Mutual repression between *msh* and Iro-C is an essential component of the boundary between body wall and wing in *Drosophila*

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Summary

During development, the imaginal wing disc of *Drosophila* is subdivided into territories separated by developmental boundaries. The best characterized boundaries delimit compartments defined by cell-lineage restrictions. Here, we analyze the formation of a boundary that does not rely on such restrictions, namely, that which separates the notum (body wall) and the wing hinge (appendage). It is known that the homeobox genes of the Iroquois complex (Iro-C) define the notum territory and that the distal limit of the Iro-C expression domain demarks the boundary between the notum and the wing hinge. However, it is unclear how this boundary is established and maintained. We now find that *msh*, a homeobox gene of the Msx family, is strongly expressed in the territory of the hinge contiguous to the Iro-C domain. Loss- and gain-of-function analyses show that *msh* maintains Iro-C repressed in the hinge, while Iro-C prevents high level expression of *msh* in the notum. Thus, a mutual repression between *msh* and Iro-C is essential to set the limit between the contiguous domains of expression of these genes and therefore to establish and/or maintain the boundary between body wall and wing. In addition, we find that *msh* is necessary for proper growth of the hinge territory and the differentiation of hinge structures. *msh* also participates in the patterning of the notum, where it is expressed at low levels.

Key words: *msh*, Iroquois complex, Wing hinge, Notum; Developmental boundary, *Drosophila*, *Dr*

Introduction

During development, the allocation of cell populations to different developmental fates relies largely on the establishment of developmental boundaries. These boundaries separate adjacent cell populations and provide cells at both sides of the boundary with positional information that governs the future patterning of the tissue. In *Drosophila*, developmental boundaries have been best characterized in the mesothorax and wings. These parts of the fly body develop from a pair of epithelial sacs, the imaginal wing discs. At metamorphosis, each imaginal disc undergoes final differentiation to give rise (from dorsal to ventral) to heminotum, dorsal wing hinge, dorsal wing blade, ventral wing blade, ventral wing hinge and mesopleura. The pair of heminota and mesopleurae form the mesothoracic body wall, while the remaining structures form the pair of wings.

In the wing disc, the best characterized developmental boundaries are associated with borders of compartments defined by cell-lineage restrictions (García-Bellido et al., 1973) (reviewed by Irvine and Rauskolb, 2001; Mann and Morata, 2000). The earliest boundary subdivides the disc into anterior (A) and posterior compartments (P). It is established by the expression of the selector genes *engrailed* and *invected* in the P compartment. It has been proposed that the selector genes confer identity to the cells of a compartment and a differential affinity that prevents cells from apposing compartments to intermingle (García-Bellido and Santamaría, 1972). The end result is a straight boundary that separates both compartments and which, after final differentiation, does not correspond with any morphological feature. A dorsoventral (DV) compartmental subdivision, orthogonal to the AP one, is established by the expression of the gene *apterous* (*ap*) in the D compartment. In the wing, this boundary runs along the wing margin and separates the dorsal and ventral wing blades. Compartment borders give rise to specialized cells that are sources of signaling 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).

Other developmental boundaries are not associated with cell lineage restrictions (reviewed by Irvine and Rauskolb, 2001; Mann and Morata, 2000; Tepass et al., 2002). This implies that cells can cross the boundary and change fates. An example is the boundary that separates the presumptive notum from the dorsal wing hinge territory (Diez del Corral et al., 1999). A selector-like role has been attributed to the genes *araucan*, *caupolican* and *mirror* (Cavodeassi et al., 1999; Cavodeassi et al., 2000; Wang et al., 2000), the three members of the Iroquois Complex (Iro-C) (Gómez-Skarmeta et al., 1996; McNeill et al., 1997). These genes, which encode related homeodomain proteins conserved from worms to vertebrates (reviewed by Cavodeassi et al., 2001), start to be expressed in the

presumptive notal region during the second instar. This expression is essential for notum specification, as clones of Iro-C^{-} cells induced early within this territory acquire the identity of the dorsal wing hinge (Diez del Corral et al., 1999). Thus, the early domain of expression of Iro-C defines the extent of the notum territory. Moreover, Iro-C genes appear to endow cells with special affinity characteristics, so that cells expressing them assort with each other, rather than with Iro-C non-expressing cells (Diez del Corral et al., 1999; Zecca and Struhl, 2002b). This specific affinity might help maintain the relatively straight and sharp notum/dorsal hinge border in the wing imaginal disc (Zecca and Struhl, 2002b). Similarly to the AP and DV boundaries, the notum/dorsal hinge boundary appears to be a source of positional information, but the molecules involved have not been identified (Diez del Corral et al., 1999).

Some progress has been made in understanding how the border of the Iro-C domain that defines the notum/dorsal hinge boundary is established. It requires the participation of two of the signaling systems that subdivide the early disc in the proximodistal axis (reviewed by Klein, 2001). Thus, on the one hand, signaling by the tyrosine kinase EGF receptor turns on the genes of the Iro-C (Wang et al., 2000; Zecca and Struhl, 2002a; Zecca and Struhl, 2002b). On the other hand, signaling mediated by the BMP2/4 homolog Dpp, which during the early/mid second larval instar is active only in the more distal territories of the disc, represses there the Iro-C and sets the distal border of the Iro-C domain (Cavodeassi et al., 2002). However, at later stages, Dpp signaling occurs in the notum territory and the border of Iro-C expression becomes refractory to it. This suggested that additional agents might control the notal-hinge boundary (Cavodeassi et al., 2002).

The *msh* (muscle-segment homeobox) gene (*Dr* – FlyBase) is a member of the conserved Msx family. It encodes a homeodomain transcription factor with an Engrailed-type repressor motif (D'Alessio and Frasch, 1996). In the embryonic mesoderm, *msh* specifies subsets of cardiac and muscle precursors and participates in cross-repressive interactions with other genes (Jagla et al., 2002). *msh* is also a neural-identity gene that is expressed in the dorsal-most region of the embryonic neuroectoderm (D'Alessio and Frasch, 1996; Isshiki et al., 1997). In the wing imaginal disc, *msh* imparts a dorsal identity to the dorsal bristles of the wing margin and to wing veins (Milán et al., 2001).

Here, we report that *msh* also participates in establishing/maintaining the notum/dorsal hinge boundary. From the second instar stage onwards, *msh* and Iro-C are expressed in adjacent domains of the imaginal wing disc, that of *msh* being distal to that of Iro-C. This situation essentially persists in the third instar, where *msh* is strongly expressed in the presumptive hinge region and Iro-C is expressed in the adjacent territory, the lateral notum. Loss- and gain-of-function analyses indicate that *msh* represses Iro-C in most of the presumptive dorsal hinge, and Iro-C prevents high level expression of *msh* in the notum, while it allows low expression of *msh* in this territory. *msh* is also necessary for proper development of the hinge and for the patterning of the notum. Moreover, the confrontation of cells expressing *msh* and Iro-C at the notum/hinge boundary appears to favor the correct growth of the notum and hinge territories.

Materials and methods

Drosophila stocks

Drosophila stocks used were: $msh^{\Delta 68}$ and *UAS-msh* (Isshiki et al., 1997), and $Df(3)iro^{DFM3}$ and *UAS-ara* (Gómez-Skarmeta et al., 1996). *UAS-mshi* was generated according to Nagel et al. (Nagel et al., 2002). The oligonucleotides used to amplify an 880 bp fragment of the first exon of the *msh* gene were: AAGTCGACGGATCCCAAGCGTGTG-ACGAACGAGCGC and AAGGTACCTCTAGAGCTGGGCGT-GGAACTCGTGGAGGC. Underlined sequences correspond to restriction sites for *Sal*I, *Bam*HI, *Kpn*I and *Xba*I that helped in the procedure. Other alleles and transgenes are described in FlyBase (http://flybase.org).

Mosaic analysis

Mitotic recombination clones homozygous for the alleles $msh^{\Delta 68}$, $Df(3L)iro^{DFM3}$ and $Chip^{e55}$ were induced by the FLP-FRT technique (Xu and Rubin, 1993) by incubating larvae for 1 hour at 37°C. Larvae were prepared as follows: flies *FRT82B* $msh^{\Delta 68}$ *arm-lacZ/TM6B* were crossed with either f^{36a} *hs-FLP; FRT82B* $P(f^{+})$ $M(3)^{w124}$*/TM6B* for clones to be examined in adults, or *hs-FLP; FRT82 ubi-GFP* $M(3)^{w124}$*/TM6B* for clones to be analyzed in imaginal discs; flies *hs-FLP; mwh* $Df(3L)iro^{DFM3}$ *FRT80B/TM6B* were crossed with *hs-FLP; ubi-GFP FRT80B*; and flies *FRT42B* $Chip^{e55}$*/Cyo* were crossed with *hs-FLP; FRT42B ubi-GFP/Cyo* flies.

Overexpression clones were obtained by crossing either a *UAS-ara* or *UAS-msh* line with *y w hs-FLP*^{122}*; act-FRT y+ FRT Gal4 UAS-GFP/SM5 Tb* (Ito et al., 1997) flies. Clones were induced by incubation of larvae at 37°C for 8 minutes (*UAS-msh*) or 15 minutes (*UAS-ara*). $Df(3L)iro^{DFM3}$ clones in individuals overexpressing *UAS-mshi* were obtained by crossing flies *hs-FLP; UAS-mshi/Cyo;* $Df(3L)iro^{DFM3}$ *FRT80B/TM6B* with flies *ap-GAL4; ubi-GFP FRT80B/TM6B*. Clones were induced (37°C, 1 hour) at 60±12 hours AEL (after egg laying) and the flies were raised at 29°C. Cuticles were prepared by boiling in KOH and mounted in ethanol/lactic acid (5:6).

Antibody and β-galactosidase staining

Imaginal discs were fixed and stained as in Xu and Rubin (Xu and Rubin, 1993). Antibodies were: rat anti-Ara, which reacts with Ara and Caup proteins (Diez del Corral et al., 1999), rabbit anti-Msh (McDonald et al., 1998) (provided by C. Doe), mouse anti-Wg, rabbit anti-Tsh, mouse anti-Nub (provided by M. S. Cohen and D.S.H.B.), rat anti-Zfh2 (Whitworth and Russell, 2003) and rabbit anti-Sc (Skeath and Carroll, 1991).

Results

Expression of *msh* and *ara/caup* in the wing disc

In the third instar wing disc, *msh* mRNA accumulates in the dorsal compartment of the wing blade region (Milán et al., 2001), and maximally in the presumptive dorsal hinge, a territory adjacent to the presumptive notum (D'Alessio and Frasch, 1996). We have compared the distribution of Msh with that of the Iro-C proteins Ara and Caup, which are co-expressed in the notum region of the disc (Gómez-Skarmeta et al., 1996). In late second/early third instar wing discs, the region of Msh accumulation abuts proximally to the Ara/Caup domain and a relatively sharp border separates their respective domains (Fig. 1A). During the third instar, Msh continues to accumulate to high levels in this region and, to low levels, in the dorsal compartment of the wing pouch and in the posterior notum territory, where it overlaps with the expression of Ara/Caup (Fig. 1B,C; for nomenclature of regions of the disc, see Fig. 1C). The high accumulation of Msh does not extend into the wing pouch, as it stops adjacent to the innermost of

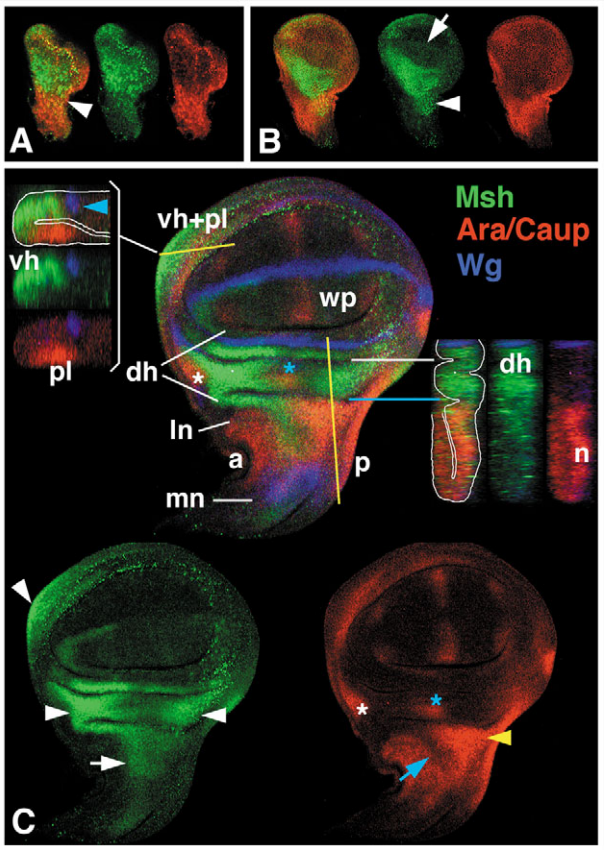


Fig. 1. Expression of *msh* and *ara/caup* in the wing disc. Images show accumulation of Msh (green), Ara/Caup (red) and Wg (blue). (A) Late second instar disc. The border between the Msh and Ara/Caup domains is relatively sharp (arrowhead). (B) Mid-third instar discs. There is low level Msh accumulation in the dorsal wing pouch (arrow) and in the posterior notum (arrowhead). (C) Late third instar disc images. Channels showing Msh and Ara/Caup expressions are separately illustrated at bottom of the panel. Yellow lines indicate the positions of *z*-axis optical sections. These are shown at left (top) and right (middle) of the panel, with separate and merged red and green channels. White curved lines highlight the profiles of the disc epithelium sections. vh, ventral hinge, pl, pleura; dh, dorsal hinge; n, notum; ln, lateral notum; mn, medial notum; wp, wing pouch; a, anterior; p. posterior; white asterisks, tegula; blue asterisks, dorsal radius. *msh* expression is maximal in the dorsal and ventral prospective hinges (white arrowheads) and lower in the posterior notum (white arrow), while that of *ara/caup* is maximal at the prospective lateral notum (yellow arrowhead). Blue arrow indicates the region of decreased Iro-C expression in the lateral notum of late third instar discs. Blue horizontal line indicates the fold that approximately coincides with the notum/hinge boundary. In the notum/dorsal hinge *z*-section, there is strong notal expression of *ara/caup* that does not overlap with the strong expression of *msh*. This expression is also contiguous but does not overlap at the *z*-section through the ventral hinge and pleura (top, left). Wg marks the wing pouch epithelium (blue arrowhead) and this is contiguous with the domain of *msh* expression. Therefore, this should correspond to the ventral hinge and *ara/caup* expression should correspond to the pleura.

the two circles of Wg expression (del Álamo Rodríguez et al., 2002). Similar to the second stage disc, the high accumulations of Msh in the hinge and of Ara/Caup in the notum are contiguous, but do not overlap (Fig. 1B,C). This was verified with a *z*-axis optical section (Fig. 1C, right). Contiguous but non overlapping expressions also occurred in the ventral hinge/mesopleura region of the disc (Fig. 1C, left).

msh is required for dorsal hinge and notum development

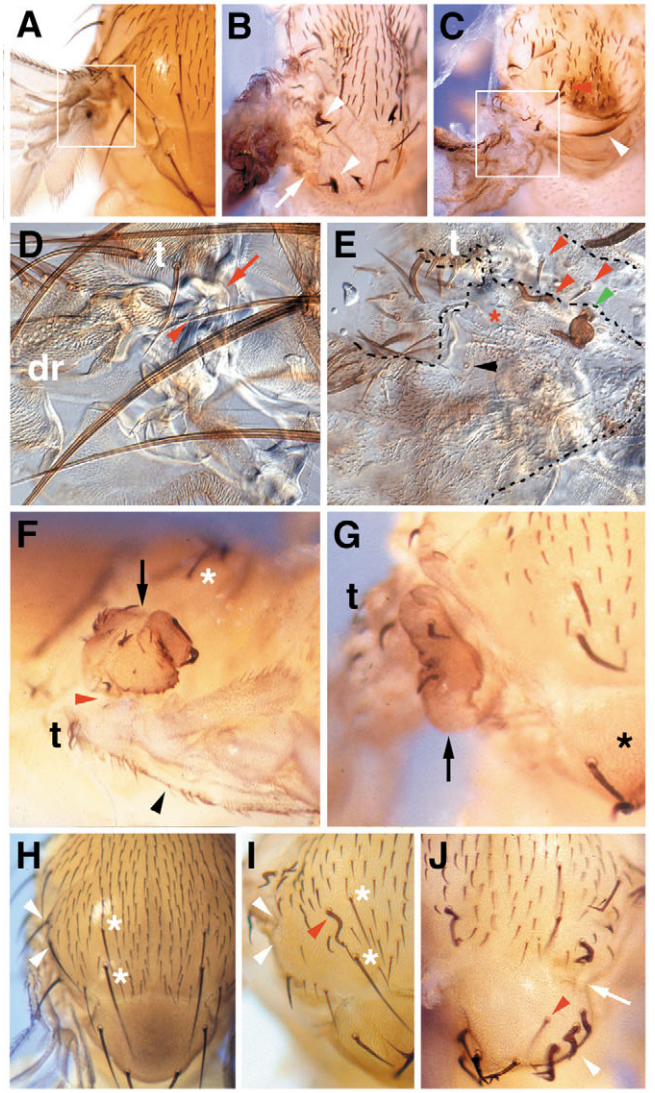
The expression of *msh* in the dorsal hinge and in the posterior notum (Fig. 1C) suggested that *msh* may function in the development of these territories. Null conditions for *msh* are embryonic lethal (Isshiki et al., 1997). Hence, we generated mitotic recombination clones homozygous for the null $msh^{\Delta 68}$ allele and examined their phenotype in adults. The *Minute* technique was used to obtain clones that comprised relatively large territories of the fly (Morata and Ripoll, 1975). Clone induction at 0-24 hours after egg-lying (AEL) was generally lethal, but at 24-48 hours AEL it was compatible with viability. In the wing disc derivatives, clone associated-defects were seen at the wing hinge, the posterior scutum and the scutellum (Fig. 2K). At the hinge, the most frequently observed anomalies were malformations ranging from small defects such as an outheld wing (not shown), to the partial loss of proximal hinge structures, or to even the complete loss of most hinge structures, namely, sclerites and the proximal dorsal radius (Fig. 2E, compare with D). In the most extreme cases, the wing was fused to the scutellum and scutum (Fig. 2B, compare with 2A) or it was displaced posteriorly (Fig. 2C). Generally, the tegula was not affected. Clone induction between 48-72 hours AEL yielded similar phenotypes, and, in addition, 19% of flies with visible clones displayed ectopic tissue carrying macro- and microchaetae, which indicated its notum identity. The ectopic notal tissue appeared dorsal to the hinge and contiguous to it (Fig. 2F,G), consistent with a transformation of hinge towards notum (see below). Taken together, these data indicated that *msh* is necessary for the correct formation of the wing hinge and that its absence can lead to formation of ectopic notal tissue.

At the notum, when $msh^{\Delta 68}$ M^+ clones were induced at 24-48 hours AEL, the most frequent anomalies were a reduction of the scutellum (Fig. 2C) and the appearance of depigmented, naked and corrugated cuticle in the lateral posterior scutum adjacent to the allula and the hinge (not shown). In addition, the msh^- clones frequently developed extra macrochaetae (Fig. 2H-J), mostly in the dorsocentral (DC) region of the notum and in the scutellum, and occasionally also induced nearby wild-type cells to differentiate as chaetae, suggesting cell non-autonomous effects (Fig. 2J). The clones also suppressed extant chaetae (Fig. 2G-I), the anterior and posterior supra-alars being the most affected, and interfered with the correct formation of the scuto-scutellar suture (Fig. 2J). Interestingly, in clones that comprised the lateral anterior notum, a region where *msh* expression has not been detected in the third instar disc, the anterior and posterior notopleural and the presutural macrochaetae were missing in 70, 10 and 15% of cases, respectively (20 heminota examined).

msh is essential for proper growth and patterning, but not for the specification, of the dorsal hinge territory

To gain insight into the function of *msh* in the hinge, we examined the effect of $msh^{\Delta 68}$ M^+ clones on the expression of

genes known to be required for development of this territory. *homothorax* (*hth*), *teashirt* (*tsh*), *zfh-2* and *wingless* (*wg*) are expressed at high levels in the presumptive hinge (Azpiazu and Morata, 2000; Casares and Mann, 2000; Klein and Martínez-Arias, 1998; Whitworth and Russell, 2003) (Fig. 1C and Fig. 3A,B,E). Their characteristic patterns of expression were not overtly modified (Fig. 3A,B,F and data not shown), which suggested that *msh* was dispensable for the specification of the dorsal hinge territory. Using as landmarks the highly resolved pattern of *scute* (*sc*; Fig. 3C) (Cubas et al., 1991; Skeath and Carroll, 1991), we observed a shortening of the distance between the *sc* proneural cluster at dorsal radius, in the hinge, and the anterior postalar cluster, in the lateral notum (Fig. 3C,D). We also observed the apparent fusion of the distal (d) and proximal (p) *sc* clusters of the tegula region (Fig. 3C,D).



K	Induction time	24/48h	48/72h	72/96h
	Anomalies(heminota)	158(119)	47(37)	15(14)
	Out-held wing	19%	15%	33%
	Hinge malformation	32%	15%	13%
	Naked cuticle	23%	32%	40%
	Scutellum reduced	26%	19%	13%
	Ectopic notum tissue	--	19%	--

These findings suggested a decrease in the size of the intervening mutant territory, an interpretation further supported by the absence of the fold of the disc that separates the notum and hinge regions (Fig. 1C) when these regions are mutant for *msh* (Fig. 3A,B, blue arrowheads). By contrast, the fold that separates the hinge from the wing pouch was unaffected by the *msh* clones (Fig. 3A,B, yellow arrowheads). These results, together with the adult phenotype of the $msh^{\Delta 68}$ M^{+} clones, indicate that *msh*, although dispensable for the specification of the dorsal hinge territory, is required for the proper growth and patterning/differentiation of this territory.

Consistently, overexpression of a *UAS-msh* transgene in clones did not impose the high levels of *tsh* expression characteristic of the third instar dorsal hinge into other territories of the disc (not shown). However, *UAS-msh* driven by *ap-Gal4* did promote expression of *zfh-2* in the dorsal compartment of the wing pouch and, to low levels, in the notum (Fig. 3G). The resulting pharate individuals showed reduced and crumpled wings, and a reduced notum; however, no hinge-like sensory organs or other structures could be discerned in the latter. Thus, although *msh* overexpression imposes some hinge-like properties to the prospective wing pouch and notum tissues (*zfh-2* expression), it seems unable to force a complete transformation of these tissues into hinge.

Fig. 2. $msh^{\Delta 68}$ M^{+} clones interfere with the development of the notum and wing hinge. Clones were visualized by the *f* marker. (A) General view of the proximal wing, wing hinge (within the square) and left heminotum of a wild-type fly. (B) Wing with an abnormally wide hinge attached to the scutum and, ectopically, to the scutellum (arrow) of a fly carrying $msh^{\Delta 68}$ clones. Arrowheads indicate mutant scutellar bristles. (C) A posteriorly displaced wing (compare with A) attached to a thorax with a reduced scutellum (white arrowhead). Positional reference: dorsocentral bristles (red arrowhead). (D,E) High magnification views of a wild-type hinge and the mutant hinge shown in C (area within a rectangle) after mounting of the cuticle. The tegula is marked (t) as a positional reference. Rectangle in A indicates approximate area of the image in D of an unrelated fly. Broken lines indicate approximate borders of clones. Sclerites 1 (arrow) and 2 (arrowhead) are clearly visible in D, but these are absent in E. Red asterisk indicates the position where they would be expected to occur. Red arrowheads indicate ectopic chaetae. Green arrowhead indicates a probable scutellar macrochaeta. dr, wild-type dorsal radius and its clusters of sensilla campaniformia; only a few dorsal radius-like sensilla are seen in E (black arrowhead). (F,G) Lateral and dorsal views, respectively, of ectopic notum structures (arrows), which developed adjacent and just above the wing hinge (red arrowhead). The presence of *f* macro- and microchaetae indicate that the tissue is mutant for *msh* and suggests its notal identity. Tegulae (t), scutellum (asterisks) and costa (arrowhead) are marked for orientation. (H,I) Wild-type notum and notum with $msh^{\Delta 68}$ M^{+} clone(s) that removed macro and microchaetae. White arrowheads indicate positions of missing supra-alar macrochaetae in I. Red arrowhead indicates an extra macrochaeta. Asterisks indicate extant dorsocentral macrochaetae. (J) Clone(s) associated with extra scutellar bristles (white arrowhead), an ectopic wild-type bristle (red arrowhead) and disruption of the scutum-scutellar suture (arrow). (K) Frequencies of anomalies (excepting modifications of the bristle pattern) associated with $msh^{\Delta 68}$ M^{+} clones induced at the indicated developmental times after egg laying. In parenthesis, number of heminota examined with one or more anomalies. Essentially all clones that comprised relatively large regions of the notum displayed one or more of the listed anomalies.

msh downregulates *ara/caup* in the wing hinge

The $msh^{\Delta 68}$ M^{+} clones derepressed the *ara* and *caup* genes of the Iro-C in the hinge territory (Fig. 4A,B). This upregulation was also observed in non-Minute $msh^{\Delta 68}$ clones induced at either 24/48 hours AEL (Fig. 4F) or 48/72 hours AEL (Fig. 4C). In all cases, derepression was cell autonomous, and did not extend into the wing pouch [the latter defined by the expression of Nubbin (Ng et al., 1995) (Fig. 4A,B) or in a triangular area that included the prospective tegula (Fig. 4B, asterisk; Fig. 4F). Similar *ara/caup* derepression was obtained by overexpressing an *msh*-interferring RNA (*UAS-mshi*; *ap-Gal4* driver, Fig. 4D).

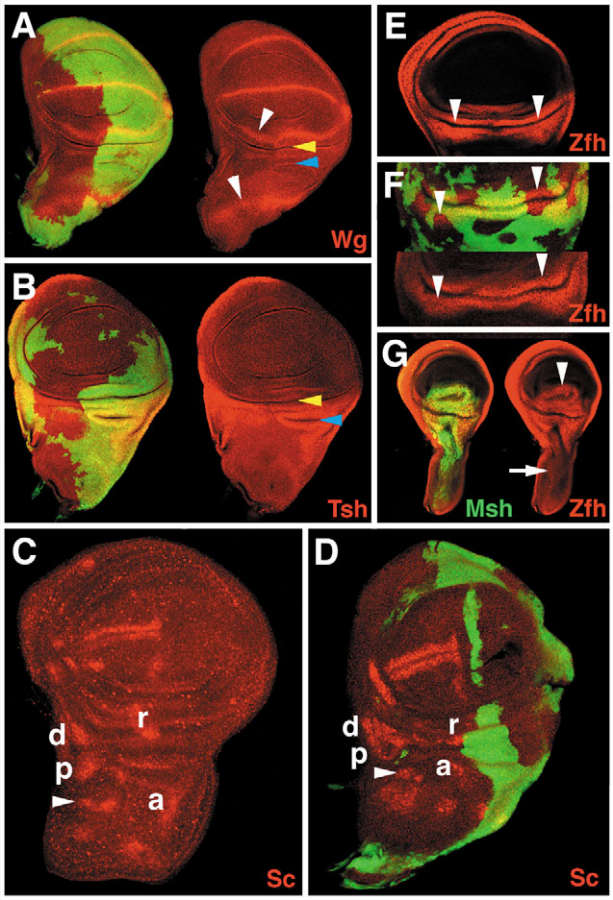


Fig. 3. *msh* is required for the proper growth and patterning of the dorsal hinge. Third instar wing discs were stained (red channel) for the indicated proteins. In A,B,D,F, $msh^{\Delta 68}$ M^{+} clones are marked by the absence of green. (A) Pattern of Wg accumulation. The expression seems only slightly decreased within the *msh* clone (white arrowheads). (B) Tsh accumulation is not appreciably modified by the *msh* clones. In A and B, the fold of the epithelium that separates the notum and hinge territories (blue arrowhead) is absent, but that which separates the hinge and the wing pouch (yellow arrowheads) is present. (C,D) Proneural clusters of Sc accumulation in a wild-type disc (C) and a disc with large $msh^{\Delta 68}$ M^{+} territories (D). d, distal tegula; p, proximal tegula; r dorsal radius; a, anterior postalar cluster. Arrowheads indicate lateroanterior proneural clusters. (E) *zfh-2* expression in the dorsal wild-type wing hinge (arrowheads). (F) *zfh-2* expression is not modified in $msh^{\Delta 68}$ M^{+} clones (arrowheads). (G) Overexpression of *UAS-msh* (green channel; *ap-Gal4* driver, at 17°C) induces ectopic expression of *zfh-2* in the wing pouch (arrowhead) and weakly in the notum territory (arrow).

As Iro-C imparts notum identity (Diez del Corral et al., 1999; Wang et al., 2000; Zecca and Struhl, 2002b), its ectopic expression in the hinge may account for the notum structures that develop in $msh^{\Delta 68}$ M^{+} clones (Fig. 2F,G). Moreover, forced expression of *ara* in the dorsal hinge interferes with its proper formation (R. Diez del Corral, PhD thesis, Universidad Autónoma de Madrid, 1998). Thus, the ectopic expression of *ara/caup* may contribute to the disappearance of the hinge structures observed in *msh* clones (Fig. 2E).

The capacity of *msh* to repress *ara/caup* was further demonstrated by overexpressing *UAS-msh* in clones in the notum. Early induction of overexpression (12-36 hours AEL) invariably repressed *ara/caup* (Fig. 4E). Late induction (60-84 h AEL) still did so if cells were located at the lateral notum, but in central and medial regions, many cells within clones or even entire clones failed to repress *ara/caup* (not shown). Expression of *UAS-msh* in the whole dorsal compartment of the disc (*ap-Gal4* driver) at 17°C reduced the size of the notum territory (Fig. 3G). At 25°C, it strongly inhibited the development of this territory (Fig. 4G) and individuals died at the pupal stages. We conclude that *msh* can repress *ara/caup* in the notum, but in the central/medial regions this capacity gradually decreases as the disc grows. Evidently, this repression requires high concentrations of Msh, such as those provided by the UAS/Gal4 system, because in the wild-type disc, Ara/Caup co-exist with low levels of endogenous Msh (Fig. 1B,C).

Iro-C downregulates *msh* in the lateral notum

We next examined whether *ara/caup* might repress *msh* in the notum by using clones that lack essentially all Iro-C function (iro^{DFM3} mutation). When induced early, these clones acquire a hinge fate and consequently upregulate *tsh* (Diez del Corral et al., 1999). Thus, as expected they expressed *msh* at levels similar to those of the hinge (Fig. 5A). However, iro^{DFM3} clones induced late in development do not undergo fate transformations (Diez del Corral et al., 1999). Still these clones, when located within the posterior lateral notum, increased autonomously the accumulation of Msh (Fig. 5B), but were unable to upregulate *tsh* (not shown). Hence, Iro-C downregulates *msh* in this region of the disc even in cells that are not transformed into hinge cells. In the anterior lateral notum, late clones do not derepress *msh* (not shown), suggesting the presence of additional repressors.

The ability of Iro-C to repress *msh* was further demonstrated with clones overexpressing *UAS-ara* in the hinge. *msh* was autonomously repressed (Fig. 5C). This result was verified by driving *UAS-ara* with *ptc-Gal4* or by constitutively activating the EGFR signaling pathway, which upregulates Iro-C in the hinge (Zecca and Struhl, 2002b) (not shown). Taken together, these data support a mutual repression between *msh* and Iro-C in the notum/hinge region of the wing disc.

As indicated above, cells within the notum that lack Iro-C function switch fate and autonomously develop as dorsal hinge (Diez del Corral et al., 1999). We now find that *msh* is necessary for proper development of the hinge. Thus, it seemed pertinent to examine the fate of cells that were simultaneously depleted of Iro-C and *msh* activities. Accordingly, we induced iro^{DFM3} clones in discs expressing *UAS-mshi* (*ap-Gal4* driver) and examined the clones in the third instar discs (adults failed to emerge). In the dorsal wing and hinge, clones appeared with

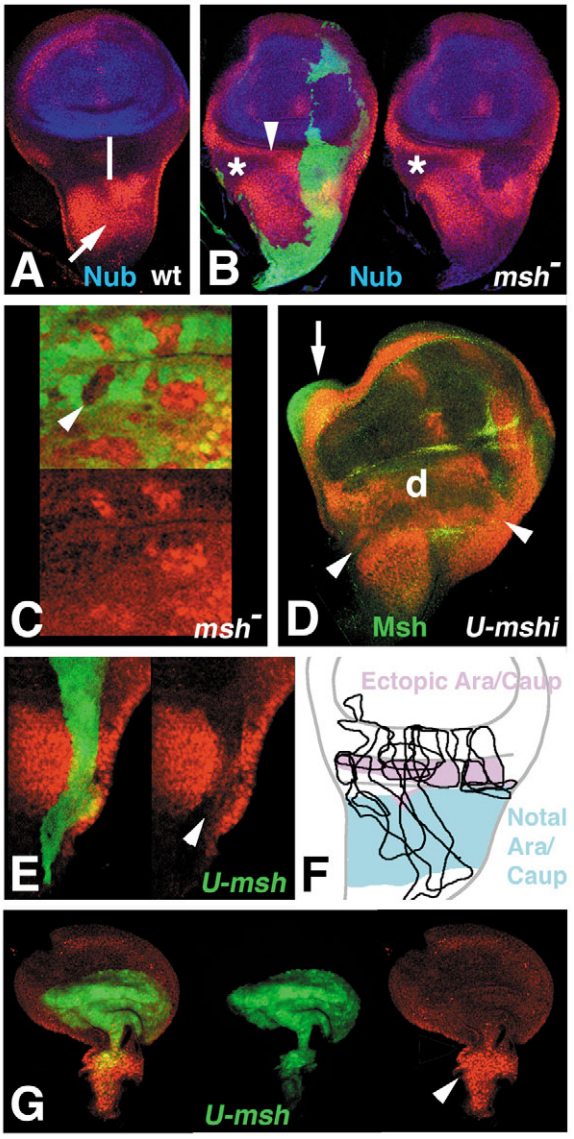


Fig. 4. *msh* downregulates *ara/caup* in the hinge territory. Red: accumulation of Ara/Caup. (A) Wild-type disc. The notum Ara/Caup domain (arrow) is widely separated (white line) from the Nub (blue) wing pouch domain. (B) Disc harboring large $msh^{\Delta 68}$ M^{+} clones (absence of green). The notal Ara/Caup domain reaches almost to the wing pouch (arrowhead). Asterisks indicate the area incapable of expressing *ara/caup*. (C) Small $msh^{\Delta 68}$ clones (absence of green), induced 48/72 hours AEL, autonomously derepress *ara/caup* at the presumptive hinge, except when located (arrowhead) in the area shown in B (asterisk). (D) Disc overexpressing UAS-*mshi* driven by *ap-Gal4*. Msh almost completely disappears from the dorsal hinge (d) and Ara/Caup accumulates there. A slightly convex line joining the arrowheads would approximately demarcate the notum/dorsal-hinge border. Arrow indicates presumptive ventral hinge and pleura with unmodified *msh* and *ara/caup* expressions. (E) Clone overexpressing *UAS-msh* (green, GFP marker, induced at 12-36 hours AEL) removed or strongly inhibited notal Ara/Caup accumulation (arrowhead). (F) Drawing of a series of non-Minute $msh^{\Delta 68}$ clones reveals the areas (purple) competent to express *ara/caup*. (G) Early overexpression of *UAS-msh*, *ap-Gal4* driver at 25°C (green, *UAS-GFP* marker) interfered with the growth of the notum territory (arrowhead; compare with wild-type disc in A, arrow). *ara/caup* expression always persisted in the proximal-most part of the notum territory, a result similarly observed with clones expressing *UAS-msh* in this location (text). This suggests that Iro-C is differentially controlled in different regions of the notum.

normal frequency (Fig. 5D). However, in the notum territory, clones did not survive or were very small, when compared with the twin wild-type clones (Fig. 5D). Thus, in this territory, cells that are neither specified as notum nor can properly develop as hinge are not viable or are outcompeted by the wild-type cells.

Other controls of *msh* at the dorsal hinge

It is known that the dorsal selector gene *ap* positively regulates *msh* in the dorsal wing blade territory (Milán et al., 2001). We investigated whether *ap* also regulates *msh* at the dorsal hinge and notum. Wing discs homozygous for the null ap^{UGO35} allele have in general a profoundly altered morphology. Some discs, however, have recognizable territories and, in both early and late third instar, had very little expression of *msh* in the presumptive hinge and notum (Fig. 5E; not shown). As expected, Iro-C expression was expanded (Fig. 5E). Clones that lack the essential Ap co-factor Chip are equivalent to reducing *ap* function (Fernández-Fúnez et al., 1998; Morcillo et al., 1997). In these clones, *msh* expression was eliminated at the dorsal hinge and wing pouch, but not at the ventral hinge, where *ap* is not expressed (Fig. 5F). Hence, *msh* is under the positive control of *ap* in the whole *ap* domain.

Because in the second instar disc, Dpp signaling confines *ara/caup* expression to the notal region of the disc (Cavodeassi et al., 2002), we examined whether this inhibition was mediated by *msh*. The expression of *msh* under loss- and gain-of-function conditions for Dpp argued against this possibility (not shown). Wg signaling is most important to form and pattern the hinge and the wing (Couso et al., 1993; Ng et al., 1996; Sharma and Chopra, 1976). Again, loss- and gain-of-function conditions for Wg did not prevent expression of *msh* in the dorsal hinge (not shown), indicating that Wg does not control *msh*.

Discussion

In *Drosophila*, the homeodomain gene *msh* is known to be involved in different processes. Thus, it participates in regional specification of muscle progenitors/founders (Nose et al., 1998); together with *vnd* and *ind*, it helps subdivide the embryonic neuroectoderm along the dorsoventral axis (reviewed in Cornell and Ohlen, 2000; Gómez-Skarmeta et al., 2003; Skeath, 1999); and it confers dorsal identity to the dorsal bristles of the anterior margin of the wing (Milán et al., 2001). We report additional functions of *msh*, namely, the formation/maintenance of the subdivision between the territories of the wing disc that will give rise to the notum (dorsal mesothoracic trunk) and the dorsal hinge (appendix), the proper growth of the dorsal hinge, and the patterning of this region and of the notum.

msh is required for dorsal hinge development

In the developing wing disc, *msh* is expressed most strongly in the territory of the dorsal hinge, the region between the notum and the dorsal wing blade territories. Removal of *msh* in clones

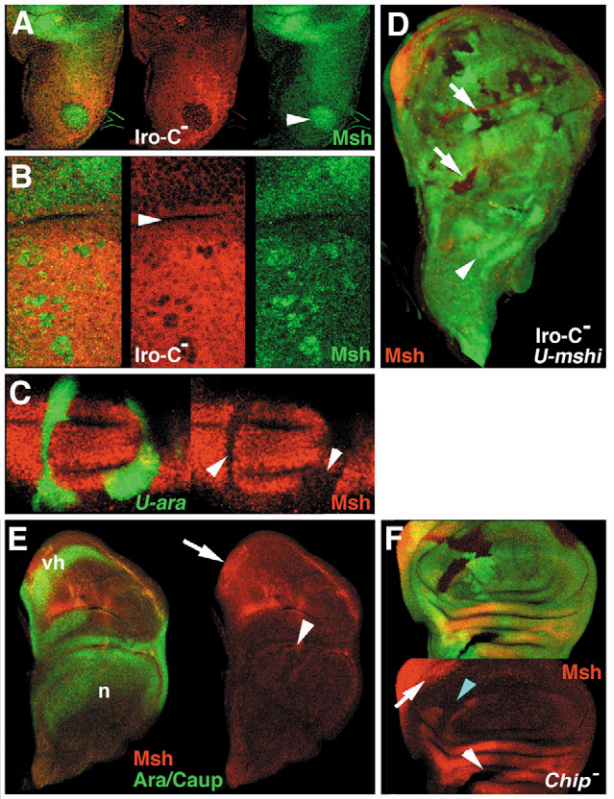


Fig. 5. Regulation of *msh* by *ara/caup* and *ap*. (A) iro^{DFM3} clone (absence of red; induced at 36-60 hours AEL). *msh* is autonomously upregulated (arrowhead). (B) Small, late-induced (60-84 hours AEL) iro^{DFM3} clones (absence of red) near the hinge border (arrowhead) similarly derepress *msh*. (C) Clones overexpressing *UAS-ara* (induced at 36-60 hours AEL) autonomously repress *msh* in the hinge territory (arrowheads). (D) iro^{DFM3} clones (absence of green) do not survive in the notum region of discs deficient for Msh (*UAS-mshi* was driven with *ap-Gal4*). Clones are found only in the dorsal hinge and in the wing pouch regions (arrows). The wild-type twin spots (bright green, arrowhead) indicate that mitotic recombination events took place in the notum territory. (E) Late third instar wing disc from a homozygous ap^{UGO35} larva. *msh* expression (red) is absent in essentially all the dorsal compartment of the disc (arrowhead), but it is present in the ventral hinge (arrow). *ara/caup* expression is shown in green. n, notum territory; vh, ventral hinge. (F) $Chip^{e55}$ clones (absence of green; induced 24-48 hours AEL) failed to activate *msh* at the dorsal hinge (arrowhead) and dorsal wing pouch (blue arrowhead), but were without effect at the ventral hinge (arrow), consistent with the independence of *msh* expression in this territory from *ap*.

results in malformations that range from small defects, such as an outheld wing, to partial or even complete loss of most hinge structures. In the latter cases, the hinge may be posteriorly misplaced and ectopically attached to the scutellum. In addition, in a fraction of flies ectopic notum tissue appears contiguous to the extant hinge. Because at least a large part of the hinge tissue is still present, we surmise that the absence of recognizable hinge structures is due to the failure of their proper differentiation. This phenotype correlates well with that observed in third instar wing discs displaying msh^- clones. Indeed, even large clones that remove *msh* from most of the dorsal hinge territory allow the specification of this territory, as demonstrated by the relatively unmodified characteristic patterns of expression of genes such as *wg*, *zfh-2*, *hth* and *tsh*, and the presence of recognizable proneural clusters of *sc* expression. Moreover, the presence in mutant hinges of relatively well resolved clusters of *sc* expression (Fig. 3D) indicate that the prepatterning of the hinge can proceed to a large extent in the absence of *msh*. We conclude that *msh* is largely dispensable for specification of the dorsal hinge territory, but it is required for the final stages of its patterning and differentiation.

Mutual repression between *msh* and Iro-C defines/maintains the notum/dorsal hinge subdivision of the wing disc

Mosaic analyses aimed at studying the patterns of cell proliferation in the wing disc disclosed the presence of the anterior, posterior, dorsal and ventral compartments of the wing with borders that imposed absolute restrictions to cell proliferation (García-Bellido et al., 1976). A border of this type was suggested to exist between the notum and dorsal hinge, as well as between the pleura and the ventral hinge (García-Bellido et al., 1976), but the complex morphology of these regions and the unavailability of appropriate cuticular markers made the proposal uncertain. In fact, analyses performed later in the wing disc, showed that clones could straddle the notum/dorsal hinge boundary, this being defined by the distal border of the Iro-C domain (Diez del Corral et al., 1999). Hence, at this boundary, the descendants of a cell would adopt their developmental fate not according to lineage, but depending on the side of the boundary they were located. The issue thus arose of how the boundary between the notum and the dorsal hinge territories would be established and maintained. Considering that the extent of the notum territory is defined by the expression of the Iro-C (Diez del Corral et al., 1999; Wang et al., 2000), this issue can be largely resolved by explaining how the distal border of the Iro-C domain of expression is defined.

So far, several genetic interactions have been identified that together permit to suggest a mechanism that partially answers this question (Fig. 6). In the second instar disc, the EGFR pathway activates *ap* and Iro-C (Wang et al., 2000; Zecca and Struhl, 2002a; Zecca and Struhl, 2002b). The distinct but overlapping domains of expression of these genes, the dorsal compartment (*ap*) and the notum territory (Iro-C), may be defined by differential sensitivity to EGFR signaling (Zecca and Struhl, 2002a) or, alternatively, in the case of Iro-C, by Dpp signaling (Cavodeassi et al., 2002). In these early stages, Dpp signaling is active only in the distal part of the disc, where it represses the Iro-C and sets its distal limit of expression. Hence the antagonistic actions of the EGFR and the Dpp pathways would define the position of the distal limit of the Iro-C domain, and therefore the position of the notum/hinge subdivision.

We now find that at approximately the time Iro-C starts to be expressed in the more proximal part of the disc, i.e. that which will become the notum, expression of *msh*, by means of *ap*, is turned on in the adjacent dorsal hinge territory (see also Milán et al., 2001). These essentially complementary patterns of expression are maintained, with some qualifications, in the third instar disc. Loss- and gain-of-function experiments show that *msh* prevents *ara/caup* from being expressed in the hinge;

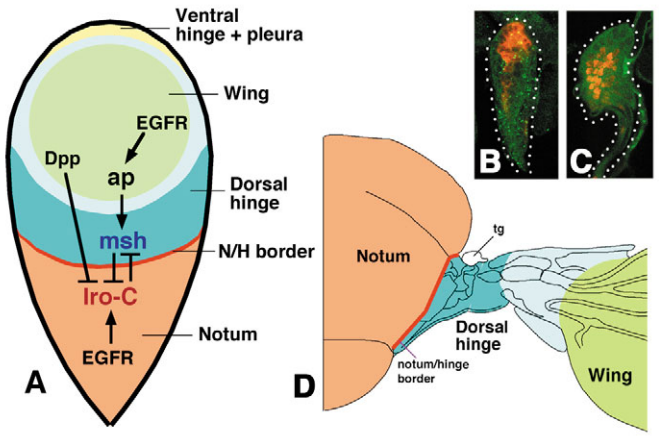


Fig. 6. Known genetic interactions that define/maintain the notum/dorsal hinge subdivision of the wing disc. (A) In the second instar, EGFR signaling activates the 'pronotum' genes Iro-C (Wang et al., 2000; Zecca and Struhl, 2002a; Zecca and Struhl, 2002b), and Dpp signaling, which is active only in the distal part of the disc, confines the expression of Iro-C to the proximal part of the disc (Cavodeassi et al., 2002), thus defining the notum territory. Also in the second instar, EGFR signaling, by means of *ap*, activates *msh* in the dorsal hinge. The proximal border of the *msh* domain abuts the Iro-C territory and the mutual repression between these genes contributes to maintain and stabilize the border between the Iro-C and the Msh territories. As discussed in the text, this border should define and/or maintain the notum/dorsal hinge subdivision of the disc (red line). (B,C) In support of this model, B and C show the expressions (red) in first/second instar discs of *dpp-lacZ* (B), which occurs mostly in the distal part of the disc, and of *ap-lacZ* (C), which takes place most strongly in a more central region. Within this region, *msh* will later be activated at high levels (Fig. 1A, and not shown). The contour of the discs has been marked with broken lines. (D) Adult structures that correspond to the domains of Iro-C (notum, orange) and *msh* (dorsal hinge, blue). The dorsal hinge has been equated to the *msh* domain. Its distal limit with the proximal wing (light blue), approximately corresponds with the inner circle of Wg expression (Fig. 1C) (del Álamo Rodríguez et al., 2002).

and *ara/caup* restrain *msh* from being expressed in the notum at the high levels typical of the hinge (although it is expressed at a low level in part of the notum). This mutual repression also occurs late during development.

How relevant is this mutual repression for the establishment of the notum/dorsal hinge territorial subdivision? As indicated above, in ~19% of flies with *msh* clones, the removal of Msh from the hinge induces extra notum tissue. In the remaining cases, this removal does not substantially affect the identity of the hinge territory. Thus, the mutual repression between *msh* and Iro-C is crucial for the notum/hinge territorial subdivision in only a small but substantial fraction of the discs. This indicates that additional agents, probably expressed in the hinge, participate in effecting the subdivision. By contrast, notum cells that lose Iro-C always change their fate to hinge cells and, consequently, depending on position, they modify the notum/hinge subdivision or create an ectopic notum/hinge boundary (Diez del Corral et al., 1999). Hence, the relevance of the *msh*/Iro-C mutual repression to define/maintain that subdivision relies mainly on its defining/maintaining the border of the Iro-C domain, and thereby preventing the expression of hinge genes within the notum territory. Thus, a 'pronotum' gene (Iro-C) and a 'hinge differentiation' gene (*msh*), despite their different positions within the genetic hierarchies that govern the development of their respective domains, cross-regulate each other and participate in the early definition of their respective territories. Our current data do not permit us to distinguish between the possibilities that the mutual repression between *msh* and Iro-C is instrumental in establishing this territorial border, or, alternatively, that it stabilizes a previous border defined by the antagonistic actions of EGFR and Dpp on the Iro-C.

The relevance of the mutual repression between Iro-C and *msh* is also manifested by their respective overexpression. Ectopic Iro-C products in the hinge impair the proper differentiation of hinge structures (R. Diez del Corral, PhD thesis, Universidad Autónoma de Madrid, 1998). High levels of Msh in the notum turn on a hinge-specific marker like *zfh-2* (Fig. 3G) and are detrimental for notum development (Fig. 4G).

In the third instar disc, the distal border of the Iro-C domain is no longer straight and displays a pronounced 'bay' where *ara/caup* are downregulated (Fig. 1C, blue arrow, red channel). This roughly coincides with the area of highest expression of *msh* in the lateral notum. *msh* is probably responsible for this downregulation of *ara/caup*, as the 'bay' disappears in *msh* clones (Fig. 4F). Moreover, the abutting domains of *msh* and Iro-C in the ventral hinge and pleura, respectively (Fig. 1C), suggest that a similar mutual repression may occur there to establish the subdivision between these neighboring regions. Finally, the removal of *msh* does not activate Iro-C in the anterior part of the hinge territory (Fig. 4B), suggesting again that agents other than *msh* and Dpp (Cavodeassi et al., 2002) help maintain Iro-C expression confined to the notum territory.

Organizing properties of the notum/hinge boundary

Iro-C^{-} clones located within the medial notum not only undergo an autonomous transformation to dorsal hinge. They also become surrounded by a fold similar to that which separates the notum and hinge territories, and they modify the expression of several markers in the surrounding wild-type tissue in a way consistent with a transformation of this tissue towards lateral notum (Diez del Corral et al., 1999). These nonautonomous effects suggest that signals emerge from the Iro-C^{-} clones, and that these signals alter the fate of the aposed notum tissue. Hence, it was inferred that, in the wild-type disc, signaling would take place across the hinge/notum boundary and this would help pattern at least the lateral notum (Diez del Corral et al., 1999). This is reminiscent of the DV and AP compartment boundaries, where signaling mediated by the diffusible molecules Wg, and Hh and Dpp, respectively, are key to stimulating the growth and pattern of the wing disc (for reviews, see Brook et al., 1996; Teleman et al., 2001; Vincent and Briscoe, 2001). However, in the hinge/notum boundary, the signaling agents have not been identified. They could be either diffusible molecules or cell-bound molecules that mediate this cell to cell communication.

Now, we find that the imaginal disc territories flanking the notum/hinge border are reduced in size when they are mutant for *msh* (Fig. 3). We do not know whether this effect is due to decreased cell proliferation, increased cell death or both, and whether it mostly affects the hinge or the lateral notum. However, it is clear that by removing *msh* and allowing Iro-C

to be expressed in the hinge, the *msh* clones suppress the confrontation of proper hinge cells with notum cells. It is tempting to speculate that this could affect the net growth of the territory by removing positional values (García-Bellido et al., 1994) and/or by suppressing or making ineffective the postulated signaling associated with the hinge/notum border. Consistently, a reduced size of the notum plus hinge region (and a simplification of the patterning) is also observed in discs overexpressing *UAS-ara* in the dorsal compartment (Diez del Corral et al., 1999) (E.V.-C. and J.M., unpublished), a condition that removes most *msh* expression from the hinge. The failure of Iro-C$^-$ clones within the notum territory to grow and survive when they are also depleted of Msh (Fig. 5D) might result from the absence of proper signaling across a boundary where wild-type notum cells confront Iro-C$^-$ *msh*$^-$ cells. Considering that the activity of the EGFR signaling pathway is necessary for notum cell proliferation (Díaz-Benjumea and García-Bellido, 1990; Simcox et al., 1996; Wang et al., 2000), it would be of interest to examine whether this pathway is involved in, or is modulated by, the presence of the notum/hinge boundary.

In *Drosophila*, the Iro-C genes and *msh* respectively participate in the DV subdivision of the eye (Cavodeassi et al., 1999; McNeill et al., 1997) and of the neuroectoderm (reviewed by Cornell and Ohlen, 2000; Gómez-Skarmeta et al., 2003; Skeath, 1999). In vertebrates, although to our knowledge no instance of mutual repression between homologs of Iro-C and *msh* has been described, members of each family participate in establishing borders by repression with other genes in the spinal cord, the brain (reviewed by Gómez-Skarmeta et al., 2003) and between rhombomeres (Lecaudey et al., 2004). Clearly, both genes are used frequently to subdivide territories and establish alternative differentiation pathways at each side of the border that separates them.

msh helps patterning the notum

Throughout the third instar, *msh* is expressed at relatively low levels in the posterior notum territory. Here, removal of *msh* most often results in impaired growth of the scutellum, absence of the scutellum/scutum suture and alterations of the bristle pattern. Interestingly, the lateral/anterior notum macrochaetae are often missing, even though they arise in a region apparently devoid of *msh* expression. This suggests that either *msh* is expressed there at very low but functional levels, or that the suppression of macrochaetae results from non-autonomous effects of the absence of Msh from neighboring territories. It should be noted that non-autonomous macrochaetae suppression is also associated with Iro-C$^-$ clones that cause notum to hinge transformations (Diez del Corral et al., 1999). This has suggested that modification of the putative signaling across the notum/hinge boundary interferes with macrochaetae patterning at the notum. It is possible that the *msh* clones might also interfere, as indicated above, with signaling from this border. If so, the presence of clusters of *sc* expression at the anterior lateral notum within large *msh* clones (Fig. 3D) suggest that this interference might occur at a stage later than the emergence of the proneural clusters.

The absence of *msh* function does not modify the expression of Iro-C in the lateral notum or the characteristic patterns of expression of *eyg* and *hth* (E.V.-C., unpublished), genes that are high in the hierarchy that control notum development (Aldaz et al., 2003; Aldaz et al., 2005). But it removes the scutum/scutellar suture and promote development of extra bristles in the dorsocentral and scutellar regions. Again, these are phenotypes suggestive of an interference with the late patterning and differentiation of these structures.

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