The Drosophila gene zfh2 is required to establish proximal-distal domains in the wing disc

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ABSTRACT

Three main events characterize the development of the proximal-distal axis of the Drosophila wing disc: first, generation of nested circular domains defined by different combinations of gene expression; second, activation of wingless (wg) gene expression in a ring of cells; and third, an increase of cell number in each domain in response to Wg. The mechanisms by which these domains of gene expression are established and maintained are unknown. We have analyzed the role of the gene zinc finger homodomain 2 (zfh2). We report that in discs lacking zfh2 the limits of the expression domains of the genes tsh, nub, rn, dve and nab coincide, and expression of wg in the wing hinge, is lost. We show that zfh2 expression is delimited distally by Vg, Nub and Dpp signalling, and proximally by Tsh and Dpp. Distal repression of zfh2 permits activation of nab in the wing blade and wg in the wing hinge. We suggest that the proximal-most wing fate, the hinge, is specified first and that later repression of zfh2 permits specification of the distal-most fate, the wing blade. We propose that proximal-distal axis development is achieved by a combination of two strategies: on one hand a process involving proximal to distal specification, with the wing hinge specified first followed later by the distal wing blade; on the other hand, early specification of the proximal-distal domains by different combinations of gene expression. The results we present here indicate that Zfh2 plays a critical role in both processes.

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Introduction

The development of both vertebrate and invertebrate appendages has been the subject of intensive study as it provides excellent systems for examining the cellular and molecular basis of pattern formation (reviewed in (Cohen, 1993; Tickle, 2003). Although evolutionary genetics studies have shown that they employ homologous genes and not strictly homologous structures, and do not share a common origin, studies suggest that vertebrate limbs and arthropod appendages share a basic developmental programme (Pueyo and Couso, 2005). As genetics studies have shown that they employ homologous genes and share a basic developmental programme (Pueyo and Couso, 2005). As most of the genes involved in limb development were first identified in Drosophila, the Drosophila imaginal discs have became attractive and powerful model systems.

The primordium of the Drosophila wing imaginal disc is established during early embryogenesis as a small group of cells that is set aside from the rest of the embryonic cells and remains quiescent during the rest of the embryonic development (Bate and Arias, 1991). During larval development these cells proliferate extensively and in the pupal stage undergo metamorphosis to give rise to an adult dorsal heminotum (the body wall) and a wing (the appendage).

In the wing disc, proximal-distal (P/D) development starts in the second instar larva with expression of the genes elbow (el) and no ocelli (noc) under the joint control of the signalling molecules Wingless (Wg) and Decapentaplegic (Dpp) (Weihe et al., 2004). el and noc (hereafter el/noc) encode zinc-finger proteins with identical expression domains that, in the wing, repress the expression of the body wall gene teashirt (tsh) and promote appendage formation (Abu-Shaar and Mann, 1998; Weihe et al., 2004; Wu and Cohen, 2002). The cells expressing el/noc form a distal region that includes the blade and the hinge of the adult wing, and the cells expressing tsh become the body wall (Azpiazu and Morata, 2000; Casares and Mann, 2000).

At the same developmental stage, activation of Notch signalling along the boundary of expression of the dorsal selector gene apterous (ap) (Blair et al., 1994; de Celis et al., 1996; Diaz-Benjumea and Cohen, 1993; Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996) induces expression of vestigial (vg) (Couso et al., 1995; Kim et al., 1996; Klein and Martinez-Arias, 1998; Williams et al., 1991, 1994). Within cells in which tsh has been repressed, Vg activates a set of genes whose products are required for P/D development (del Álamo Rodríguez et al., 2002). nubbin (nub) (Ng et al., 1995), rotund (rn) (St. Pierre et al., 2002), defective proventriculus (dve) (Koelzer et al., 2003; Nakagoshi et al., 2002), nab (Clements et al., 2003; Terriente Félix et al., 2007) and
Four-jointed (fj) (Cho and Irvine, 2004) are expressed in nested circular domains of different cell diameters centered in the distal-most region of the wing disc (Fig. 1A). Activation of these various genes takes place at different developmental stages: first, vg is activated at the boundary of ap expression (Klein and Martinez-Arias, 1998; Williams et al., 1991, 1993); then nab and rn are activated by Vg in middle/late second instar larvae (del Álamo Rodríguez et al., 2002); thereafter dve, nab, and fj are also activated by Vg in early third instar (Cho and Irvine, 2004; Koelzer et al., 2003; Terriente Félix et al., 2007); finally activation of the vg quadrant enhancer permits amplification of the range of vg expression (Zecca and Struhl, 2007a,b).

From an early stage of larval development these patterns of expression generate ring-like domains defined by different combinations of gene products. nab and rn encode transcription factors containing POU and zinc-finger domains, respectively (Ng et al., 1995) (St. Pierre et al., 2002), dve encodes a novel type of homeobox protein originally shown to be required for development of the larval proventriculus (Fuss and Hoch, 1998) (Nakagoshi et al., 1998), nab encodes a transcriptional co-factor that repress Rn transcriptional activity (Clements et al., 2003; Terriente Félix et al., 2007), and fj encodes a type II transmembrane protein (Villano and Katz, 1995).

The ring of cells that express nab and rn but do not express nab activates transcription of the vg inner ring (IR) (Fig. 1A) (Couso et al., 1993). A specific enhancer, the spade enhancer (Couso et al., 1994; Neumann and Cohen, 1996), drives vg expression in the IR. Its activation requires a non-cell autonomous signal coming from the adjacent vg-expressing cells (del Álamo Rodríguez et al., 2002; Liu et al., 2000). Wg, in turn, promotes cell proliferation and increases the number of cells in the different P/D domains (Neumann and Cohen, 1996) (Klein and Arias, 1999). As a result of the cell proliferation, the ring of cells that expresses vg moves proximally, farther from the vg-expressing cells. At this stage, continued wg expression is controlled by a different mechanism that requires the gene homothorax (hth) (Azpiazu and Morata, 2000; Casares and Mann, 2000; del Álamo Rodríguez et al., 2002; Liu et al., 2000). Although the different domains of nab, rn, dve and nab expression all depend on Vg, it is not known how they are generated and maintained, since strict clonal

![Fig. 1. Temporal and spatial patterns of expression of zfh2, nab, rn, dve, nab and wg in wild-type wing discs. (A) (left) A third instar larva wing imaginal disc labelled with Wg antibody, and (right) the expression domains along the P/D axis of the genes analyzed in this work. Vertical bars indicate domains of expression; the horizontal bar indicates the wing margin. The presumptive regions of the main areas of the adult mesothorax are indicated. N: notum, H: wing hinge, W: wing blade, OR: wg outer ring, IR: wg inner ring, WM: wing margin, D: dorsal wing, V: ventral wing. (B, B') Rn (rn-lacZ; blue), Zfh2 (red) and Nab (green) expression in late second (B) and third (B') instar larvae. (C, C') Rn (blue), Dve (red) and Nab (green) expression in late second (C) and third (C') instar larvae. (D, D') Wg (blue), Zfh2 (red), and Dve (green) (D-D'); and Wg (blue), Zfh2 (red), and Nab (green) (E-E') expression in late second (D, D) and early (D', D') instar larvae. Dve and Nab expression are always complementary to that of Zfh2, and delimit distally expression in the IR of Wg, which is co-expressed with Zfh2 (arrows). Optical cross-sections of a third instar larval disc are shown on the right. (F, F') Wg (blue), Rn (rn-lacZ; red) and Nab (green) expression in late second (F) and third (F') instar larva. The IR is always expressed in the cells that express Rn and do not express Nab. Single channels of the area selected in panel F are shown separately. An optical cross-section of a disc is shown on the right. (G, G') Zfh2 (red) and Vg (green) expression in early (G) and late (G') third instar larvae. Arrows indicate the gap between the two expression domains. Here and in all later figures: white bars indicate the relative sizes of the discs; dorsal is up and anterior left; the blue channel, when presented as a single channel, is shown in black and white.
After three subcutaneous immunizations with 0.7 mg of protein each time, the rabbits (Nagel et al., 2002), to yield gagccg-3′zfh2

Generation of UASZfh2RNAi

Generation of anti-Nub antibody

same result. Thus, to illustrate El/Noc expression, we only show the results with the subcloned in Bluescript vector to form BH-INTzfh2. The RNAi construct was injected into y w;FL眼睛 embryos.

Production of genetic mosaics

Mis-expression experiments using the ActSc-y->Gal4 UASGFP chromosome were carried out by inducing FRT/FLP recombination with heat shock for 12 min at 34.5 °C. Loss-of-function clones were induced by 1 h heat shock at 37 °C. The experiments involving UASzfh2RNAi were performed with two copies of the transgene, and the larvae were grown at 29 °C.

Preparation of adult cuticle

Pharate adults were extracted from their pupal capsules and dissected in a 1:3 glycerol/ethanol solution; the wings were expanded in 10% KOH, dehydrated in ethanol and mounted in eupalar.

Results

Fig. 1A shows a third instar larva wing disc stained with anti-Wg antibody and a schematic representation of the expression patterns of the genes relevant to this work. To better understand the role of nub, rn, nub, dve and zfh2 we examined their patterns of expression at different stages of development of the disc. Expression of nub and rn was first detected in the wing disc in middle/late second instar larvae as a small group of cells that correspond to the distal-most region of the wing. At this early stage the nub expression domain was already slightly broader than that of rn, and zfh2 was expressed in a domain that included the nub and rn domains (Fig. 1B, and Supplementary Fig. S1A). Later on zfh2 expression occurred in a ring of cells that partially overlapped with the cells expressing nub and rn (Fig. 1B’ and Supplementary Fig. S1A’). This late domain of zfh2 expression corresponds to the cells that would form the adult wing hinge (Whitworth and Russell, 2003).

Expression of dve and nab started in late second/early third instar larvae as a small group of cells in the centre of the n-expression domain (Fig. 1C and Supplementary Fig. S1B) (Koelzer et al., 2003; Terriente Félix et al., 2007). The dve-expression domain was initiated earlier and was one or two cells broader than that of nab, but several cells narrower than that of rn (Fig. 1C, C’ and Supplementary Fig. S1B, B’). Double staining for zfh2/dve or zfh2/nab revealed a close spatial and temporal correlation between distal loss of zfh2 expression and activation of dve and nab expression. Hence, throughout larval development the expression domain of zfh2 was almost complementary to those of dve and nab (Figs. 1D, E and Supplementary Fig. S1C, C’). The limits of expression of dve and nab were not sharp; their expression faded away proximally and zfh2 expression faded away distally, so that, at the boundary, cells expressed low levels of both.

During third instar larval development, wg was expressed distally in a stripe of cells that would form the adult wing margin and, proximally, in two rings, the inner (IR) and outer (OR) ring (Couso et al., 1993) (Fig. 1A). wg IR expression started in early third instar larvae as a ring of cells that co-expressed zfh2, nub and rn but did not express dve or nab. Hence, the limit of expression of the IR coincided proximally with the proximal limit of rn expression and distally with both the distal limit of zfh2 and the proximal limits of dve and nab (Figs. 1A, F). These patterns of expressions remained unchanged during the rest of larval development (Fig. 1F and Supplementary Fig. S1D). It has been suggested that expression of dve and nab depends on a non-autonomous signal from vg-expressing cells (Koelzer et al., 2003; Terriente Félix et al., 2007). Although in early third instar larvae the proximal limit of vg expression coincided with those of dve and nab, the dve and nab domains broadened as development progressed. Thus, at the end of larval development, there was a gap between the proximal limit of vg expression and the distal limit of zfh2 expression, which always abutted the dve and nub domain (Fig. 1G, G’).

Materials and methods

Fly stocks

The following fly strains were used: enGal4, cGal4, UASGFP; dppMS209R20Gal4 (Wildier and Perrimon, 1995); UASrn, n-lacZ, ribGal4 and Df(3R)vinD10 (St. Pierre et al., 2002); zfh2UASflanked (gift from I. Rodriguez); nub1β (Ng et al., 1995); nub1α-lacZ, UASnub (Terriente Félix et al., 2007); UASdsve (Koelzer et al., 2003); UASvgo (Bourouis, 2002); LPS10Gal4, nubMS209R20 (Calleja et al., 1996); ribGal4887F, y w hsFLP, ActSc-y->Gal4 UASGFP (Gos et al., 1997); dveGFP970H; Df(2R)w; this is a small deletion that completely removes the gene; Exelixis); UASnub (Wu and Cohen, 2000); UASnubRNAi (Zirin and Mann, 2007). The following strains were used: ribGFP2O (Hoodless et al., 1996) and UASnub (Nellen et al., 1996). The nubα-lacZ allele was generated by imprecise excision of nubGal4MS209R20, it is homozygous lethal with a strong phenotype in double heterozygotes nubR5 nubR5; it is homozygous lethal with a strong phenotype in double heterozygotes uasGFP and, as a consequence, activation of wg in the IR by Rs is repressed. Our results suggest that appendage development occurs by a combination of two different strategies. On one hand, specification involves a proximal to distal progression: body wall, wing hinge, and wing blade. On the other hand, there is early specification of the P/D domains defined by different combinations of gene expression. These early-defined domains are later expanded by Wg-promoted cell proliferation. Our results indicate that Zfh2 plays a crucial role in both strategies.

Immunostaining

Imaginal discs were fixed and stained by standard techniques for confocal microscopy. The specific antibodies used were: mouse anti-β-galactosidase (1:2000) (Promega #Z3781); rat anti-Ds (1:5000) (Yang et al., 2002); rabbit anti-Dve (1:200) (Nakagoshi et al., 1998); rat anti-FJ (1:1000) (Strutt et al., 2004); rabbit anti-Nab (1:1000) (Terriente Félix et al., 2007); rat anti-El (1:100), guinea pig anti-Noc (1-100) (Weihe et al., 2004); rabbit anti-Nab (1:1000); rabbit anti-Tib (1:30) (Wu and Cohen, 2000); guinea pig anti-Vg (1:100); mouse anti-Wg (1:25) (D.S.H.B. #404D) and rat anti-Zfh2 (1:100) (Forlini et al., 1991). All experiments carried out so far indicate that el and noc share the same regulatory regions (Weihe et al., 2004) and our own results). Several experiments performed using either anti-El or anti-Noc antibodies gave the same result. Thus, to illustrate El/Noc expression, we only show the results with the anti-Noc antibody, which gave better resolution.

Generation of anti-Nub antibody

Two rabbits were immunized with purified complete Nub-6His-tagged protein. After three subcutaneous immunizations with 0.7 mg of protein each time, the rabbits were bled and sera tested on imaginal discs. Both sera gave rise to the same expression pattern. We confirmed that the antibodies recognized Nub by immunolabeling dppGal4/UASnub wing discs. In addition, the expression patterns revealed by the antibodies were identical to those obtained with the nubGal4-UASGFP line.

Generation of UASzfh2RNAi

The Gal4 inducible construct for RNA interference of zfh2 was made as follows: a 456 bp fragment from the zfh2 coding sequence was amplified by PCR with 5′-cagcatcattctcccgtgtagcgcgc-3′ upper primer and 5′-ctccgacgccgcctgtctgaccttcc- lower primer. The BamHI-KpnI PCR fragment was cloned in phBBS vector (Nagel et al., 2002), to yield phBBSzfh2. Then the BamHI-SacI fragment of phBBSzfh2 was subcloned in Bluescript vector to form BS-INTzfh2. Finally, the Sall-KpnI fragments from pBBS-zfh2 and BS-INTzfh2 were cloned together at the KpnI site of pUAS vector, generating pHASzfh2. The RNAi construct was injected into y w;FL眼睛 embryos.

Restrictions have not been found (Irvine and Rauskolb, 2001; Zirin and Mann, 2007).

In this report we investigate the role of another gene involved in P/D development: zfh2 finger homedomain 2 (zfh2). zfh2 encodes a 3005 amino acid protein containing sixteen zinc-fingers and three homeodomain motifs, and immunoblot analysis suggests that a single polypeptide is produced (Fortini et al., 1991). It has been suggested that zfh2 is required for P/D wing development, but no clear function has been assigned to it (Whitworth and Russell, 2003). Here we show that it plays an important role in establishing the different ring domains along the P/D axis. In wing discs lacking Zfh2 the expression domains of nab, dve, rn, and nub are almost coincident, and expression of wg in the wing hinge is lost. We analyze the underlying mechanisms and conclude that Zfh2 is required to delimit expression of nab and dve in the wing hinge. In discs lacking Zfh2 expression of these genes is proximally expanded and, as a consequence, activation of wg in the IR by Rs is repressed. Our results suggest that appendage development occurs by a combination of two different strategies. On one hand, specification involves a proximal to distal progression: body wall, wing hinge, and wing blade. On the other hand, there is early specification of the P/D domains defined by different combinations of gene expression. These early-defined domains are later expanded by Wg-promoted cell proliferation. Our results indicate that Zfh2 plays a crucial role in both strategies.

Materials and methods
**Fig. 2.** Adult phenotypes and patterns of gene expression in wing discs lacking Zfh2 function. (A-B′) Wild-type (A, A′) and LP30Gal4–UASzfh2RNAi (B, B′) adult wings. (A′, B′) Magnified views of the wing hinges. Most of the hinge area is deleted; the tissue that remains is very disorganized and only a few sclerites can be identified. Two-arrowed bars indicate the affected area. Black bars indicate the relative sizes of the wings. (C–C′) Wg (blue), Nab (red), and en–GFP (green) expression in enGal4–UASzfh2RNAi UASGFP wing discs of late (C) and early (C′) third, and late second (C′), instar larvae. Note that both the IR and the OR are lost (arrowheads). Nab expression is proximally expanded (arrows in panels C and C′), and more cells express nab in the posterior (p) compartment in early discs (arrow in panel C′). (D) Wg (blue), Nab (red) and ci–GFP (green) expression in a ciGal4–UASzfh2RNAi UASGFP wing disc of a late second instar larva. More cells express nab in the anterior compartment (arrow). (E) Dve (red) expression in an enGal4–UASzfh2RNAi UASGFP wing disc of a third instar larva. Dve expression is proximally expanded (arrow). (F) Vg (red) and Fj (green) expression in an Lp30Gal4–UASzfh2RNAi disc. Expression is not altered. (G) Rn (m-lacZ; red), Nab (green) and en–GFP (blue) expression in an enGal4–UASzfh2RNAi UASGFP wing disc. Nab is mis-expressed in all the m-expressing cells (arrow). Optical cross-sections of the anterior (a) and the posterior (p) compartments are shown. (H) Schematic representation of the results shown in this figure. Vertical bars represent domains of expression in wild-type (black) and zfh2RNAi-carrying (grey) wing discs. Horizontal bar indicates the wing margin. (a) anterior and (p) posterior compartment.

zfh2 is required to specify domains in the proximal-distal axis of the wing hinge.

We sought to analyze the function of Zfh2 in P/D wing development. No null alleles for zfh2 are available. However several P-element insertions in this gene have similar wing phenotypes, and homozygotes for any of them are poorly viable and have a deletion in the wing hinge (Whitworth and Russell, 2003). This phenotype is similar to that caused by loss of wg expression in the IR but affects more proximal tissue (Couso et al., 1994). It has been reported that wg expression is not altered in a zfh2 mutant background (Whitworth and Russell, 2003). In mutants for the allele zfh2MS209R20, generated by mobilization of the zfh2MS209 p-element insertion, we observed that expression of nab expanded proximally and wg expression in the IR was partially lost (Supplementary Fig. S2A). In order to obtain a stronger zfh2 mutant phenotype we generated UASzfh2RNAi transgenic flies (see Materials and methods). To express the construct we first used as driver a Gal4 insertion in zfh2 (LP30Gal4); its pattern of expression, detected by UASGFP, was identical to that revealed by anti-Zfh2 antibody. The expression of Zfh2 in LP30Gal4–2xUASzfh2RNAi was variable; most discs showed no expression and some showed weak expression (Supplementary Fig. S2B). Most of the transgenic individuals died as late pupae before eclosion; only a minority developed into adults and these did not expand their wings. The LP30Gal4–UASzfh2RNAi wings displayed a stronger zfh2 phenotype than those previously reported. There was a larger deletion in the wing hinge and the remaining tissue was very disorganized with only a few sclerites identifiable (compare Figs. 2A, A′ and B, B′). In these discs, both wg IR and OR expression were mostly lost, although the phenotype had lower penetrance in the OR (Supplementary Fig. S2C). This phenotype was better observed when we drove the UASzfh2RNAi with engrailed-Gal4 (enGal4), which is only expressed in the posterior compartment. In those discs, wg expression in both the IR and the OR was lost in the posterior compartment (Fig. 2C).

We next analyzed the expression of dve and nab. In enGal4–UASzfh2RNAi late third instar wing discs, nab expression was proximally expanded in the posterior compartment (arrow in Fig. 2C). This expansion was also observed in discs of mid and early third instar larvae, where the effect was even stronger (Fig. 2C′, C′). We confirmed these results by mis-expressing zfh2RNAi in the anterior compartment under the control of cubitus interuptus-Gal4 (ciGal4–UASzfh2RNAi). In this experiment, we observed an increased number of nab-expressing cells in the anterior compartment (Fig. 2D). The same expansion of nab expression was also observed in clones of cells expressing zfh2RNAi (Act5C–Gal4–UASzfh2RNAi UASGFP), although with lower penetrance, probably due to the low efficiency of the RNAi-induced knock-down. In these discs, when observed, both the mis-expression of nab and the repression of wg were cell autonomously restricted to cells of the clones (Supplementary Fig. S2D–E). dve expression was also expanded proximally in both enGal4–UASzfh2RNAi and ciGal4–UASzfh2RNAi wing discs (Fig. 2E and not shown). As both dve and nab expression depends on Vg, we next examined vg expression...
In Lp30Gal4-UASzf2RNAi wing discs and found it was not affected (Fig. 2F).

We next looked at the expression of the gene four-jointed (fl). It has been reported that fj influences the expression of wg in the wing hinge (Cho and Irvine, 2004). We did not observe changes in the expression of fj in Lp30Gal4-UASzf2RNAi wing discs (Fig. 2F).

Rn is a transcriptional activator required for activation of the wg IR, and functions as a transcriptional repressor in combination with Nab, thereby switching off wg IR expression (Terrientê Félix et al., 2007). We tested whether the loss of Wg in zf2 discs is due to the expansion of nab expression in the IR domain, and found that this was indeed the case. In enGal4-UASzf2RNAi wing discs the expression patterns of rm and nab almost coincided (Fig. 2G). This was also observed in zf2RNAi-expressing clones (ActSC-Gal4-UASzf2RNAi UASGFP), where we observed that nab expression was not altered and that the expansion of nab expression within the clone cells was restricted to the rm-expressing cells (Supplementary Fig. S2E).

We next looked at the expression of genes whose wild-type expression domains expand more proximally than the IR of wg: nub, el and tsh. In enGal4-UASzf2RNAi wing discs, the expression domains of nub, rm and dve almost coincided, especially in the dorsal compartment. This could be easily seen in optical cross-sections (Figs. 3A, B) and in earlier discs (Fig. 3C). We also examined the expression of tsh and el/noc. These two genes display complementary patterns of expression and the boundary between them defines the notum/wing hinge boundary. In enGal4-UASzf2RNAi wing discs el/noc expression was partially lost in the hinge, although its expression in the wing margin was not affected (3D). The expression of tsh was expanded distally. We observed a partial rescue of el/noc expression in the posterior cells located close to the anterior compartment (arrowhead in Fig. 3D). We also looked at the expression of the gene dachous (ds). ds encodes a large protocadherine (Brodsly and Steller, 1996; Clark et al., 1995); its expression domain is very similar to that of zf2 (Rodriguez, 2004), and it reported to be required for correct activation of wg in the IR (Cho and Irvine, 2004). However expression of ds did not seem to be altered in enGal4-UASzf2RNAi wing discs (Fig. 3E).

In summary, taking as a reference the proximal limit of the rm expression domain, the absence of zf2 had the effect of expanding proximally the expression domains of nab and dve and reducing nub expression. Thus, in zf2 discs, the expression domains of nab, rm, dve and nab were almost identical, and wg expression in both the IR and the OR was lost. These results are summarized in Figs. 2H and 3F.

Zfh2 represses the expression of nab

The results presented so far suggest that Zfh2 plays a role in P/D development. We next wished to study the phenotypic effects of mis-expressing zf2. The molecular characteristics of zf2 do not permit mis-expression experiments, since the large size of the gene has prevented the isolation of a full-length cDNA. However, it has been reported that zf2 is distally mis-expressed in nab discs (Whitworth and Russell, 2003). We confirmed this result in wing discs of the strong regulatory allele nab1. In nab1 discs zf2 was mis-expressed in many of the cells of the wing pouch (Fig. 4A). In these discs nab expression was lost or reduced in the areas where zf2 was mis-expressed, but expression of dve, vg and fj was not affected (Figs. 4A-D). To confirm that the loss of nab in nab1 discs is due to zf2 mis-expression, we distally expressed UASzf2RNAi (nab1 elGal4pros15; UASzf2RNAi). Under these conditions wild-type expression of nab in the pouch was restored (Fig. 4E). These results suggest that activation of nab expression in early third instar larvae requires distal repression of zf2. In effect, a detailed examination of nab expression indicated that nab was strongly expressed in the distal wing, weakly expressed in the notum, and not expressed at all in the zf2 expression domain (Fig. 4F).

In nab discs expression of wg is lost in the IR and expanded in the wing margin (Fig. 4G) (Ng et al., 1995). Strikingly, we observed that expression of zf2RNAi in nab discs driven by dveGal4 (nab1 dveGal4/ nab1; UASzf2RNAi) restored the normal expression of wg in both the IR and the wing margin (Fig. 4H). This suggests that these phenotypes are caused by zf2 mis-expression. It is worth noting that the rescue of the IR is non-cell autonomous, since the IR was
We next wanted to analyze the mechanism that drives the expansion of nab and dve expression in discs lacking zfh2. We observed that in enGal4-UASzfh2RNAi wing discs both the nab and dve-expression domains expanded into the rn expression domain (Fig. 2G), but vg expression was not affected. Both nab and dve depend on Vg (Koelzer et al., 2003; Terriente Félix et al., 2007). Hence, some additional factor may activate them outside the vg-expression domain. To see whether this factor was Rn we made use of the dppGal4 driver to mis-express rn in a stripe of cells through the centre of the wing disc (dppGal4-UASrn UASGFP). In this experiment both nab and dve were ectopically activated in the dpp domain in a cell-autonomous manner (Figs. 5A, B). To obtain additional insight we looked at the expression of nab and dve in rn zfh2 discs (rn

If the expansion of the dve and nab expression domains requires Rn, the rn mutant phenotype should be epistatic over zfh2 one. This is what happened; in that background neither nab nor dve were detected outside the vg-expression domain (Fig. 5C), which proves that the proximal expansion of nab and dve-expression domains observed in zfh2 discs requires Rn. Thus, we conclude that in discs lacking zfh2, Rn is able to activate dve and nab.

Since rn expression also depends on Vg we considered the possibility that in wild-type discs Rn mediates the activation of dve and nab by Vg. However, discs homozygous for a null allele of rn (rn

Nevertheless, we observed a reduction in nab expression levels in clones of cells mutant for rn (Supplementary Fig. S2F), indicating that, in the distal wing, both Rn and Vg activate nab expression. Finally, we tested the possibility that Dve mediates the activation of nab, but nab expression was unaffected in dve mutant clones (data not shown).

Nub and Dpp signalling are required for distal repression of zfh2 expression

The domains of gene expression in the P/D axis are not maintained by lineage. This suggests that a cross-regulatory genetic network maintains the expression domains. In this context, and to have a better understanding of zfh2 function, we attempted to analyze the mechanisms that delimit zfh2 expression.

After the second instar larval stage, expression of zfh2 is not observed in the distal-most region of the wing disc. It has been proposed that zfh2 expression is distally repressed by the combined action of Nub and Vg (Whitworth and Russell, 2003). However, this idea does not explain the fact that nab, vg and zfh2 are co-expressed in the lateral-most regions of the wing disc (Figs. 5G, G'). In order to further analyze the mechanisms that regulate zfh2 we first analyzed clones homozygous for the lethal allele nab5, since previous analyses had been carried out using the weak allele nab2. Out of 42 nab5 clones found in the distal wing disc, zfh2 was mis-expressed in 32 clones; in 18 cases zfh2 was expressed in all the cells of the clone; and in 14 cases expression was either weak, or affected only the lateral-most cells of the clones. Most of the clones showing complete or partial zfh2 mis-expression were located laterally, and the clones showing no expression were located centrally (Figs. 6A, A').

As mentioned above, nab is expressed together with zfh2 in the wing hinge, suggesting that Nub has a co-repressor. Since we observed that zfh2 de-repression in nab clones was stronger in clones located more laterally than in clones located in the centre of the disc, we considered Dpp signalling a likely candidate. We therefore made clones of a strong allele of the Dpp receptor tkv. These clones have poor viability but we found that they mis-expressed zfh2 in the centre of the wing disc, suggesting that Dpp signalling is involved in zfh2 repression (arrowhead in Fig. 6B). We confirmed this result by mis-expressing Dpp and finding that Dpp was able to repress zfh2 activated outside the dve-expression domain, as happens in wild-type discs (Fig. 1D). Most of the individuals carrying this genetic combination died as late pupae, but we were able to recover several adult flies (six individuals) and expand their wings. The sizes of the wings indicated that the nab phenotype was partially suppressed (see Discussion) (Figs. 4I, J).
Nevertheless, since nub and dpp were expressed along with zfh2 in the centre of the disc, there must be an additional factor whose expression is restricted to the distal area where zfh2 is repressed. Indeed Vg mis-expression repressed zfh2 (dppGal4 UASvg) (Supplementary Fig. S2G) (Whitworth and Russell, 2003). It seems therefore that distal repression of zfh2 requires the joint action of Nub, Vg and Dpp signalling.

The expression patterns of zfh2 and vg are not complementary (Fig. 1G’), which suggests that some gene downstream of Vg actually represses zfh2. dve and nab are obvious candidates as their expression domains abut that of zfh2. We therefore mis-expressed nab and dve in the zfh2 expression domain. zfh2 expression turned out not to be affected in clones of nab-expressing cells (Act5C-Gal4 UASnab) (data not shown) but it was cell autonomously repressed in clones of dve-expressing cells (Act5C-Gal4 UASdve) (Fig. 6D). Interestingly, these clones were round with smooth borders, suggesting that their cells have altered affinity and minimize contact with their neighbouring cells. The above observations imply that the distal repression of zfh2 expression observed in late second instar larvae is caused by activation of dve, acting in concert with Nub and Dpp signalling. To confirm this inference we made dve loss-of-function clones. However zfh2 expression proved to be unaltered in the dve mutant clones (Fig. 6E). These clones were made using a small deficiency that removes the gene, and we confirmed by immunoassay that dve expression was not detectable in the mutant cells. We then generated dve Minute+ clones in mid/late first instar larvae (40±12 h A.E.L.). These clones have a proliferative advantage over the rest of the cells, so that by the stage at which dve is activated (late second instar larvae), there should be

Fig. 5. Zfh2 represses the activation of dve and nab by Rn. (A-B) Nab (A) and Dve (B) expression in dppGal4 UASrn UASGFP wing discs. Both Nab and Dve are mis-expressed (arrows). (C) Wg (blue) and Nab (red) expression in rm35 Lp30Gal4 UASzfh2RNAi UASGFP wing discs. The proximal expansion of Nab expression observed in zfh2 discs is not observed in these discs. In this experiment Nab expression is restricted to the domain of Vg expression, as detected by a fold in the epithelium coincident with the limit of Vg expression. (D-E) Wg (green) and Dve (red) (D) or Nab (red) (E) in rm35 wing discs. The IR is missing and expression of Nab and Dve is reduced proximally. In panel D the distal expression of Dve is out of focus.

Fig. 6. The combined action of Nub, Vg and Dpp signalling limits distal Zfh2 expression. (A-A’) Zfh2 (red) expression in nubR5 clones labelled for the lack of green staining. Zfh2 is mis-expressed in all the mutant cells in the lateral clones (arrows), but the central clones do not show Zfh2 mis-expression (arrowhead). (B) Zfh2 (red) expression in thy1AC clones (lack of green staining). In these clones Zfh2 expression is expanded both proximally (arrow) and distally (arrowhead). (C) Zfh2 (red) expression in vgBEGal4 UASdppGFP. Zfh2 is repressed (arrows). (D) Zfh2 (red) expression is repressed in the clones of dve-expressing cells (green) (Act5C-Gal4 UASdve UASGFP). (E-F) Zfh2 (red) is not mis-expressed in dve Minute+ clones (arrows in panel E) or in dve Minute clones (arrows in panel F). Clones are identified by the absence of green staining (UbiGFP) (E) or by antibody against Dve (green) (F). In panels A, B, D high magnifications of the selected areas are shown on the right.
a significant number of cells that will never express dve in the clones. zfh2 was not distally de-repressed even under these conditions (Fig. 6F).

The combined action of Tsh and Dpp signalling defines the proximal limit of the zfh2 expression domain

We next ask how the proximal limit of the zfh2 expression domain is defined. It has been suggested that zfh2 expression is first activated by Wg and thereafter maintained by autoregulation (Whitworth and Russell, 2003). However, expression of zfh2 was not affected in rm20 wing discs, where Wg IR expression is lost (Fig. 5D), or when Wg signalling was compromised by overexpression of the ubiquitously expressed serine-threonine protein kinase encoded by shaggy (sgg) (Diaz-Benjumea and Cohen, 1994; Ruel et al., 1993; Siegfried et al., 1992) (Fig. 7A). It has been reported that expression of elinoc in late wing discs depends on Wg (Weilhe et al., 2004), and we noted that expression of elinoc was absent in the experiment of Fig. 7A, indicating that Wg signalling was being prevented. Moreover, suppression of zfh2 expression did not affect the expression of zfh2Gal4 (LP30Gal4-UASzfh2RNAi UASGFP), suggesting that there was no autoregulation.

The proximal limit of zfh2 expression is coincident with the distal limit of the tsh domain in the centre of the disc, whereas they are co-expressed laterally in late discs (Fig. 7B). It was therefore to be expected that the combined action of Tsh and Dpp signalling would repress zfh2. To test this notion we activated the Dpp pathway ectopically by clonal mis-expression of the tkv receptor (Act5C-Gal4-UASTkv UASGFP). Only the proximal-most clones, which probably express tsh, were able to repress zfh2 (Fig. 7C). To confirm this result we generated larger clones by mis-expression of the activated version of tkv (Act5C-Gal4-UASTkvOP UASGFP) (Hoodless et al., 1996). These clones repressed zfh2 only in the tsh-expressing cells (Fig. 7D, D′), suggesting that Dpp signalling is able to repress zfh2 expression but requires Tsh. We performed three additional experiments to confirm this idea. First, we made enGal4-UASTshRNAi wing discs and found that zfh2 was proximally expanded (Fig. 7E, compare with wild-type zfh2 expression in Fig. 7B). Second, we made clones of tsh-expressing cells in the zfh2 expression domain, and observed that ectopic expression of tsh was able to repress zfh2, although only partially (Fig. 7F). Third, we found that in tkv mutant clones zfh2 was mis-expressed proximally (arrow in Fig. 6B). Together these observations suggest that the combined action of Tsh and Dpp signalling defines the proximal limit of zfh2 expression.

Discussion

In this report we analyzed the contribution of the gene zfh2 to P/D wing development, and the genetic mechanisms that regulate its expression. The evidence that we present indicates on one hand that zfh2 expression is delimited proximally and distally by complex regulatory mechanisms, and, on the other, that P/D wing development depends on two different strategies, and zfh2 is involved in both.

Two different mechanisms delimit the expression of zfh2 proximally and distally

zfh2 expression in the wing disc shows a dynamic pattern (Whitworth and Russell, 2003). It has been reported that zfh2 is first activated by the joint action of Wg and Dpp signalling and later

Fig. 7. The combined action of Tsh and Dpp signalling defines the proximal limit of zfh2 expression. (A) Zfh2 (red) expression is not affected in clones of sgg-expressing cells, identified by green labelling (Act5C–Gal4–UASsgg UASGFP). (B) Zfh2 (red) and Tsh (green) expression in a third instar larva wing disc. Zfh2 and Tsh expression are complementary in the centre of the disc (arrow) but overlap laterally (arrowhead). An optical cross-section of the centre of the disc is shown on the right. (C) Zfh2 (red) expression in clones of tkv-expressing cells (green) (Act5C–Gal4–UASTkv UASGFP). Zfh2 expression is lost but only in the proximal-most clones (arrows) that presumably lie in the tsh-expressing domain. Distal clones with no effect on Zfh2 expression are indicated (arrowheads). (D, D′) Tsh (blue) and Zfh2 (red) expression in clones expressing an activated version of the tkv receptor (green) (Act5C–Gal4–UASTkvOP UASGFP). Zfh2 is lost from the clones that lie in the Tsh domain (arrows), but not from the distal-most clones (arrowhead). (E) Zfh2 (red) and enGFP (green) expression in enGal4–UASTshRNAi UASGFP. Zfh2 expression is proximally expanded in the posterior compartment (arrow). (F) Zfh2 (red) expression in clones of tsh-expressing cells (green) (Act5C–Gal4–UASTsh). Zfh2 is partially lost in these clones. The high level of tsh expression in these clones probably compensates for the low level of Dpp signalling in the lateral clones. In the lateral-most clones the effect is weaker. In panels C–F high magnifications of the selected areas are shown on the right.
repressed by the combined action of Nub and Vg (Whitworth and Russell, 2003). The experiments presented here indicate first, that Dpp signalling is involved in distal repression of zfh2, and second, that Vg is not a direct repressor. Lateral mis-expression of dpp repressed zfh2 expression, and clones mutant for the tkv receptor, in which Dpp signalling is compromised, de-repressed zfh2 in the centre of the wing.

On the other hand, vg mis-expression in the centre of the wing disc repressed zfh2, but the zfh2 and vg-expression domains are not complementary, and Vg is supposed to be a type of co-activator protein (MacKay et al., 2003). This suggested that a gene downstream of Vg might mediate zfh2 repression. We tested Dve and Nab as candidates for this repression. The domains of expression of both genes are always complementary to that of zfh2, but only dve mis-expression repressed zfh2, and dve lack-of-function clones did not de-repress zfh2. These observations support the view that distal repression of zfh2 requires a complex mechanism involving Dpp signalling, Nub, and a gene downstream of Vg. If Dve is also involved, we would have to invoke an additional factor redundant with it. dve encodes a putative transcription factor containing a novel class of homeodomain that is intermediate between POU and otd class homeodomains (Nakagoshi et al., 1998). Alignment with other known homeodomain proteins in the fly identified 16 genes (Nakagoshi et al., 1998). Of these, the gene Cf1a/vvl/dfr (Anderson et al., 1995) is the only one with a pattern of wing expression capable of providing the same function as dve. However, in our hand, lack-of-function and mis-expression experiments failed to indicate that Cf1a/vvl/dfr plays any role in repressing zfh2 expression (data not shown).

We also examined the mechanism that delimits proximal zfh2 expression. Our findings indicate that zfh2 is proximally repressed by the combined action of Tsh and Dpp signalling: first, tsh and zfh2 always had complementary expression domains, less in lateral areas of late discs, where Dpp signalling is low or null; second, loss of Tsh expanded zfh2 proximally, and tsh-expressing clones downregulated zfh2; and third, clones of a constitutively active form of the Tkv receptor repressed zfh2, while loss-of-function clones of tkv de-repressed zfh2. It has been shown that Tsh can bind directly to DNA to repress expression of its targets (Alexandre et al., 1996), and that the Dpp-downstream transcription factor Mad acts in combination with other transcription factor (Xu et al., 1998). Thus, in the absence of additional molecular data, we propose that Tsh and Mad act together to repress zfh2.

Wing development progresses from proximal to distal

We have presented evidence that, in wing disc development, the early domain of cells that lack Tsh corresponds to the presumptive wing hinge. We have also presented evidence that the presumptive
wing-blade domain is specified later and requires distal repression of zfh2 (Whitworth and Russell, 2003).

First, in discs lacking zfh2 the wing hinge was not specified. This could be observed in early discs, in which the wg IR was not activated and the various domains of gene expression characteristic of the hinge were not formed, and in the adult wing, in which most of the hinge domain was deleted. Second, in nub discs, in which zfh2 was distally mis-expressed, the wing blade was strongly reduced. The partial rescue of the nub phenotype obtained by distal expression of Zfh2RNAi suggests that the mis-expression of zfh2 is, if not the unique, a main cause of the phenotype. These findings suggest that maintaining expression of zfh2 in the distal-most domain of the wing has dramatic consequences for development of the wing blade. So far, we have only identified one Zfh2-target gene, nab. But the lack of nab expression observed in the wing blade of nub discs does not explain its phenotype (Terriente Félix et al., 2007). Thus, a better understanding of the causes of the nub phenotype will require the identification of other genes whose distal expression is controlled by Zfh2.

It is worth noting that although the wing hinge domain is specified earlier, it is not required for the subsequent specification of the wing blade. Thus, the lack of a wing hinge observed in zfh2 mutants or in the spade alleles of wg (Couso et al., 1994), does not affect the development of more distal structures.

\textit{Zfh2 is required to establish proximal-distal nested domains}

The results that we have presented here indicate that Zfh2 plays an important role in the specification of nub, rn, dve and nab domains.

We have observed that in discs lacking Zfh2 the domains of expression of nub, rn, dve and nab were coincident, and that expression of vg and fi was not affected (Fig. 8A). We also observed that: in discs lacking Zfh2 expression of dve and nab was expanded proximally to all the rn-expressing cells; \( \text{rn} \) mis-expression activated expression of both dve and nab; expression of dve and nab was restricted to the vg-expressing cells in \( \text{rn} \) mutant discs, and in \( \text{rn} \) mutant clones nab expression was reduced in the vg-expression domain. Together, all these findings strongly indicate: first, that Rn activates the expression of nab and dve, and second, that Zfh2 represses their activation. In doing so, Zfh2 delimits the domain of expression of these two genes, which allows activation of the wg IR (Fig. 8B).

We also observed that the expression of wg in both the IR and the OR was lost in discs lacking Zfh2. It is known that the activation of the wg IR requires Rn and that Nab represses Rn transcriptional activity (Terriente Félix et al., 2007). This would explain the loss of the wg IR, since in discs lacking Zfh2 all the \( \text{rn} \)-expressing cells express nab. The cause of the loss of the OR is more difficult to understand, since the mechanisms that control its expression are not known.

In discs lacking Zfh2 the \( \text{nub} \) expression domain narrowed and \( \text{tsh} \) expression was distally expanded to the \( \text{rn} \)-expression domain. In addition, the expression of el/nac in the wing hinge was lost. It is known that el/nac represses \( \text{tsh} \) and that its expression in a ring of cells in the wing hinge requires Wg. Thus, the loss of the wg OR would expand distally the expression of \( \text{tsh} \), which would repress \( \text{nub} \). Why does \( \text{tsh} \) expression not extend further distally than the \( \text{rn} \)-expression domain? Two observations can provide an answer: first, it has been reported that the Polycorn group of genes maintains distal repression of \( \text{tsh} \) (Zirin and Mann, 2004). Second, clonal mis-expression of \( \text{dve} \) represses \( \text{tsh} \) expression (data not shown). Either of these observations would explain why the distal expansion of \( \text{tsh} \) does not go beyond the limit of the \( \text{rn}/\text{dve} \)-expression domain.

\textit{Two different strategies drive the proximal-distal development of the wing}

The results that we have presented indicate that the development of the wing discs in the P/D axis involves two different strategies, both of which require Zfh2 (Fig. 8C). On one hand, proximal to distal progression specifying first the proximal-most domain of the appendage (wing hinge), defined by zfh2 expression, followed by the distal-most domain (wing blade), where zfh2 expression is repressed. On the other hand, progressive specification of the P/D domains, whose maintenance requires a complex regulatory genetic network that involves Zfh2 (Fig. 8B). Our findings have revealed the complexity of the mechanisms involved in P/D wing development, but further analysis is needed to understand how the generation of these domains of gene expression is related to the complex morphology of the adult wing hinge.

Vertebrate limb development in P/D axis is poorly understood. The classical model suggest that limb mesenchymal cells form successively more distal domains, and the different cell fates along the P/D axis are determined by a clock-like mechanism that records the time that mesenchymal cells spend in the progress zone. More recently, an alternative model has been suggested according to which the main regions along the P/D axis are specified at the same time in the early limb, and then expand at different times to form the complete limb (reviewed in (Tabin and Wolpert, 2007)). The results that we present here for \textit{Drosophila} are compatible with both models.

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\textbf{Appendix A. Supplementary data}


\textbf{References}


