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Repression of Teashirt marks the initiation of wing development

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SUMMARY

The wing imaginal disc comprises the primordia of the adult wing and the dorsal thoracic body wall. During second larval instar, the wing disc is subdivided into distinct domains that correspond to the presumptive wing and body wall. Early activity of the signaling protein Wingless has been implicated in the specification of the wing primordium. Wingless mutants can produce animals in which the wing is replaced by a duplication of thoracic structures. Specification of wing fate has been visualized by expression of the POU-homeodomain protein Nubbin in the presumptive wing territory and by repression of the homeodomain protein Homothorax. We report that repression of the zinc-finger transcription factor Teashirt (Tsh) is the earliest event in wing specification. Repression of Tsh by the combined action of Wingless and Decapentaplegic is required for wing pouch formation and for subsequent repression of Hth. Thus, repression of Tsh defines the presumptive wing earlier in development than repression of Hth, which must therefore be considered a secondary event.

Key words: Imaginal disc, Teashirt, Homothorax, Wing, Pattern formation, *Drosophila melanogaster*

INTRODUCTION

The appendages of *Drosophila* develop from invaginations of the larval ectoderm to form imaginal discs. In leg and wing imaginal discs the distal-most tip of the appendage is formed by cells in the center of the disc, with peripheral tissue contributing to more proximal structures. The secreted signaling proteins Wingless (Wg) and Decapentaplegic (Dpp) instruct cells to adopt distal identity. Distal leg segments are lost in *wg* or *dpp* mutants (Diaz-Benjumea et al., 1994; Held et al., 1994). The combined activity of Wg and Dpp controls the expression of several transcription factors in specific domains along the proximodistal (PD) axis of the leg, thereby determining PD cell fates (Lecuit and Cohen, 1997). The initial subdivision of the disc into leg and body wall depends on activity of the homeodomain protein Distal-less (Dll), which is required at early stages for formation of all leg structures (Cohen et al., 1989; Cohen and Jürgens 1989). Specification of the leg also requires repression of the homeodomain protein Homothorax and the zinc-finger protein Teashirt (Abu-Shaar and Mann, 1998; Wu and Cohen, 1999; Wu and Cohen, 2000). Wg and Dpp activity are required for both activating Dll and repression of Homothorax and Teashirt.

Wg and Dpp are also required for wing development, but how they specify distal wing fate is less well understood. The wg^1 mutant provides the most striking evidence for an early role of Wg in specification of the wing (Sharma and Chopra 1976; Morata and Lawrence 1977). wg^1 mutant flies often replace the wing with a duplication of the thorax. This is reflected in the imaginal discs by loss of expression of the wing pouch marker Nubbin and uniform expression of the body-wall marker Teashirt throughout the disc (Ng et al., 1996). Using temperature-sensitive alleles of *wg*, it has been possible to show that this requirement is fulfilled during second instar (Couso et al., 1993; Williams et al., 1993; Ng et al., 1996). Wg is initially expressed in a ventroanterior wedge in the wing disc. At this stage, Wg represses expression of the neuregulin-like ligand Vein in the ventral region of the disc (Schnepp et al., 1996; Wang et al., 2000). Vein activates the EGF receptor and controls the localized expression of Apterous, to specify dorsal cell identity. Vein does not regulate Wg expression but does suppress wing development in the notum, apparently by blocking responsiveness of notum cells to Wg. Ectopic expression of Wg in the notum causes transformation to wing fate (Ng et al., 1996). Activation of Apterous in turn leads to activation of Notch signaling in cells along the dorsoventral (DV) boundary and to induction of *wg* and the *vestigial* boundary enhancer (Diaz-Benjumea and Cohen 1995; Couso et al., 1995; Rulifson and Blair, 1995; Kim et al., 1995; Kim et al., 1996; de Celis et al., 1996; Doherty et al., 1996).

The antagonistic interaction between Vein and Wg in early stages is necessary for the separation of wing and notum, but is not sufficient to explain how the wing field is specified. Vein limits the ability of Wg to induce wing fate in the notum, but does not explain how the size and shape of the wing field are defined by ventrally expressed Wg. Control of Vestigial expression has been proposed to be an important step in this process (Klein and Martinez-Arias, 1998). To date the earliest positively expressed marker for wing fate is the POU-homeodomain protein Nubbin, which is induced in the

presumptive wing field under Wg control in late second instar (Ng et al., 1995; Ng et al., 1996). At this stage, Vestigial is expressed in both presumptive wing and body wall territories. Wg activity also represses expression of Teashirt and Homothorax. Repression of Tsh is important for the establishment of distal wing fate, as ectopic expression of Tsh blocks the Wg expression at the DV boundary and interferes with wing pouch development (Casares and Mann, 2000). Repression of Hth in the distal region is also important. Ectopic expression of Hth in the wing pouch causes defects in the wing (Ryoo et al., 1999; Azpiazu and Morata, 2000; Casares and Mann, 2000). We examine the earliest stages of wing specification with reference to the expression of Nubbin, Tsh and Hth. We find that repression of Tsh in response to Wg and Dpp signaling occurs well in advance of both the onset of Nubbin expression and repression of Hth, which must therefore be considered secondary events in determination of the wing.

MATERIALS AND METHODS

Antibodies

Mouse anti-Nubbin and rat anti-Nubbin (Averof and Cohen, 1997), anti-Vestigial (Williams et al., 1993), rabbit anti-Hth (Kurant et al., 1998) and mouse anti-Wg (Brook and Cohen, 1996) were used as previously described. Rat anti-Hth was raised against His-tagged Hth protein [the construct was a gift from Dr A. Salzberg (Kurant et al., 1998)]. Mouse and rabbit anti-Tsh antibodies are described in (Ng et al., 1996; Wu and Cohen, 2000).

Fly stocks

nub^{1} is a regulatory mutant of *nubbin* that affects expression in the wing disc (Ng et al., 1995), *vg*^{83b27r} is a null allele of *vestigial* (Williams et al., 1993). *act>CD2>Gal4* is described in (Pignoni and Zipursky, 1997). *ap*^{UG035} is a null allele of *ap* (Cohen et al., 1992). *UAS-arm*^{S10} was described in (Pai et al., 1997). *UAS-tkv** (Lecuit et al., 1996) and *UAS-Tsh* (Wu and Cohen, 2000) have been described previously. *wg*^{1} is a disc specific regulatory allele (Sharma and Chopra 1976). *wg*^{17B40lacZ} is a lethal allele caused by insertion of a P-element *lacZ* reporter (FlyBase).

Clonal analysis

nubbin mutant clones were induced at 72- to 96-hour-old larvae of genotype *wHSflp; 30A nub*^{1} *FRT40A/arm-lacZ FRT40A*. 30A homozygotes are viable and do not have defects in the wing. *vestigial* mutant clones were induced at 48-72 hours AEL in larvae of genotype *wHSflp; FRT42 vg*^{83b27r}*/FRT42 arm-lacZ.*

Flip-out clones

arm^{S10}-expressing clones were induced at 24-48, 48-72 and 72-96 hours (first, second or third instar stages) in larvae of the genotype *y w act>CD2>Gal4/y w HSflpI; UAS-arm*^{S10}*/UAS-GFP. tkv**-expressing clones were induced in second and in third instar between 48-72 hours in larvae of the genotype: *y w act>CD2>Gal4/y w HSflpI; UAS-tkv*/UAS-GFP.*

RESULTS

Expression of Teashirt, Nubbin, Vestigial, Wingless and Homothorax in the nascent wing primordium

To understand the early specification of the wing field within the imaginal disc we have examined the patterns of expression of Wg, Tsh, Hth, Vestigial and Nubbin at very early stages. Vestigial is expressed in every cell of the wing disc primordium in the embryo (Williams et al., 1993). In wing discs from early and mid second instar larvae, Vestigial expression has begun to retract from the presumptive notum, but is expressed in both cell layers in the ventral part of the disc (Fig. 1A). At this stage, Hth is expressed in every cell. By contrast, Tsh is expressed in the presumptive body wall but has already begun to be repressed in the future wing pouch (Fig. 1A).

These early gene expression patterns are best visualized by comparing the horizontal optical sections (Fig. 1A) with

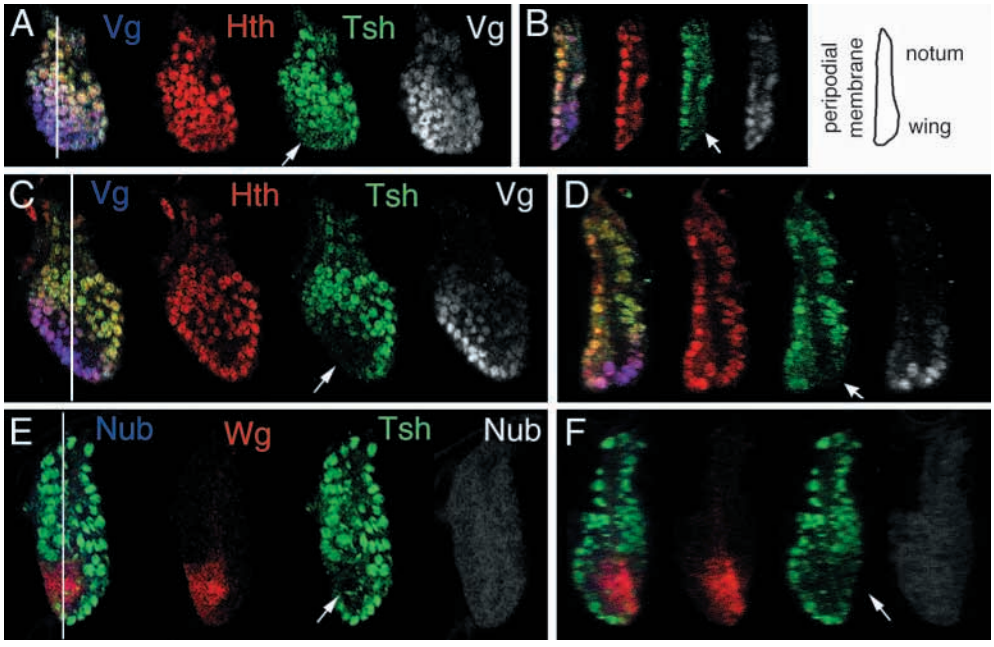

Fig. 1. Early expression of Tsh, Hth, Vestigial, Wg and Nubbin. (A) Expression of Vestigial (blue), Hth (red) and Tsh (green) proteins in an early second instar wing disc. The overlay is show on the left. Single channels are shown on the right. Tsh expression is low in the area indicated by the arrow. The white line indicates the position of the optical cross section shown in B. Cells expressing low levels of Tsh appear purple in the overlay on the left. The diagram on the right indicates the orientation of the optical cross sections. (C) Expression of Vestigial, Hth and Tsh in a mid second instar wing disc. Tsh is repressed in presumptive wing (arrow). The white line indicates the position of the optical cross section shown in D. (E) Expression of Tsh, Nubbin (blue) and *wg-lacZ* (anti-β-gal, red) in a mid second wing disc. Tsh is repressed in presumptive wing (arrow) where *wg-lacZ* is expressed. Nubbin expression is not detected at this stage (white in single channel). (F) Cross-section of E.

optical cross sections that cross the two cell layers (Fig. 1B). The cross sections in Fig. 1B are oriented with the cells that form the peripodial layer on the left side of the lumen. Cells on the right side will form the majority of the wing and body wall. Until mid third instar the epithelium is cuboidal. Later, the peripodial layer becomes a very thin squamous epithelium and the other layer becomes a thick pseudostratified epithelium, which is highly folded (see Fig. 2E). By mid second instar, the patterns have resolved further and the subdivisions become more clear (Fig. 1C,D). Tsh is repressed in the presumptive wing territory. Vestigial is expressed in a larger area, foreshadowing its expression along the DV boundary in the body wall, as well as in the wing. Hth continues to be expressed in all cells. These observations indicate that repression of Tsh and restriction of Vestigial expression are the earliest markers of wing specification. These changes occur well before Hth repression is evident. Although Tsh and Vestigial end up in approximately reciprocal patterns by late second instar, the dynamics of their expression does not suggest that they regulate each other's expression. At the earliest stages they overlap considerably. Tsh then begins to be repressed in a small subset of the region where Vestigial is robustly expressed (Fig. 1A,B).

Wg activity has been implicated in specification of the wing field in the disc (Ng et al., 1996; Wang et al., 2000). The levels of Wg protein expression are too low to be detected by antibody labeling during the second instar, so we made use of a *wg-lacZ* reporter gene to visualize *wg* gene expression. *wg-lacZ* is expressed in the region of the wing disc where Tsh is repressed, during mid second instar (Fig. 1E,F). Repression of Tsh occurs before expression of Nubbin can be detected. By late second instar, signaling between dorsal and ventral compartments induces Wg and Vestigial expression in cells adjacent to the DV boundary (Williams et al., 1993). At this stage Nubbin begins to be faintly detectable within the domain of Tsh repression (Fig. 2A). By early third instar, Vestigial is expressed in a band centered on the DV boundary that extends from the wing primordium into the body wall (Fig. 2B,C). Repression of Hth begins in the presumptive wing and Nubbin expression broadens. Hth and Nubbin still overlap at this stage. Nubbin expression becomes stronger in the presumptive wing pouch during mid and late third instar (Fig. 2D).

As Hth expression retracts from the wing pouch, it resolves into three distinct domains in the proximal region (Fig. 2D,E). Two of the Hth domains overlap with the proximal rings of Wg expression that are observed in the presumptive wing hinge region (Fig. 2F) (Neumann and Cohen, 1996a). It has been reported previously that Hth is regulated by Wg at these late stages (Azpiazu and Morata, 2000; Casares and Mann, 2000). Both rings of Wg expression are distal to the Tsh expression domain (Fig. 2G). The proximalmost ring of Hth, which is regulated by secreted Wg, overlaps the edge of the Tsh domain (arrow Fig. 2E). At this stage, Vestigial and Nubbin expression are centered on the stripe of Wg expression at the DV boundary. Vestigial expression is limited to the distal wing pouch and does not extend as far as the first ring of Hth

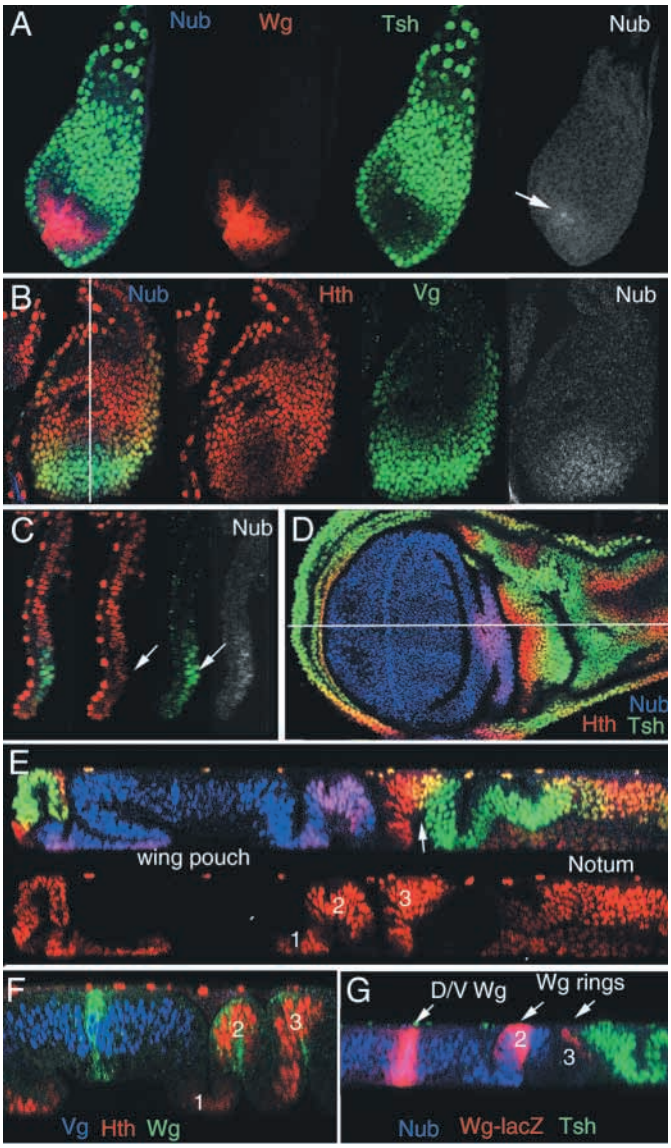

Fig. 2. Expression of Tsh, Hth, Vestigial, Wg and Nubbin at later stages of wing development. (A) Tsh protein (green) is repressed in *wg-lacZ* expressing cells (anti-β-gal, red) in a late second instar disc. Nubbin is faintly detectable (blue in overlay, white in single channel image). (B) Hth (red), Vestigial (green) and Nubbin (blue/white) expression in an early third instar disc. Hth begins to be repressed in the wing pouch. Nubbin is expressed in wing pouch cells. (C) Cross-section of B. Hth is still expressed in some Vestigial-expressing cells (arrow), suggesting that Hth is repressed after Vestigial is expressed. (D) Nubbin, Hth and Tsh expression in a late third instar wing disc. The magnification of D is 50% that of the other panels. The spatial relationships in the proximal regions are better illustrated in the cross-section shown in E. Nubbin is expressed in the wing pouch. Hth expression at this stage is different from continuous expression pattern at earlier stages shown in B,C. Hth is expressed in three separate domains as labeled as 1, 2, 3. Nubbin expression overlaps with Hth domain 1 and 2. Tsh overlaps partly with Hth domain 3 (yellow, arrow). Tsh is expressed proximal to Hth ring 3. Tsh and Hth are co-expressed in the notum. (F) Cross-section of a late third instar wing disc showing Hth, Vestigial (blue) and Wg (green). Vestigial is expressed in the wing pouch. The proximal rings of Wg expression overlap with Hth domain 2 and 3. (G) Cross of a mid-late third instar disc. The *wg-lacZ* rings (anti-β-gal, red) are distal to Tsh expression. 2, 3 denote the positions of Hth rings 2 and 3, which overlap the two rings of *wg* expression. Nubbin overlaps Hth ring 2 and the first *wg* ring.

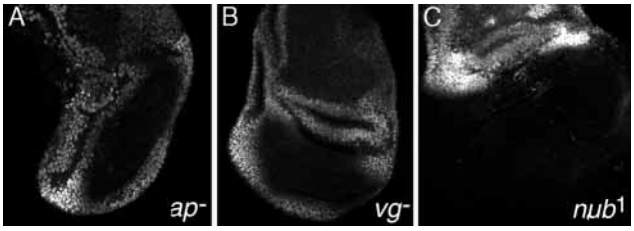

Fig. 3. Tsh is repressed normally in *vestigial*, *apterous* and *nubbin* mutant wings. (A) Tsh expression in an ap^{UG035} mutant wing disc. (B) Tsh expression in a vg^{83b27r} mutant wing disc. (C) Tsh expression in a nub^{1} mutant wing disc.

expression (Fig. 2F). Nubbin extends more proximally, overlapping the first and second rings of Hth and the first ring of Wg expression (Fig. 2G). Tsh expression is proximal to the outer ring of Wg expression, which runs through the base of the wing hinge (Neumann and Cohen, 1996a). Thus, the border of Tsh expression coincides with border between wing and the body wall, whereas Hth is expressed in rings in the wing hinge as well as more proximally in the notum.

Vestigial and Nubbin do not repress Tsh in the wing pouch

The nascent wing pouch is first marked by the repression of Tsh during the second instar. This appears to occur before Wg expression is activated at the DV boundary (Fig. 1). This would suggest that Wg expression at the DV boundary is unlikely to be responsible for repression of Tsh. To test this, we examined Tsh expression in *apterous* null mutant discs. *apterous* activity is required to initiate signaling between dorsal and ventral compartments and to induce Wg expression along the DV boundary. Earlier expression of Wg in the ventral anterior wedge is not affected in *apterous*⁻ mutant discs (Ng et al., 1996). Tsh expression is repressed normally in the rudimentary wing pouch of *apterous* mutant discs (Fig. 3A), indicating that Wg expression at the DV border is not required in order for Tsh to be repressed. Vestigial expression is also not induced at the DV border in *apterous* mutant discs (Williams et al., 1993), suggesting that Vestigial may not be required for Tsh repression. Consistent with this, we find that Tsh expression is repressed normally in the wing pouch of *vestigial* null mutant wing discs (Fig. 3B). Likewise, clones of cells mutant for *vestigial* did not show ectopic expression of Tsh, though they did show ectopic expression of Hth (Fig. 4A). The *vestigial* mutant clones were induced in early second instar, prior to specification of the wing pouch. Comparable results were obtained with clones induced later in development, during second or early third instar and examined in mature third instar discs (not shown) (Azpiazu and Morata, 2000). We also tested clones expressing Vestigial for repression of Tsh. As expected, clones expressing Vestigial did not affect the level of Tsh expression, but reduced Hth levels to some extent (Fig. 4B). Taken together, these observations indicate that Vestigial contributes to repression of Hth, but not to repression of Tsh.

As *nubbin* is also required for normal development of the wing blade, we examined the role of Nubbin in repressing Tsh. Fig. 3C shows that Tsh is repressed normally in the wing pouch in nub^{1} mutants. nub^{1} is a regulatory allele that strongly reduces Nubbin protein expression in the wing pouch (Ng et al., 1995). Likewise, Tsh remains repressed in nub^{1} mutant clones, although Hth can be ectopically expressed in lateral and proximal clones (Fig. 4C). Thus repression of Tsh is an early event that does not require the activity of Vestigial or Nubbin.

Wg and Dpp repress Tsh at early stages

Repression of Tsh appears to be the first identifiable step in specification of wing fate. Previous reports have indicated that ectopic expression of Tsh in the wing pouch interferes with wing development. For example, Wg expression at the DV boundary was strongly reduced by Tsh expression, whereas ectopic expression of Hth had a much weaker effect on Wg expression (Casares and Mann, 2000). Ectopic expression of Tsh in the wing pouch also induced Hth expression in some cells (Casares and Mann, 2000; Azpiazu and Morata, 2000). Wg expression at the DV boundary is necessary for growth and patterning of the wing pouch (Zecca et al., 1996; Neumann and

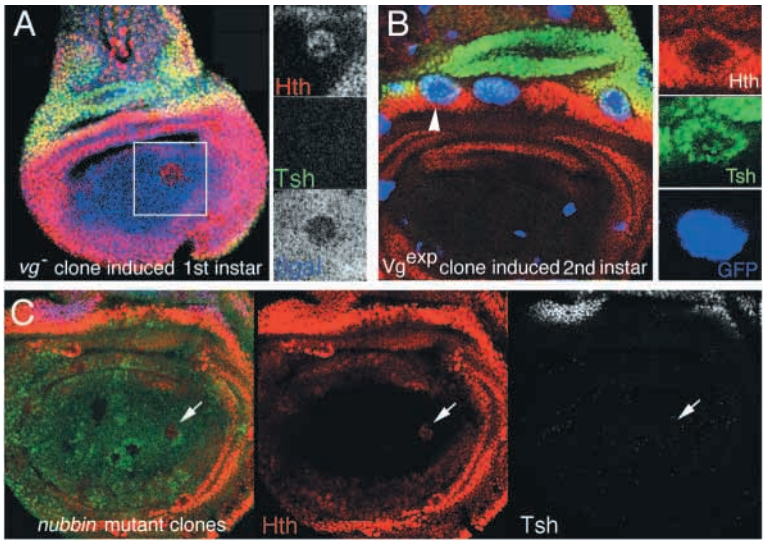

Fig. 4. Tsh and Hth expression in genetic mosaics. (A) vg^{83b27r} mutant clones were induced between 48 and 72 hours after egg laying. Early third instar larvae were examined 2 days after clone induction, so that the majority of clones examined were induced in early second instar. Clones were labeled by absence of β-gal (blue). Hth (red) was ectopically expressed in the clone. Tsh (green) was not. Higher magnification views of the boxed region are shown separately for each channel at right. 11 of 13 clones examined expressed ectopic Hth, but Not Tsh. The relatively large size of the clone shown here may reflect some growth prior to wing pouch specification. (B) Clones expressing Vestigial (Act>Gal4 UAS-Vg UAS-GFP) induced during second instar and examined in mature third instar discs. Vestigial-expressing clones marked by co-expression of GFP (blue). Hth (red) levels were reduced in the mutant cells. Tsh (green) levels were unaffected relative to adjacent wild-type cells. Note that the Vg-expressing clones tend to sort out from the disc (arrowhead) and so the nuclei of the cells are not uniformly in the same focal plane. (C) nub^{1} mutant clones induced in early third instar 72-96 hours after egg laying. Clones were labeled by absence of β-gal. Hth was ectopically expressed in a nub^{1} clone whereas Tsh was not (arrow).

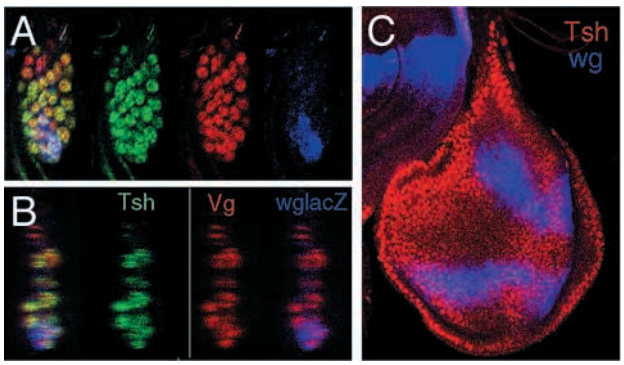

Fig. 5. Tsh is not repressed in *wg* mutant discs in second instar. (A,B) Second instar $wg^{1}/wg^{17en40lacZ}$ wing disc labeled to visualize Tsh (green), Vestigial (red) and wg-*lacZ* (blue). (B) An optical section perpendicular to the lane of the section in A. (C) Third instar disc of the same genotype labeled to visualize Wg (blue) and Tsh (red). Note the uniform expression of Tsh and the symmetric duplication of the characteristic notum stripe of Wg expression.

Cohen, 1996a; Neumann and Cohen, 1997), so repression of Tsh is necessary to allow subsequent wing development.

How is Tsh repressed in the wing during early wing development? We have previously reported that ectopic expression of Wg can repress Tsh in the notum and lead to ectopic wing formation and that wg^{1} mutants cause uniform expression of Tsh throughout the mature wing disc (Ng et al., 1996). This suggested that Wg activity is required in second instar to repress Tsh. To verify this, we examined Tsh expression in early $wg^{1}/wg^{17en40lacZ}$ discs and found that Tsh was not repressed (Fig. 5A,B). We note that $wg^{17en40lacZ}$-expressing cells also expressed Tsh in these discs. This indicates Tsh is indeed ectopically expressed in *wg*-expressing cells. Tsh was not repressed at all in two out of six second instar $wg^{1}/wg^{17en40lacZ}$ wing discs. Tsh was incompletely repressed in two out of six wing discs and repressed normally in two out of six discs. This is consistent with the frequency with which adult wings were missing and replaced by duplicated notum structures in this genotype (26/80 possible wings were affected in 40 $wg^{1}/wg^{17en40lacZ}$ adult flies). Replacement of the wing by a duplication of notum structures is obvious in mature third instar discs (Fig. 5C).

At early stages, Wg and Tsh are expressed in nearly complementary regions of the disc (Fig. 1E,F), suggesting that Wg might repress Tsh directly. To test this, we induced clones of cells expressing a constitutively active form of Armadillo (UAS-Arm^{s10}) in first instar discs and examined their effects on Tsh expression in early third instar discs. Tsh expression was repressed in Arm^{S10}-expressing clones (Fig. 6A). Homothorax was not repressed. Note that these discs were examined before the onset of Wg and Hth expression in the secondary rings that form in the wing hinge. Arm^{S10}-expressing clones induced in second instar and examined in slightly older third instar discs also showed repression of Tsh (Fig. 6B). Hth levels were increased in some clones in these older discs, perhaps reflecting regulation of the Hth rings by Wg, as described previously (Casares and Mann, 2000).

As already noted, Vestigial and Nubbin do not appear to be required for repression of Tsh. We examined Arm^{S10}-expressing clones for ectopic expression of Vestigial and Nubbin to see whether their ectopic expression correlated with

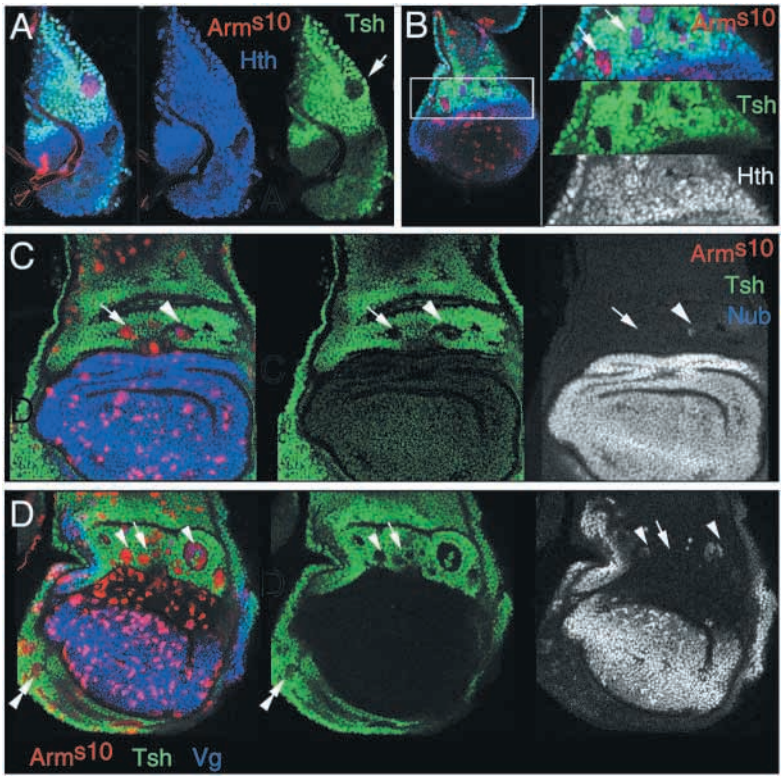

Fig. 6. Tsh is repressed by Wg signaling. arm^{s10}-expressing clones were induced in *y w act>CD2>Gal4/y w HSflpI; UAS-*arm^{S10} */UAS-GFP* larvae 24-48 hours after egg laying (A), 48-72 hours after egg laying (B,C) and 72-96 hours after egg laying (D). arm^{s10}-expressing clones were labeled by co-expression of GFP (red). (A) Optical section of an early third instar wing disc containing a clone induced in first instar. Tsh (green) was repressed autonomously in the clone. Hth (blue) was not. Tsh and Hth channels shown separately at right. (B) Optical section of a mid third instar wing disc containing multiple arm^{s10}-expressing clones. Higher magnification views of the boxed region are shown on the right. Tsh (green) was repressed in arm^{s10}-expressing clones (e.g. arrows). Hth (blue, or white in single channel) expression was not reduced in these clones. In some clones, Hth was upregulated. (C) Optical section of a late third instar wing disc containing multiple arm^{s10}-expressing clones labeled with anti-Tsh and anti-Nubbin (blue, or white in single channel). Most clones repressed Tsh but did not express Nubbin (e.g. arrow). Some clones expressed low levels of Nubbin (arrowhead). (D) Optical section of a late third instar wing disc containing multiple arm^{s10}-expressing clones labeled with anti-Tsh and anti-Vestigial (blue, or white in single channel). Vestigial was expressed in most clones although not in every cell of the clone (arrowheads). Some clones do not express Vestigial (arrow). Tsh was repressed in cells lacking ectopic Vestigial expression.

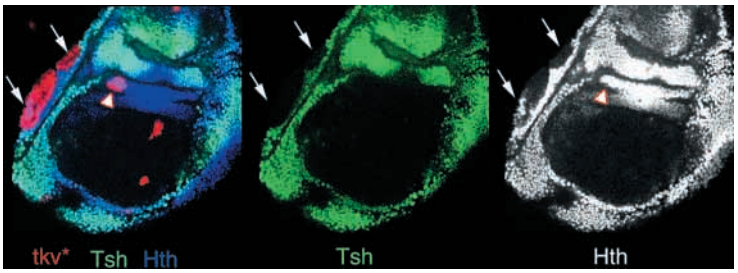

Fig. 7. Tsh is repressed by Dpp signaling. Clones expressing activated Thickveins were induced in larvae of the following genotype: *y w act>CD2>Gal4/y w HSflpI; UAS-tkv* /UAS-GFP.* Clones were induced at 48-72 hours after egg laying. An optical section of a late third instar wing disc is shown. Tsh (green) expression is repressed in *tkv**-expressing clones (arrows). Hth (blue, or white in single channel) is repressed in some cells of the lateral clones. Hth is not repressed by clones in the Hth ring 3 (arrowhead). Cells that express both proteins appear pink.

repression of Tsh. Some Arm^{S10}-expressing clones expressed low levels of Nubbin, others did not (Fig. 6C). Tsh was repressed in these clones regardless of whether Nubbin was expressed. Vestigial was upregulated in Arm^{S10}-expressing clones in the wing pouch and was ectopically expressed in some but not all Arm^{S10}-expressing clones elsewhere (Fig. 6D). Ectopic Vestigial was observed in the hinge region, but did not correlate with repression of Tsh. This confirms the observation that Tsh is not ectopically expressed in *nubbin* or *vestigial* mutant clones and support the view that Vestigial and Nubbin are unlikely to mediate the repressive effects of Wg signaling on Tsh expression.

vein is another candidate for mediating the effects of Wg signaling on Tsh expression. *vein* is a ligand for the EGF receptor that is normally expressed in the presumptive notum region and is required for the specification of dorsal and notum fate (Wang et al., 2000). Specification of the wing pouch by Wg correlates with repression of *vein* expression in the ventral region of the wing disc. We note that although *vein* is not expressed in the ventral pleural region of the wing disc, Tsh expression is also repressed in Arm^{S10}-expressing clones in this region (Fig. 6D, double arrowhead). Thus, we consider it unlikely that the repression of Tsh by Wg signaling is mediated by repression of Vein. This view is supported by the observations of Wang et al. (2000), who found that Tsh is expressed in the notum of $Egfr^{ts}$ and vn^{ts} mutant wing discs.

In view of the finding that Wg and Dpp work together to induce Dll and repress Hth in the leg disc, we wondered whether Dpp signaling is also required for Tsh repression in the wing. To test this, we expressed a constitutively active form of the Dpp receptor Thickveins in clones of cells (Tkv*) (Lecuit et al., 1996). Tsh expression was repressed in some Tkv*-expressing clones in the lateral region of the disc when clones were induced in early second instar (Fig. 7) (larvae dissected 3 days after clone induction). Hth expression was also repressed by Tkv* at this stage, but to a lesser extent. Clones induced in late second-early third instar larvae did not repress Tsh (not shown). These observations suggest that Dpp signaling cooperates with Wg signaling in repression of Tsh in the nascent wing pouch.

The observations presented so far indicate that Wg and Dpp activity are required during second instar to specify wing identity and to repress Tsh expression. To ask whether repression of Tsh is necessary for wing pouch formation, we assessed the effects of preventing repression of Tsh in the early stages of wing specification. Tsh was misexpressed using $scalloped^{Gal4}$. $scalloped^{Gal4}$ is expressed all cells of the wing imaginal disc primordia in the embryo and so directs broad misexpression of genes in the early wing disc. Tsh expression suppressed formation of the wing pouch (Fig. 8A). In optical cross section, Hth expression is evident in all cells of the disc, indicating suppression of wing pouch fate. Thus, forced expression of Tsh can override the effects of Wg and Dpp signaling in repression of Hth. This indicates that repression of Tsh is a necessary primary response to these cues in early wing patterning. The effects of ectopic Tsh were less severe than the wg^{1} mutation, in that Tsh did not cause complete symmetric duplication of the notum, but suppressed formation of the wing pouch. To examine the effects of Tsh on Vg and Nubbin expression, we made use of dpp^{Gal4} to direct Tsh expression in a stripe bisecting the wing pouch. Casares and Mann (Casares and Mann, 2000) have previously reported that Tsh repressed Vg expression. Their experiment addressed repression of Vg at the DV boundary, where it is under Notch control. By eliminating Vg and Wg expression at the DV boundary as well, this has secondary effects on the Wg-dependent expression of Vg in the wing pouch. When expressed using dpp^{Gal4}, to avoid repression of Wg at the DV boundary, we observed little direct repression, if any, of Vg by Tsh in the wing pouch (Fig. 8B). However, Nubbin was repressed in Tsh-expressing cells (Fig. 8B).

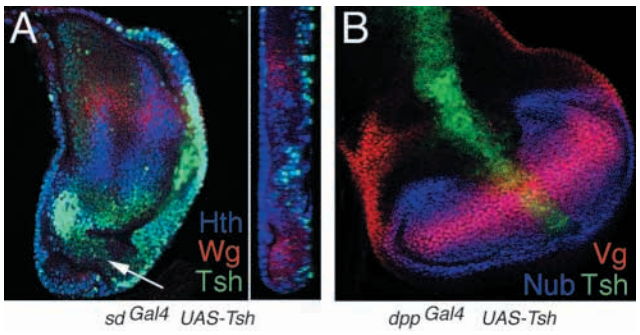

Fig. 8. Repression of Tsh is required for wing pouch formation. (A) $scalloped^{Gal4}$ *UAS-Tsh* wing disc labeled to visualize Wg (red), Hth (blue) and Tsh (green). Note the absence of the wing pouch and the lack of the DV boundary stripe and rings of Wg expression. The folding of the discs makes it difficult to get all nuclei in the same horizontal optical section. The optical cross section at right shows that Hth was expressed in all nuclei, indicating that wing pouch specification has been blocked (B) dpp^{Gal4} *UAS-Tsh* wing disc labeled to visualize Vg (red), Nub (blue) and Tsh (green). Nub was partially repressed in the dorsal and ventral compartments. We also noted stronger repression in a part of the dorsal territory. The basis for this difference is not clear, but we noted weak Tsh expression in much of the stronger repression domain. Perhaps this reflects intrusion of tissue from the hinge region due to ectopic Tsh expression.

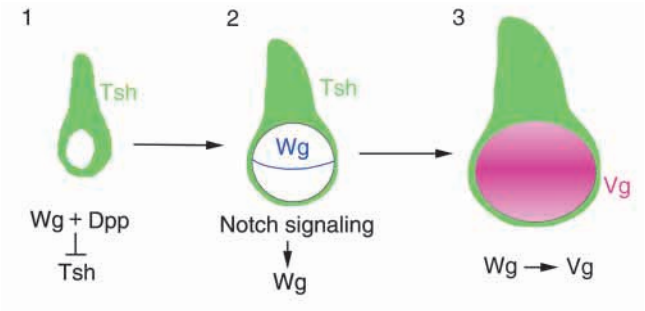

Fig. 9. The three major stages of wing development: (1) repression of Tsh by Wg and Dpp signaling; (2) Loss of Tsh allows Notch-dependent activation of Wg and Vestigial (Vg) boundary enhancer expression at the DV boundary; and (3) long-range Wg gradient induces several genes required for wing growth and patterning, e.g. Vg.

DISCUSSION

Wing patterning can be subdivided into at least three discrete stages (Fig. 9). The earliest observable changes in gene expression patterns that indicate specification of the wing field are repression of Tsh and retraction of Vestigial expression. Wg signaling represses Tsh expression at this stage. Dpp contributes to repression of Tsh. At present, we have only been able to address directly the time at which Dpp acts by comparing the effects of clones induced at different stages. Clones induced in late second or early third instar are ineffective, whereas clones induced in early second instar are able to repress Tsh. Interestingly, Wg and Dpp cooperate to repress Hth in the wing pouch, even though this occurs somewhat later that repression of Tsh (Casares and Mann 2000; Azpiazu and Morata, 2000). These observations support the view that Wg and Dpp act in conjunction to specify the wing field in a manner analogous to the way they cooperate in leg patterning.

The combined actions of Wg and Dpp are responsible for both dorsoventral and proximodistal patterning of the leg (Brook and Cohen, 1996; Jiang and Struhl, 1996; Thiesen et al., 1996; Lecuit and Cohen, 1997). The wing makes use of a different strategy from the leg to control DV patterning and outgrowth from the body wall. After the wing field is established, interaction between D and V cells lead to localized activation of Notch signaling, initially in ventral cells (Ng et al., 1996). Subsequently Notch is activated in cells on both sides of the DV boundary (Diaz-Benjumea and Cohen, 1995; Rulifson and Blair, 1995; de Celis et al., 1996; Doherty et al., 1996; Neumann and Cohen, 1996b). Three separate mechanisms have been implicated in limiting Notch activation to cells adjacent to the boundary (Axelrod et al., 1996; Panin et al., 1997; Fleming et al., 1997; Michelli et al., 1997; de Celis and Bray, 1997; Neumann and Cohen, 1998). Localized Notch activity turns on the vestigial boundary enhancer and Wg expression in cells adjacent to the DV boundary (Diaz-Benjumea and Cohen, 1995; Kim et al., 1995; Neumann and Cohen, 1996b). Subsequently, the long-range Wg morphogen gradient regulates downstream genes, including *Achaete-scute, Dll*, the *vestigial* quadrant enhancer, the Wg receptor DFz2 and possibly other genes, to control wing development (Zecca et al., 1996; Neumann and Cohen, 1996a; Neumann and Cohen, 1997; Cadigan et al., 1998). Wingless also acts at this stage to regulate growth in the wing hinge and in the wing pouch (Neumann and Cohen, 1996a; Johnston and Edgar, 1998). This is mediated in part through Vestigial and its co-factor Scalloped, which are required for cell survival in the wing pouch (Kim et al., 1996; Halder et al., 1998; Liu et al., 2000).

We have presented evidence that repression of Tsh in the earliest phase of wing specification appears to be required for subsequent Notch-dependent induction of Wg at the DV boundary. Casares and Mann (Casares and Mann, 2000) have reported that clones of cells lacking Hth activity cause outgrowth of extra wing tissue along the DV boundary. Our results suggest that this is unlikely to be due to an early role of Hth in specification of the size of the wing field because Hth is expressed in the early presumptive wing well after Tsh is repressed. Instead, Hth appears to act in the second stage in conjunction with Tsh to limit the region in which Notch can activate Wg at the DV boundary. Wg expression at the DV boundary extends proximally into the domain of Hth expression in the anterior wing hinge but does not extend into the Tsh domain. In ectopic expression experiments, Hth has a limited ability to repress Notch-dependent activation of Wg on its own, but is able to do so when co-expressed with Tsh (Casares and Mann, 2000). These observations support the view that Hth cooperates with Tsh during later stages to repress Wg activation. We note that this does not exclude a role for Hth as a co-factor in conjunction with Tsh at earlier stages, but if so, the positional information would seem to derive from regulation of Tsh expression.

Interestingly, Hth and Tsh can also repress the *vestigial* quadrant enhancer, which depends on Wg and Dpp signaling in phase 3 (Casares and Mann, 2000). Homothorax, Tsh and Vestigial appear to form a loop of mutual repression at this stage, as Vestigial also represses expression of Hth (Azpiazu and Morata, 2000). Together, these observations suggest that Wg and Dpp have a complex regulatory interaction with Hth. Their activities repress it in the pouch, perhaps through activation of Vestigial and Scalloped. At the same time, the outer rings of Wg expression are required for Hth expression in the wing hinge (Casares and Mann, 2000; Azpiazu and Morata, 2000). We suggest that regulation of Hth may be secondary to regulation of Tsh in specification of the wing field.

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REFERENCES

Abu-Shaar, M. and Mann, R. (1998). Generation of multiple antagonistic domains along the proximodistal axis during *Drosophila* leg development. *Development* **125**, 3821-3830.

Averof, M. and Cohen, S. M. (1997). Evolutionary origin of insect wings from ancestral gills. *Nature* **385**, 627-630.

Axelrod, J. D., Matsuno, K., Artavanis-Tsakonas, S. and Perrimon, N. (1996). Interaction between wingless and Notch signaling pathways mediated by Dishevelled. *Science* **271**, 1826-1832.

Azpiazu, N. and Morata, G. (2000). Function and regulation of homothorax in the wing imaginal disc of Drosophila. *Development* **127**, 2685-2693.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Brook, W. J. and Cohen, S. M. (1996). Antagonistic interactions between wingless and decapentaplegic responsible for dorsal-ventral pattern in the Drosophila Leg. *Science* **273**, 1373-1377.

Cadigan, K. M., Fish, M. P., Rulifson, E. J. and Nusse, R. (1998). Wingless repression of Drosophila frizzled 2 expression shapes the Wingless morphogen gradient in the wing. *Cell* **93**, 767-777.

Casares, F. and Mann, R. S. (2000). A dual role for homothorax in inhibiting wing blade development and specifying proximal wing identities in Drosophila. *Development* **127**, 1499-1508.

Cohen, B., McGuffin, M. E., Pfeifle, C., Segal, D. and Cohen, S. M. (1992). apterous, a gene required for imaginal disc development in Drosophila encodes a member of the LIM family of developmental regulatory proteins. *Genes Dev.* **6**, 715-729.

Cohen, S. M., Bronner, G., Kuttner, F., Jurgens, G. and Jackle, H. (1989). Distal-less encodes a homoeodomain protein required for limb development in Drosophila. *Nature* **338**, 432-434.

Cohen, S. M. and Jürgens, G. (1989). Proximal-distal pattern formation in *Drosophila*: cell autonomous requirement for *Distal-less* gene activity in limb development. *EMBO J.* **8**, 2045-2055.

Couso, J. P., Bate, M. and Martinez Arias, A. (1993). A *wingless*-dependent polar coordinate system in the imaginal discs of Drosophila. *Science* **259**, 484-489.

Couso, J. P., Knust, E. and Martinez Arias, A. (1995). Serrate and wingless cooperate to induce vestigial gene expression and wing formation in Drosophila. *Curr. Biol.* **5**, 1437-1448.

de Celis, J. F. and Bray, S. (1997). Feed-back mechanisms affecting Notch activation at the dorsoventral boundary in the Drosophila wing. *Development* **124**, 3241-3251.

de Celis, J. F., Garcia-Bellido, A. and Bray, S. J. (1996). Activation and function of *Notch* at the dorsal-ventral boundary of the wing imaginal disc. *Development* **122**, 359-369.

Diaz-Benjumea, F. J., Cohen, B. and Cohen, S. M. (1994). Cell interactions between compartments establishes the proximal-distal axis of *Drosophila* limbs. *Nature* **372**, 175-179.

Diaz-Benjumea, F. J. and Cohen, S. M. (1995). Serrate signals through Notch to establish a Wingless-dependent organizer at the dorsal/ventral compartment boundary of the *Drosophila* wing. *Development* **121**, 4215-4225.

Doherty, D., Fenger, G., Younger-Shepherd, S., Jan, L.-Y. and Jan, Y.-N. (1996). Dorsal and ventral cells respond differently to the *Notch* ligands *Delta* and *Serrate* during *Drosophila* wing development. *Genes Dev.* **10**, 421-434.

Fleming, R. J., Gu, Y. and Hukriede, N. A. (1997). Serrate-mediated activation of Notch is specifically blocked by the product of the gene fringe in the dorsal compartment of the Drosophila wing imaginal disc. *Development* **124**, 2973-2981.

Halder, G., Polaczyk, P., Kraus, M. E., Hudson, A., Kim, J., Laughon, A. and Carroll, S. (1998). The Vestigial and Scalloped proteins act together to directly regulate wing-specific gene expression in Drosophila. *Genes Dev.* **12**, 3900-3909.

Held, L. I. J., Heup, M. A., Sappington, J. M. and Peters, S. D. (1994). Interactions of *decapentaplegic*, *wingless* and *Distal-less* in the *Drosophila* leg. *Roux' Arch. Dev. Biol.* **203**, 310-319.

Jiang, J. and Struhl, G. (1996). Complementary and mutually exclusive activities of Decapentaplegic and Wingless organize axial pattern during Drosophila limb development. *Cell* **86**, 401-409.

Johnston, L. A. and Edgar, B. A. (1998). Wingless and Notch regulate cell-cycle arrest in the developing Drosophila wing. *Nature* **394**, 82-84.

Kim, J., Irvine, K. D. and Carroll, S. B. (1995). Cell recognition, signal induction, and symmetrical gene activation at the dorsal-ventral boundary of the developing Drosophila wing. *Cell* **82**, 795-802.

Kim, J., Sebring, A., Esch, J. J., Kraus, M. E., Vorwerk, K., Magee, J. and Carroll, S. B. (1996). Integration of positional signals and regulation of wing formation by *Drosophila vestigial* gene. *Nature* **382**, 133-138.

Klein, T. and Martinez-Arias, A. (1998). Different spatial and temporal interactions between Notch, wingless, and vestigial specify proximal and distal pattern elements of the wing in Drosophila. *Dev. Biol.* **194**, 196-212.

Kurant, E., Pai, C. Y., Sharf, R., Halachmi, N., Sun, Y. H. and Salzberg, A. (1998). Dorsotonals/homothorax, the Drosophila homologue of meis1, interacts with extradenticle in patterning of the embryonic PNS. *Development* **125**, 1037-1048.

Lecuit, T., Brook, W. J., Ng, M., Calleja, M., Sun, H. and Cohen, S. M. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the Drosophila wing. *Nature* **381**, 387-393.

Lecuit, T. and Cohen, S. M. (1997). Proximal-distal axis formation in the Drosophila leg. *Nature* **388**, 139-145.

Liu, X., Grammont, M. and Irvine, K. D. (2000). Roles for scalloped and vestigial in regulating cell affinity and interactions between the wing blade and the wing hinge. *Dev. Biol.* **228**, 287-303.

Micchelli, C. A., Rulifson, E. J. and Blair, S. S. (1997). The function and regulation of cut expression on the wing margin of Drosophila: Notch, Wingless and a dominant negative role for Delta and Serrate. *Development* **124**, 1485-1495.

Morata, G. and Lawrence, P. A. (1977). The development of wingless, a homeotic mutation of Drosophila. *Dev. Biol.* **56**, 227-240.

Neumann, C. J. and Cohen, S. M. (1996a). Distinct mitogenic and cell fate specification functions of wingless in different regions of the wing Development. *Development* **122**, 1781-1789.

Neumann, C. J. and Cohen, S. M. (1996b). A hierarchy of cross-regulation involving *Notch*, *wingless*, *vestigial* and *cut* organizes the dorsal/ventral axis of the Drosophila wing. *Development* **122**, 3477-3485.

Neumann, C. J. and Cohen, S. M. (1997). Long-range action of Wingless organizes the dorsal-ventral axis of the Drosophila wing. *Development* **124**, 871-880.

Neumann, C. J. and Cohen, S. M. (1998). Boundary formation in the Drosophila wing: the POU-domain protein Nubbin attenuates Notch activity. *Science* **281**, 409-413.

Ng, M., Diaz-Benjumea, F. J. and Cohen, S. M. (1995). Nubbin encodes a POU-domain protein required for proximal-distal patterning in the Drosophila wing. *Development* **121**, 589-599.

Ng, M., Diaz-Benjumea, F. J., Vincent, J. P., Wu, J. and Cohen, S. M. (1996). Specification of the wing by localized expression of wingless protein. *Nature* **381**, 316-318.

Pai, L. M., Orsulic, S., Bejsovec, A. and Peifer, M. (1997). Negative regulation of Armadillo, a Wingless effector in Drosophila. *Development* **124**, 2255-2266.

Panin, V. M., Papayannopoulos, V., Wilson, R. and Irvine, K. D. (1997). Fringe modulates Notch-ligand interactions. *Nature* **387**, 908-913.

Pignoni, F. and Zipursky, S. L. (1997). Induction of Drosophila eye development by decapentaplegic. *Development* **124**, 271-278.

Rulifson, E. J. and Blair, S. S. (1995). Notch regulates wingless expression and is not required for reception of the paracrine wingless signal during wing margin neurogenesis in Drosophila. *Development* **121**, 2813-2824.

Ryoo, H. D., Marty, T., Casares, F., Affolter, M. and Mann, R. S. (1999). Regulation of Hox target genes by a DNA bound Homothorax/Hox/Extradenticle complex. *Development* **126**, 5137-5148.

Sharma, R. P. and Chopra, V. L. (1976). Effect of the wingless (wg1) mutation on wing and haltere development in Drosophila melanogaster. *Dev. Biol.* **48**, 461-465.

Schnepp, B., Grumbling, G., Donaldson, T. and Simcox, A. (1996). Vein is a novel component in the Drosophila epidermal growth factor receptor pathway with similarity to the neuregulins. *Genes Dev.* **10**, 2302-2313.

Theisen, H., Haerry, T. E., O'Connor, M. B. and Marsh, J. L. (1996). Developmental territories created by mutual antagonism between Wingless and Decapentaplegic. *Development* **122**, 3939-3948.

Wang, S. H., Simcox, A. and Campbell, G. (2000). Dual role for Drosophila epidermal growth factor receptor signaling in early wing disc development. *Genes Dev.* **14**, 2271-2276.

Williams, J. A., Paddock, S. W. and Carroll, S. B. (1993). Pattern formation in a secondary field: a hierarchy of regulatory genes subdivides the developing Drosophila wing disc into discrete subregions. *Development* **117**, 571-584.

Wu, J. and Cohen, S. M. (1999). Proximal distal axis formation in the Drosophila leg: primary subdivision into proximal and distal domains by Homothorax, Teashirt and Distal-less expression. *Development* **126**, 109-117.

Wu, J. and Cohen, S. M. (2000). Proximal distal axis formation in the Drosophila leg: distinct functions of Teashirt and Homothorax in the proximal leg. *Mech. Dev.* **94**, 47-56.

Zecca, M., Basler, K. and Struhl, G. (1996). Direct and long-range action of a Wingless morphogen gradient. *Cell* **87**, 833-844.