of the disc. This marks the second phase of P-D elaboration where the appendage anlage is split into proximal wing and blade. We note that, at this time, *hth* and *tsh* remain coexpressed in the notum, where *zfh-2* is not expressed. The pattern of *zfh-2* expression at this stage suggests that it is still influenced by Wg signalling since it remains restricted to areas of high Wg expression. During L3, the division of the wing disc into three distinct domains is maintained and refined as the individual domains undergo their characteristic patterning. At this time, Hth and Wg are upregulated in the proximal wing anlage, where their activities are interdependent, while *zfh-2* expression persists but becomes independent of Wg activity.

Our data further support a qualitative difference in the activity of Wg in the proximal wing compared with wing blade. In addition to the activation of different effectors, previous investigations have shown that ectopic Wg expression in the proximal wing causes large overgrowth of proximal tissue, but similar overexpression in the wing blade produces no overgrowth (Klein and Martinez Arias, 1998; Neumann and Cohen, 1996a; Russell, 2000). This indicates that a different mitogenic response to Wg signalling is activated in the wing pouch compared with proximal wing. One intriguing observation, previously noted (Casares and Mann, 2000), is the apparent ability of membrane-tethered Wg to elicit a response at a distance from the source. It is proposed that, in the embryo at least, this may be mediated by the transport of Wg in secretory vesicles (Pfeiffer et al., 2000). Whether this is the case in the wing disc, and whether these observations highlight a difference between proximal tissue and wing pouch tissue in the ability to transport Wg, remains to be determined. Taken together, these data highlight the distinction between proximal wing and wing blade as separate subregions of the wing disc, a difference which is achieved in part by a temporally regulated differential response to Wg signalling.

Therefore, our observations suggest a model in which the wing disc is sequentially partitioned in a proximal to distal direction: notum, proximal wing, and finally wing blade (see Fig. 9). This view of temporal specification of PD identities is supported by transplantation experiments where L2 wing disc fragments can only differentiate proximal wing structures, whereas L3 disc fragments can produce wing blade elements (Bownes and Roberts, 1979; Karlsson, 1981). In support of the more general applicability of our findings, a recent study on PD patterning in the *Drosophila* leg has shown that Wg and Dpp act early to establish the PD axis, but later are not required (Galindo et al., 2002). These data appear strikingly similar to our results and suggest an important common mechanism for PD axis elaboration that has previously been unappreciated. Our investigation also serves to emphasize the importance of considering the development of the imaginal disc as an extremely dynamic field, with respect to rapid changes in both size and patterning.

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