

Dpp signalling is a key effector of the wing-body wall subdivision of the *Drosophila* mesothorax

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SUMMARY

During development, the imaginal wing disc of *Drosophila* is subdivided along the proximal-distal axis into different territories that will give rise to body wall (notum and mesothoracic pleura) and appendage (wing hinge and wing blade). Expression of the Iroquois complex (Iro-C) homeobox genes in the most proximal part of the disc defines the notum, since Iro-C⁻ cells within this territory acquire the identity of the adjacent distal region, the wing hinge. Here we analyze how the expression of Iro-C is confined to the notum territory. Neither Wingless signalling, which is essential for wing development, nor Vein-dependent EGFR signalling, which is needed to activate Iro-C, appear to delimit Iro-C expression. We show

that a main effector of this confinement is the TGFβ homolog Decapentaplegic (Dpp), a molecule known to pattern the disc along its anterior-posterior axis. At early second larval instar, the Dpp signalling pathway functions only in the wing and hinge territories, represses Iro-C and confines its expression to the notum territory. Later, Dpp becomes expressed in the most proximal part of the notum and turns off Iro-C in this region. This downregulation is associated with the subdivision of the notum into medial and lateral regions.

Key words: Iroquois complex, Dpp signalling, Imaginal wing disc, Notum development, *Drosophila melanogaster*

INTRODUCTION

A salient problem in development is how groups of equivalent cells are allocated to different developmental fates. In *Drosophila*, the study of imaginal discs has provided substantial insights. During the larval stages, each one of the pair of imaginal wing disc grows from approximately 40–50 cells to 50,000 cells. Later, during metamorphosis, the discs undergo eversion and differentiation to form the mesothoracic body wall (notum and mesopleura) and the dorsal mesothoracic appendages (the pair of wings, each composed of wing hinge and wing blade). However, the allocation of cells to the different territories that give rise to these morphologically distinct structures occurs much earlier, during late first or second instar, when the discs have at most a few hundred cells.

Still, the earliest subdivisions of the wing discs do not correspond to these territories but to compartments defined by cell lineage restrictions (García-Bellido et al., 1973) (reviewed by Mann and Morata, 2000). The first subdivision, into anterior (A) and posterior compartments (P), is inherited from the embryo and it is established by the expression of the selector genes *engrailed* and *invected* in the P compartment. A dorsal-ventral (DV) compartmental subdivision, orthogonal to the AP one, occurs during the early-mid second instar. This subdivision is established by the expression of the gene *apterous* (*ap*) in the D compartment. The expression of a

selector gene confers identity to the cells of a compartment. Cells from apposing compartments do not intermingle because of differential affinities. In addition, compartment borders are sources of signalling molecules that organize both cell proliferation and patterning of the entire disc (for reviews, see Brook et al., 1996; Teleman et al., 2001; Vincent and Briscoe, 2001).

According to current thinking (reviewed by Klein, 2001), the subdivision of the wing disc along the proximal-distal axis into body wall (notum) and appendage (wing) is effected, during the early second instar, by the Wingless (Wg) and the EGFR signalling pathways. The Wg molecule accumulates in the most distal part of the disc (Couso et al., 1993; Ng et al., 1996) and instructs cells to repress the ubiquitously expressed zinc-finger transcription factor gene *teashirt* (*tsh*) (Wu and Cohen, 2002) and activate wing-specific genes like *nubbin* (*nub*), *vestigial* (*vg*) and *scalloped* (*sd*) (Ng et al., 1996; Williams et al., 1993), thus specifying the wing blade territory. Specification of the medial region of the disc, which will give rise to the dorsal wing hinge, requires Wg, *homothorax* and *tsh*, although it is unclear how the actions of these genes are integrated (Azpiazu and Morata, 2000; Casares and Mann, 2000; Klein and Martínez-Arias, 1998). More is known about the specification of the proximal region of the disc that gives rise to the notum. Here, the neuregulin Vein (Vn) molecule is expressed, activates the tyrosine kinase EGF receptor, and this autonomously turns on notum genes like the three members of

the Iroquois Complex (Iro-C) (Simcox et al., 1996; Wang et al., 2000; Zecca and Struhl, 2002a; Zecca and Struhl, 2002b). The Iro-C genes, *araucan* (*ara*), *caupolican* (*caup*) and *mirror* (*mirr*), encode related homeodomain proteins conserved from worms to vertebrates (reviewed by Cavodeassi et al., 2001). Their expression in the most proximal region of the second instar wing disc is essential for notum specification, since clones of Iro-C⁻ cells induced early within this territory acquire the identity of the adjacent distal region, namely, the proximal wing hinge and differentiate structures characteristic of this region (tegula, sclerites, etc) (Diez del Corral et al., 1999). Thus, the early domain of expression of Iro-C defines the extent of the notum territory.

An antagonistic interaction between the Wg and the EGFR pathways might explain the confinement of Iro-C expression to the proximal region of the wing disc. Thus, it has been proposed that, in the second instar disc, Wg signalling represses *vn*. Since Wg signalling is strongest in the distal part of the disc, it should restrict expression of *vn* to the proximal part of the disc (Wang et al., 2000). Then, activation of the EGFR pathway would be maximal in this territory and this would activate Iro-C within it. Hence, the early domain of expression of Iro-C, and therefore the prospective notum territory, would ultimately be delimited by the negative input of Wg emanating from the distal region of the disc.

In this work, we further analyze the control of the early expression of Iro-C in the proximal region of the wing disc. Our findings argue against Wg signalling being the main negative regulator of Iro-C expression. Moreover, while we confirm that the Vn/EGFR signalling pathway is necessary for Iro-C activation, our data, in agreement with recent findings (Zecca and Struhl, 2002a), indicate that the availability of Vn does not restrict Iro-C expression to the prospective notum. This appears to be accomplished, instead, by signalling mediated by the BMP2/4 homolog Dpp. *dpp* is expressed in a stripe of A cells abutting the AP compartment boundary from very early larval stages (Burke and Basler, 1996), an expression essential for growth and patterning of the wing disc in its AP axis (reviewed by Affolter et al., 2001; Dahmann and Basler, 1999; Podos and Ferguson, 1999; Serrano and O'Farrell, 1997). However, Dpp had not been implicated, until now, in the initial territorial subdivision of the disc along the proximal-distal axis. We find that during the early-mid second larval instar, the Dpp pathway is active only in the wing and hinge territories. This activity defines, by repression, the distal border of the Iro-C domain and confines the expression of Iro-C to the notum territory. Later, *dpp* becomes expressed in the most proximal part of the notum and turns off Iro-C in this region. This downregulation is associated with the subdivision of the notum into medial and lateral regions (Calleja et al., 2000).

MATERIALS AND METHODS

All the mutant alleles and transgenes are described in FlyBase (<http://flybase.bio.indiana.edu:82/>). *wg^{CX3/wg^{CX4}}* larvae were raised at 18°C, a condition in which all wing discs showed the double notum phenotype. These discs, at second instar, did not have detectable Wg protein. *MS248-Gal4* driver (Sánchez et al., 1997) is expressed in the prospective notum at least from second instar discs (see Fig. 2H,I).

Mitotic recombination clones and misexpression experiments

Mitotic recombination clones homozygous for the null *tkv^{Δ12}* allele were induced by the FLP-FRT technique (Xu and Rubin, 1993) at 24±12 hours after egg laying (AEL) for 1 hour at 37°C. The clones were revealed by staining with anti-β-galactosidase antibody in larvae of the genotype *y w hsFLP122; tkv^{Δ12} FRT40A/Minute(2L) arm-lacZ FRT40A*. Clones of cells overexpressing *tkv^{QD}* were obtained by treating *y hsFLP122; Actin>y⁺>Gal4, UASlacZ/+; UAS-tkv^{QD}/+* larvae for 8 minutes at 37°C either at 36±12 hours AEL (after egg laying) or 72±12 hours AEL. The clones were revealed by staining with anti-β-galactosidase antibody. Similarly, clones of cells overexpressing *Axin* were obtained by treating *y hsFLP122; Actin>y⁺>Gal4, UAS-GFP/+; UAS-Axin/+* larvae for 5 minutes at 37°C at 36±12 hours AEL and larvae were developed at 25°C. Larvae expressing transgenes by means of the Gal4 system were raised at 29°C (*UAS-dpp, UAS-brinker, UAS-dad, UAS-vn*), 25°C (*UAS-dTCFA* and *UAS-raf^{DN}*), or 18°C (*UAS-ras1^{V12}*).

Histochemistry

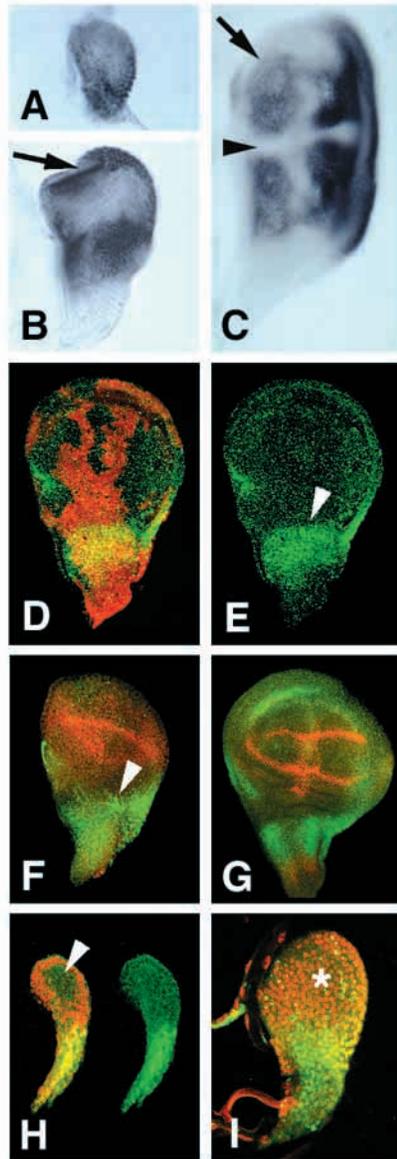
Imaginal discs were dissected and stained as described previously (Gómez-Skarmeta et al., 1995). Primary antibodies were: rabbit and mouse anti-β-galactosidase (Cappel and Promega); rat anti-Iro-C (Diez del Corral et al., 1999), which reacts with the Araucan and Caupolican proteins; rabbit anti-pMad (Tanimoto et al., 2000); mouse anti-Omb (a gift from G. O. Pflugfelder); mouse anti-Wg, mouse anti-Nub and rabbit anti-Tsh (kindly provided by M. S. Cohen); guinea pig anti-Senseless (a gift from H. J. Bellen); mouse anti-Ptc (from I. Guerrero). Secondary antibodies were from the Jackson Laboratory and Amersham. Probe to detect *vn* mRNAs was prepared using the DIG RNA labelling kit (Roche), as described by the supplier. Whole-mount in situ hybridizations were performed essentially as described previously (Jiang et al., 1991).

RESULTS

Wg and Vn/EGFR signalling are unlikely candidates to delimit early notal Iro-C expression

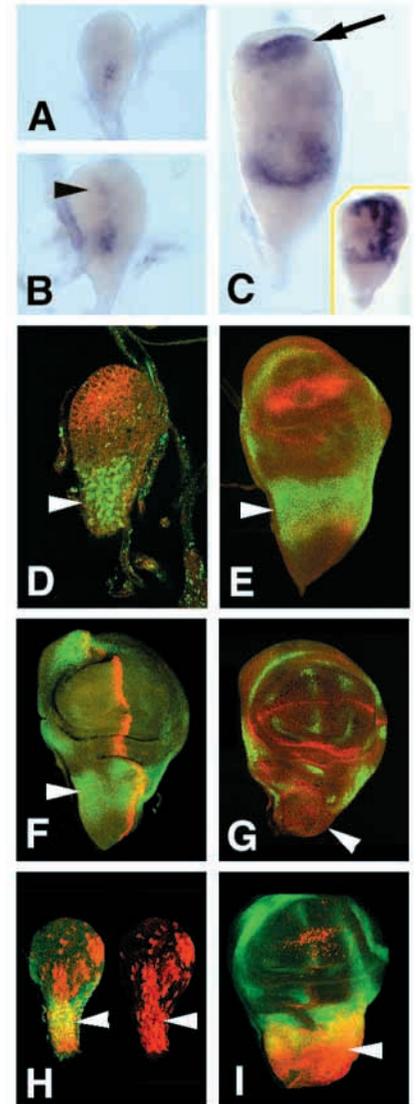
Wg signalling, emanating from the distal-most part of the disc, appears to repress *vn* and thereby confine *vn* expression to the proximal part of the disc (Wang et al., 2000). Since Vn/EGFR signalling is a requisite for Iro-C expression (Wang et al., 2000; Zecca and Struhl, 2002a), it seemed possible that Wg might ultimately be responsible for restricting expression of Iro-C to the notum territory. Moreover, it is well known that early depletion of Wg (as in *wg^{CX3/wg^{CX4}}* discs) prevents wing development and promotes formation of a second ectopic notum (Couso et al., 1993; Ng et al., 1996), a result that could imply the breakdown of the regulation that confines Iro-C expression to the proximal region of the disc and, therefore, the expansion of the domain of Iro-C expression. However, when examining second instar *wg^{CX3/wg^{CX4}}* discs from larvae raised at 18°C (a condition in which all wing discs showed, at the third instar, the double notum phenotype), we found that Iro-C expression was still restricted to the proximal part of the disc (Fig. 1A). This suggested that such an expansion had not occurred. Later, in the early third instar, a separate expression of Iro-C, clearly distinguishable from an expansion of the extant Iro-C domain, was turned on in distal regions of the disc (Fig. 1B). During the third instar, this distal expression evolved revealing the formation of the ectopic notum (Fig. 1C). When the notum duplication was complete, the Iro-C expressions in the extant and ectopic nota were similar to that of a wild-type notum (compare

Fig. 1. Wg signalling does not delimit the notal Iro-C domain. (A-C) Domains of Iro-C expression in second, early third and late third instar $wg^{CX3/wg^{CX4}}$ wing discs, respectively. Arrows indicate ectopic nota; arrowhead, residual hinge territory. (D,E) Early third instar disc with clones of cells overexpressing *UAS-Axin* (red). The clones did not affect the distal border of Iro-C expression (arrowhead in green channel image, E). (F) Early third instar disc expressing *UAS-dTCFΔ* driven by *dpp^{disk}-Gal4*. The distal border of Iro-C expression (green) was not appreciably disturbed. Arrow points to region of maximal *UAS-dTCFΔ* expression. Red channel, Wg counterstaining. (G) An older disc similarly expressing *UAS-dTCFΔ* showed abnormal pattern of Wg (red, compare with Fig. 2G) in the wing pouch, indicating the activity of the construct. Control flies expressing this or the *UAS-Axin* construct showed typical *wg* insufficiency phenotypes. (H) Second instar wild-type disc stained for Tsh (red) and Iro-C (yellow or green; green channel shown at right). Note the absence of Tsh protein from the prospective wing pouch (arrowhead). (I) Older late second instar $wg^{CX3/wg^{CX4}}$ wing disc, stained as in H. Tsh is almost not removed from the distal part of the disc (asterisk), indicating the failure of the residual Wg to specify the wing territory (Wu and Cohen, 2002). Compare also with Fig. 4I.



with Fig. 4E) and were separated by a narrow gap of Iro-C non-expressing tissue (Fig. 1C), presumably a residual hinge (Diez del Corral et al., 1999; Gómez-Skarmeta et al., 1996). This further indicated that the depletion of Wg did not modify the distal border of the Iro-C domain and that this residual hinge would be independent of Wg signalling. Still, it should be stressed that $wg^{CX3/wg^{CX4}}$ is a strong hypomorphic mutant combination and that, conceivably, residual Wg activity might still confine Iro-C expression to the proximal regions of the disc. However, this residual activity was essentially incapable in repressing *tsh* in the distal part of the disc, the domain closest to the normal Wg source (Fig. 1I, compare with 1H and Fig. 4I),

Fig. 2. Vn/EGFR signalling does not delimit the notal Iro-C domain. (A-C) Domains of *vn* expression in second, early third and mid third instar $wg^{CX3/wg^{CX4}}$ wing discs, respectively. Arrowhead in B indicates initial ectopic *vn* expression; arrow in C, *vn* expression in ectopic nota. Inset: late third instar $wg^{CX3/wg^{CX4}}$ wing disc showing symmetrical domains of *vn* expression (reduced magnification). (D,E) Extent of the Iro-C domain (green; arrowheads) is essentially not affected in *UAS-vein*; *ap-Gal4* (misexpression in the D compartment) second and third instar discs, respectively (compare with Fig. 4A,E). Wg (wing pouch marker) is in red. In parallel experiments, no significant effects were observed using the *C765*, *tsh-Gal4*, *omb-Gal4*, *dpp^{disk}-Gal4* and *MS1096* drivers. In E, the relatively large extent of the Wg domain in the anterior-posterior axis indicates that *ap* was already expressed in the dorsal compartment (Ng et al., 1996) and, therefore, that the *ap-Gal4* driver was active in this disc. (F) Third instar disc overexpressing *UAS-ras1^{V12}* in the anterior compartment (*ptc-Gal4* driver). Extent of notal Iro-C domain (green; arrowhead) is unaffected. Red: Ptc marker. (G) Notal Iro-C expression (green) is inhibited and notum territory is reduced (arrowhead, compare with E or F) in *UAS-raf^{DN}/MS-248Gal4* discs. Red: Wg marker. Inhibition of Iro-C was also observed using the driver *dpp^{disk}-Gal4*. (H,I) Expression of the *MS-248Gal4* driver, as revealed by *UAS-lacZ* (red; separate red channel is shown in H on the right), in second and late third instar wing discs, respectively. Counterstaining: Iro-C protein (green). Note strong expression of driver in the proximal region of the discs (arrowheads).



suggesting that it was ineffective in the patterning of the disc (*tsh* repression is the earliest known effect of Wg signalling in the wing disc) (Wu and Cohen, 2002). We further verified that Wg depletion did not expand the Iro-C domain by examining its distal border in clones of cells (induced in the first larval instar) that overexpressed the Wg signalling pathway antagonist Axin (Willert et al., 1999), or by expressing the dominant negative form of the pathway effector dTCF (van de Wetering et al., 1997)

with the *dpp^{disk}-Gal4* driver. In both cases, the border was not significantly affected (Fig. 1D-G). Taken together, these and the above observations make Wg signalling an unlikely candidate for Iro-C repression.

Prompted by the above results, we examined whether the domain of *vn* expression was expanded in *wg^{CX3}/wg^{CX4}* discs. The results resembled those obtained by monitoring Iro-C. In the second instar mutant discs, the *vn* domain was similar to that of a wild-type disc (Fig. 2A) (Simcox et al., 1996; Wang et al., 2000). Later, a separate domain of expression appeared in the distal part of the disc (Fig. 2B,C), which in the late third instar became a faithful mirror-image duplication of the extant domain (Fig. 2C, inset). We concluded that the depletion of Wg signalling did not expand the extant *vn* domain, but permitted formation of a second, distal *vn* domain that corresponded to the ectopic notum.

With the aid of the Gal4 system, we confirmed that EGFR activity was necessary to activate notal Iro-C expression (Wang et al., 2000; Zecca and Struhl, 2002a; Zecca and Struhl, 2002b). Indeed, blocking EGFR signalling in the notum territory (*UAS-raf^{DN}/MS-248Gal4*) inhibited Iro-C expression (Fig. 2G). This suggested that the availability of EGFR activity might define the distal limit of the Iro-C domain. However, overactivation of the EGFR pathway in proximal/intermediate regions of the disc by misexpressing *UAS-vn* or *UAS-ras^{V12}* (a constitutive activation of the EGFR pathway) with several different drivers did not expand the Iro-C domain (Fig. 2D-F, compare with Fig. 4A,E). The inability of the misexpression of *vn* to modify the Iro-C domain of late third instar discs has recently been reported (Zecca and Struhl, 2002a). We concluded that the EGFR signalling pathway, as activated by the Vn ligand, while important for Iro-C activation is not the main candidate to delimit the domain of Iro-C expression and, therefore, the notum territory.

The Dpp pathway is mostly active in the distal regions of the early wing disc

In third instar wing discs, the expression of *dpp* in both proximal and distal territories (see below) does not suggest a function in regulating the domain of Iro-C. However, in the second instar disc *dpp* is expressed in distal regions but it is absent from the Iro-C domain (Fig. 3A,B) (see also Burke and Basler, 1996; Masucci et al., 1990). Dpp is a diffusible molecule and, therefore, we determined its range of activity by monitoring the phosphorylated form of the Mad protein (pMad), an intermediate of the Dpp transduction pathway (Tanimoto et al., 2000). pMad accumulated in the cells near the source of Dpp, but it was reduced or absent within the Iro-C domain (Fig. 3C,D). Another useful indicator of Dpp activity is the type I TGF β receptor Thick veins (Tkv), since its expression is negatively regulated by Dpp signalling (Lecuit and Cohen, 1998). In addition, high levels of Tkv can limit Dpp diffusion and help to confine the region in which the pathway will be activated (Lecuit and Cohen, 1998). We find that the Iro-C domain is located within a region of high accumulation of Tkv (Fig. 3E,F), a result compatible with Dpp activity being strongly reduced or absent from that domain.

The Dpp pathway negatively regulates Iro-C in the early wing disc

The complementary territories of Iro-C and *dpp* signalling

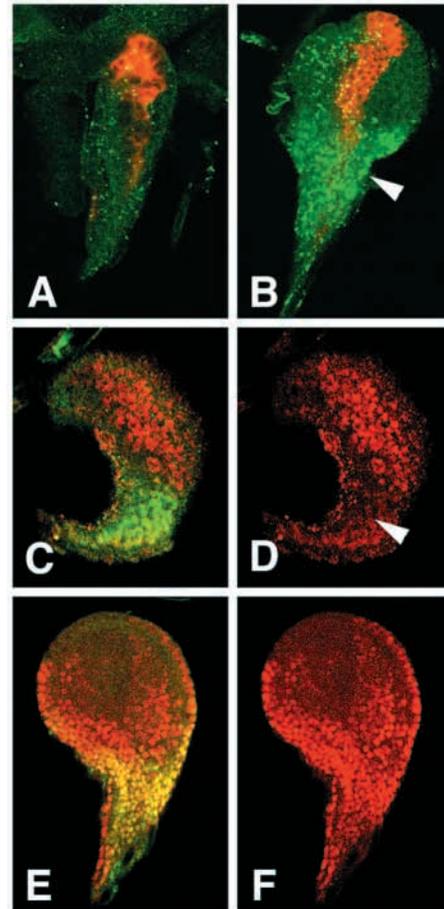


Fig. 3. Complementary domains of Iro-C expression and Dpp activity in the second instar wild-type wing disc. Iro-C protein, green; Dpp pathway markers, red. (A,B) *dpp* expression (revealed by *dpp-lacZ*) precedes that of Iro-C and it does not occur or it only occurs at very low levels within the Iro-C domain, the presumptive notum (arrowhead). (C,D) Dpp pathway activity (pMad protein) is reduced within the Iro-C domain (arrowhead). (E,F) Iro-C (yellow and green) is expressed within the domain of high accumulation of *tkv-lacZ* (red and yellow).

activity (pMad) suggested that the Dpp pathway might repress Iro-C at the early stages of wing disc development. We therefore manipulated the levels of Dpp signalling and monitored the expression of Iro-C. In the strong hypomorphic *dpp^{d12}/dpp^{d14}* combination, the Iro-C domain comprised most cells of the early wing disc (Fig. 4B) and its distal border was very close to a small area that corresponded to the wing pouch (Fig. 4B, arrow), as identified by the Nubbin (Nub) marker (Ng et al., 1995). Since the Iro-C and the Nub domains are well separated in wild-type discs of similar age (Fig. 4A, arrowhead), this suggested that the Iro-C domain was distally expanded in the *dpp^{d12}/dpp^{d14}* discs and covered at least part of the hinge/proximal-wing territory. However, it might be argued that the expansion of the Iro-C territory was an illusion caused by the apposition of an essentially normal notum to a hinge/wing territory dwarfed by reduced Dpp signalling (Serrano and O'Farrell, 1997). This was not the case. By following the development of these discs, we observed that Iro-C proteins were gradually removed from part of the putative

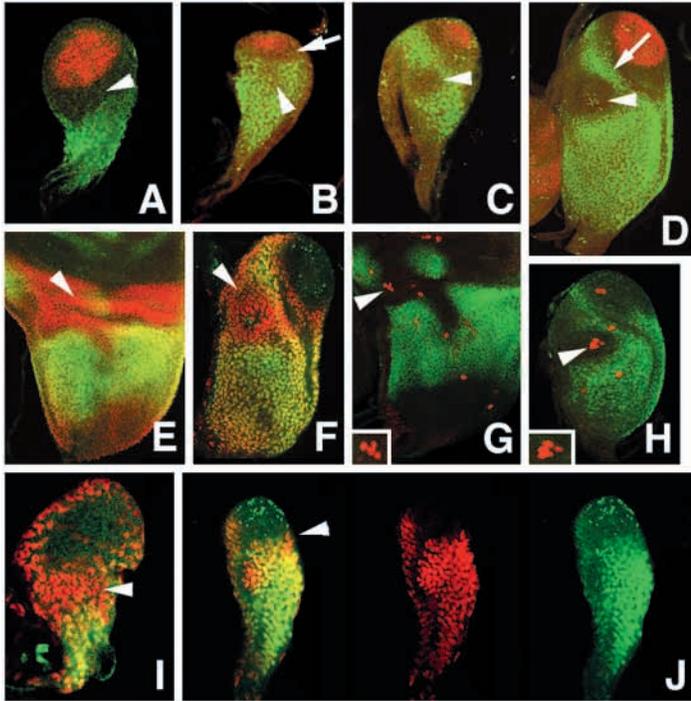


Fig. 4. Insufficiency of *dpp* expands the Iro-C domain (green). (A) Second instar wild-type wing disc: notum (green, Iro-C), wing pouch (red, Nub protein) (Ng et al., 1995), hinge (unlabelled territory, arrowhead). (B-D) Second, early third and late third instar *dpp^{d12/dpp^{d14}}* discs stained as in A. Iro-C ectopically accumulates in the hinge territory (arrowhead in B); later it is removed from part of it (arrowheads in C,D). (E,F) Tsh in wild-type and *dpp^{d12/dpp^{d14}}* late third instar discs, respectively, accumulates most strongly in the hinge territory (arrowheads). (G,H) Sensory organ mother cells (anti-Senseless antibody, red) in wild-type and *dpp^{d12/dpp^{d14}}* late third instar discs, respectively. Presumptive hinge groups (arrowheads) are shown at higher magnification in insets. (I,J) Tsh (red) and Iro-C in wild-type (I) and *dpp^{d12/dpp^{d14}}* (J, merged and separate channels) second instar discs. To emphasize detail, discs are reproduced at different magnifications.

ectopic domain (arrowheads, Fig. 4B-D). The region in which Iro-C was gradually switched off was identified as hinge territory by two criteria. First, it accumulated the Tsh protein very strongly (Fig. 4F), similarly to a wild-type hinge (Fig. 4E). And second, it developed a group of several sensory organ precursor cells (Fig. 4G,H); such characteristic groups develop in the hinge, but never in the notum. However, ectopic Iro-C expression was maintained in other distal regions (Fig. 4D, arrow). Consistent with the distal expansion of Iro-C in second instar *dpp^{d12/dpp^{d14}}* discs, the Iro-C domain was coextensive with that of Tsh, which includes the territory fated to become hinge (Fig. 4J, arrowhead). This coexpression was never observed in wild-type discs (Fig. 1H, Fig. 4E and 4I, arrowhead). Note that the gradual removal of Iro-C protein from the prospective hinge in *dpp^{d12/dpp^{d14}}* discs (Fig. 4B-D) indicates that, even under conditions of strong Dpp insufficiency, the distal border of the Iro-C domain can be generated, at least in part. This could be due to residual Dpp signalling and/or to additional uncharacterized factors, which would normally contribute to maintain and refine this border. To help distinguish between these alternatives, we examined

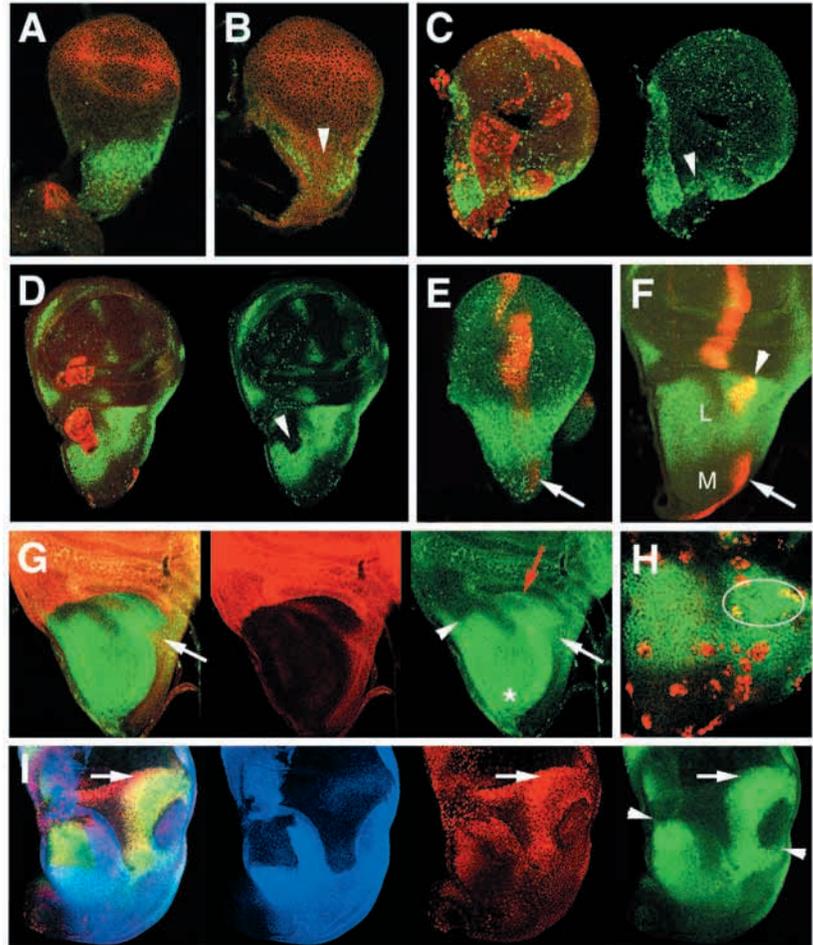
the effect of the complete loss of reception of the Dpp signal by generating, during the first instar, clones mutant for the null *tkv^{a12}* allele. Owing to the difficulty of detecting cell clones in second instar discs, we were forced to examine them in third instar discs. In these *tkv^{a12}* clones, the domain of Iro-C expression appeared distally expanded, as detected by comparison with the domain of Tsh expression (Fig. 5I, compare with Fig. 4E). This, however, was not the case for clones located in the more anterior part of the disc (Fig. 5G, arrowhead). Note again that this region coincides with that in which Iro-C is first expressed and later removed in *dpp^{d12/dpp^{d14}}* discs (Fig. 4B-D). This suggests that after the initial restriction of Iro-C by Dpp signalling, additional factors contribute to maintain the anterior part of the Iro-C border.

We next increased Dpp signalling by misexpressing *UAS-dpp* in the proximal region of the disc (*MS248-Gal4* driver; Fig. 2H,I), and found that it downregulated Iro-C in a large part of the notum territory (compare Fig. 5A and 5B). Misexpression in cell clones of a constitutively activated form of Tkv (*UAS-tkv^{QD}*) also suppressed Iro-C expression autonomously (Fig. 5C,D), although not completely in some regions (see below). We conclude that Dpp signalling must be absent (or strongly reduced) from the notum territory for Iro-C expression. Consistently, misexpression of the Dpp pathway antagonists *UAS-brinker* or *UAS-daughters against dpp* (Affolter et al., 2001) within this territory (*MS248-Gal4*) did not detectably affect the expression of Iro-C in second instar discs (not shown).

The Dpp pathway downregulates Iro-C in the medial notum

During the third instar, after Iro-C has specified the prospective notum, *dpp* is turned on in this territory and helps effect its patterning (Mullor et al., 1997; Sato and Saigo, 2000; Tomoyasu et al., 2000) (Fig. 5E,F, arrows). The activation of *dpp* in the proximal-most region of the prospective notum is accompanied by a gradual removal of Iro-C (Fig. 5E,F), a repression essential to specify the medial versus the lateral notum (Calleja et al., 2000). Dpp was responsible for this downregulation, since it was prevented by decreasing (*dpp^{d12/dpp^{d14}}* mutant; compare Fig. 4D with 4E,G) or abolishing (clones mutant for a null *tkv* allele; Fig. 5G) Dpp signalling. In contrast, constitutive activity of the Dpp pathway in cell clones autonomously inhibited Iro-C in the lateral notum, except in a region overlapping or very close to an endogenous source of Dpp (compare Fig. 5F with 5H; see also 5C, arrowhead). Thus, while in the medial notum there is a correspondence between Dpp expression and Iro-C repression, this correlation does not hold everywhere in the lateral notum, where the appearance of Dpp expression may not result in turning off Iro-C (Fig. 5F, arrowhead; Fig. 5H, circled). Interestingly, *vn* is also maximally expressed in the region of overlap of *dpp* and Iro-C expressions (Simcox et al., 1996) (our unpublished data), and might antagonize, through the activation of EGFR signalling, the repression of the Iro-C genes by the Dpp pathway. We conclude that, in the third instar disc, the levels of Dpp signalling are critical to establish the medial-lateral subdivision of the notum by its negative regulation of Iro-C in the medial region. This negative regulation should be mediated by *pannier* (Calleja et al., 2000),

Fig. 5. Dpp pathway inhibits Iro-C (green). (A,B) Early third instar wild-type and *MS-248 Gal4; UAS-dpp* discs, respectively. Iro-C is downregulated in the notum territory (arrowhead). Wing pouch marker: Wg (red). (C,D) *UAS-tkv^{QD}* (red, *lacZ* marker) autonomously inhibits Iro-C in cell clones. Inhibition fails in a region of the lateral-posterior notum (arrowhead in C). Inhibition is also observed in late third instar discs (D, arrowhead). (E,F) Early and late third instar wild-type wing discs. *dpp-lacZ* expression (red) increases during the third larval instar in the most proximal region of the notum (arrows) and Iro-C is gradually inhibited (M, medial notum; L, lateral notum). (G) *tkv^{a12}* cells (absence of red label) ectopically express Iro-C in the medial notum (asterisk, compare with F). In addition, Iro-C expression is increased within the clone, as compared with the contiguous wild-type territory (arrow). Arrowhead points to the anterior part of the border of the Iro-C domain, which is not modified by the presence of the clone. In contrast, expansion of the Iro-C domain has occurred in the region of the posterior border (red arrow; see also I). (H) Late induced *UAS-tkv^{QD}* clones (*lacZ* marker, red) do not express Iro-C, except at the region within or close to a lateral Dpp source (circled, compare with F). (I) *tkv^{a12}* clone (absence of blue marker) showing a distal expansion of Iro-C expression (arrow), as indicated by the overlap with the Tsh marker (red). Imaginary line between arrowheads would run along the normal border of the Iro-C domain.



which is activated by *dpp* in the medial notum (Sato and Saigo, 2000; Tomoyasu et al., 2000).

DISCUSSION

During larval development, the wing imaginal disc is subdivided in the proximal-distal axis into territories that will give rise to notum, dorsal wing hinge, wing blade, ventral wing hinge and mesothoracic pleura. While many aspects of the genetic control of this subdivision remain obscure, it is clear that activation of the Wg signalling pathway in cells located at the distal part of the disc during the early second instar (Couso et al., 1993; Ng et al., 1996) promotes specification of the wing territory. This is accomplished by activation of wing-specific genes in the distal region of the disc (Ng et al., 1996; Williams et al., 1993) and the repression of more 'proximal' genes like *tsh* (Wu and Cohen, 2002). Wg signalling is also important to define the wing hinge territories (Klein and Martínez-Arias, 1998). With regards to notum specification, it has been proposed that Wg signalling would antagonize and thereby restrict *vn* expression to the proximal region of the disc (Wang et al., 2000). This would lead to high EGFR activity and Iro-C activation in this territory. Hence, Wg signalling would ultimately be responsible for defining the notum territory. This conclusion was derived from the observation that the domain

of *vn* expression was apparently expanded into the distal regions in *wg¹/wg^{CX4}* second instar discs. Our results, however, do not support a role for Wg signalling as the main effector of the notum/wing subdivision. Using the stronger hypomorphic combination *wg^{CX3}/wg^{CX4}* (Lindsley and Zimm, 1992) under conditions in which all wing discs develop into double nota, we find that *vn* and the notum-defining Iro-C genes are still confined to the proximal region of the second instar disc. Moreover, although these discs lack expression of wing-specific genes and do not repress *tsh* in their distal region (Ng et al., 1996; Williams et al., 1993; Wu and Cohen, 2002) (our data), this region is apparently normal in size and morphology. Only later, at the beginning of the third instar, ectopic domains of expression of both *vn* and Iro-C appear, well separated from their extant domains, within the distal region. Evidently, they are associated with the generation of an ectopic notum in mirror-image disposition to the extant one. We conclude that Wg signalling, while essential for wing and wing hinge identity to the distal regions of the disc (Ng et al., 1996; Williams et al., 1993), is not a key effector of the notum/appendage territorial subdivision. Our results indicate that a main effector of this subdivision is the Dpp signalling pathway.

Indeed, we find that in the second instar disc *dpp* is expressed only in its distal regions and that the activity of the Dpp pathway, as measured by pMad accumulation, is reduced in or absent from the proximal part of the disc, where Iro-C is

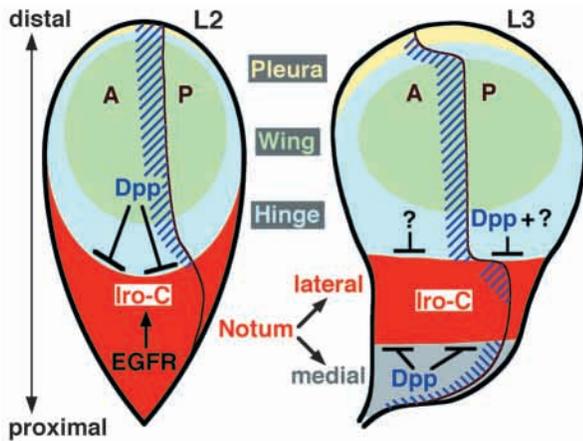


Fig. 6. Dpp signalling sequentially establishes the notum-hinge boundary in second instar wing disc (L2) and the medial-lateral subdivision of the notum in third instar wing disc (L3). A and P, anterior and posterior compartment. Dpp source is hatched in blue. During the second instar, Iro-C is activated by Vn/EGFR in the most proximal part of the wing disc (Wang et al., 2000; Zecca and Struhl, 2002a; Zecca and Struhl, 2002b). The distal border of the Iro-C domain, which defines the notum-hinge boundary (Diez del Corral et al., 1999), is established by repression by Dpp signalling, which at this stage only functions in the distal parts of the disc. In the third instar, *dpp* is expressed in the notum territory and again negatively regulates Iro-C in the medial notum. In the third instar disc, additional, uncharacterized factors (question marks) help maintain the notum-hinge border of Iro-C expression, since this border is generated, at least in part, under conditions of strong depletion of Dpp signalling (Fig. 4D, Fig. 5G).

expressed. When Dpp signalling is reduced or abolished in *dpp^{d12}/dpp^{d14}* discs or *tkv^{a12}* clones, respectively, Iro-C expression is distally expanded. Conversely, increased *dpp* expression or constitutive activation of the pathway (*UAS-tkv^{QD}* clones) repress Iro-C expression within the prospective notum. These data, together with previous findings (Wang et al., 2000), suggest that the Iro-C domain of expression, and hence the notum territory of the wing disc, is defined during the early larval stages by two antagonistic signals: Vn/EGFR, which activates Iro-C, and Dpp, which constrains its expression to the proximal region of the disc by repressing it in the neighbouring (hinge) territory (Fig. 6). While we have confirmed that Vn/EGFR is necessary for Iro-C activation, the localised expression of *vn* does not appear to be instrumental in confining Iro-C expression to this territory, since ectopic Vn does not significantly expand the Iro-C domain. After submission of this manuscript, Zecca and Struhl (Zecca and Struhl, 2002b) similarly reported that misexpression of *vn* does not modify Iro-C expression. However, these authors additionally show, using overexpressing cell clones (Zecca and Struhl, 2002b), that a constitutively activated form of EGFR can autonomously activate Iro-C in particular subregions of the prospective hinge, and that Ras^{V12}, a presumably stronger activation of the pathway, can do so anywhere within the prospective wing hinge and even in some clones within the wing blade. While we have not observed ectopic activation of Iro-C with *UAS-ras^{V12}* expressed with several Gal4 lines, probably because we have used milder overexpressing conditions (i.e., larvae were cultured at 18°C, since in our

hands they died at higher temperatures), these observations indicate that, at least in third instar wing discs, sufficiently strong activation of the EGFR/Ras pathway is able to activate Iro-C almost anywhere in the disc. Zecca and Struhl (Zecca and Struhl, 2002a; Zecca and Struhl, 2002b), based on these observations and in the absolute necessity of EGFR activity for Iro-C expression and notum development (Simcox et al., 1996; Wang et al., 2000; Zecca and Struhl, 2002a; Zecca and Struhl, 2002b), propose that this pathway would be maximally activated in the proximal region of the disc and would thus turn on and maintain Iro-C expression in the prospective notum. However, direct comparative measurements of the activity of the pathway (for instance, by examining the levels of phosphorylated MAP kinase) (Gabay et al., 1997) in the prospective notum and in other region of the second and third instar wing disc have not been performed. Thus, it is not clear whether there is indeed a gradation of EGFR pathway activity along the proximal-distal axis of the disc (Wang et al., 2000; Zecca and Struhl, 2002a; Zecca and Struhl, 2002b) and, if present, whether it does help effect the notum/hinge subdivision. Regardless of its presence and functional significance, our data indicates that the Dpp pathway, by negatively regulating the expression of Iro-C, is an important player in establishing the early distal border of the Iro-C domain and, therefore, the notum-hinge subdivision. In the wild type, this repression would be sufficient to counteract the activation by the EGFR pathway and prevent Iro-C expression in the hinge. However, strong experimental overactivity of the EGFR pathway would overrule the repression by Dpp and allow ectopic expression of Iro-C in the hinge and wing (Zecca and Struhl, 2002a; Zecca and Struhl, 2002b). Whether these two pathways act antagonistically and in parallel on the Iro-C or negatively regulate each other is not known. Note, finally, that in the early *dpp^{d12}/dpp^{d14}* discs, Iro-C expression, while occurring in an expanded domain, is still excluded from the prospective wing territory (Fig. 4B). This suggests the presence of additional repressors and/or the absence of essential activators within this domain.

Later in development, even under conditions of complete loss of Dpp signal reception (*tkv^{a12}* clones), the distal border of the Iro-C domain can be generated at least in the anterior part of the disc (Fig. 4D, Fig. 5G). This is most likely due to additional uncharacterized factors, independent of Dpp signalling, that would normally contribute to maintain and refine this border (Fig. 6). In the posterior part of the disc, loss of the reception of the Dpp signal leads to a distal expansion of the Iro-C domain, detectable even in third instar discs. While this could reflect a continuous requirement for Dpp signalling throughout the second and third instars for maintaining the Iro-C border, it is also possible that this signal is only required during the early establishment of the border and that its early disruption is irreversible in later stages. So, these results are still compatible with the idea that additional unknown factors are helping maintain this part of the border.

Signalling pathways in notum development

From very early in the development of the wing disc, the Hedgehog (Hh) signalling pathway is active in a stripe of anterior cells adjacent to the AP compartment border. This stripe extends from the proximal-most part of the disc to the

tip, as revealed by Hh targets like *ci* and *ptc* (Dahmann and Basler, 1999; Podos and Ferguson, 1999; Serrano and O'Farrell, 1997) (our unpublished data). Surprisingly, we have observed that in these early discs *dpp*, another target of Hh, is not activated in their proximal region. This suggests the presence of uncharacterized negative regulators that block transcription of *dpp* in this territory. Although the complementary domains of Iro-C and pMad (Fig. 3C,D) might suggest that Iro-C could be one of these negative regulators, we find that misexpression of the Iro-C gene *arauca* does not downregulate *dpp* (F. C., unpublished).

Hh, Dpp and Wg signalling pathways intervene in the patterning of both the body trunk and the appendages of *Drosophila*. However, it is clear that the cellular responses to these signals are different in the mesothoracic body wall versus the wing and the legs (reviewed by Morata and Sánchez-Herrero, 1999). Indeed, in the wing disc, the pathways are essential for the specification and growth of the wing, but not so for the notum, which can develop to a large extent in the absence of these signals. Hh, Dpp and Wg are required for late events such as the notal medial/lateral subdivision and bristle patterning (Morata and Sánchez-Herrero, 1999). Our findings reinforce this view since the Dpp pathway appears to be inactive within the notum territory during its specification (it only helps delimit its extent). Thus, to our knowledge, only the EGFR pathway is indispensable for the specification and growth of the notum.

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