tailup, a LIM-HD gene, and Iro-C cooperate in Drosophila dorsal mesothorax specification

Joaquín de Navascués and Juan Modolell*

The LIM-HD gene tailup (tup; also known as islet) has been categorised as a prepatterning gene that antagonises the formation of sensory bristles on the notum of Drosophila by downregulating the expression of the proneural achaete-scute genes. Here we show that tup has an earlier function in the development of the imaginal wing disc; namely, the specification of the notum territory. Absence of tup function causes cells of this anlage to upregulate different wing-hinge genes and to lose expression of some notum genes. Consistently, these cells differentiate hinge structures or modified notum cuticle. The LIM-HD co-factors Chip and Ssdp are also necessary for notum specification. This suggests that Tup acts in this process in a complex with CHIP and Ssdp. Overexpression of tup, together with araucan, a ‘pronotum’ gene of the iroquois complex (Iro-C), synergistically reinforces the weak capacity of either gene, when overexpressed singly, to induce ectopic notum-like development. Whereas the Iro-C genes are activated in the notum anlage by EGFR signalling, tup is positively regulated by Dpp signalling. Our data support a model in which the EGFR and Dpp signalling pathways, with their respective downstream Iro-C and tup genes, converge and cooperate to commit cells to the notum developmental fate.

KEY WORDS: tailup, islet, Notum development, EGFR, Dpp, Drosophila

INTRODUCTION

The imaginal wing discs of Drosophila, the precursors of the wings and most of the mesothorax, are a classical system in which to study the allocation of different subsets of cells to diverse developmental fates, i.e. body wall (dorsal mesothorax) or appendage (wing). Although we still lack a comprehensive picture of the genetic processes governing the development of the wing disc, genes and signalling pathways have been identified that define the proximal-most part of the disc as the notum territory (reviewed by Calleja et al., 2002; Mann and Morata, 2000). The EGFR signalling pathway plays a major role, as its absence prevents formation of the notum (Simcox et al., 1996; Wang et al., 2000; Zecca and Struhl, 2002b). In the notum anlage, EGFR signalling activates the genes of the iroquois complex (Iro-C), a cluster of three related homeodomain genes. Consistently, these cells differentiate hinge structures or modified notum cuticle. The LIM-HD co-factors Chip and Ssdp are also necessary for notum specification. This suggests that Tup acts in this process in a complex with CHIP and Ssdp. Overexpression of tup, together with araucan, a ‘pronotum’ gene of the iroquois complex (Iro-C), synergistically reinforces the weak capacity of either gene, when overexpressed singly, to induce ectopic notum-like development. Whereas the Iro-C genes are activated in the notum anlage by EGFR signalling, tup is positively regulated by Dpp signalling. Our data support a model in which the EGFR and Dpp signalling pathways, with their respective downstream Iro-C and tup genes, converge and cooperate to commit cells to the notum developmental fate.

*Author for correspondence (e-mail: jmodol@cbm.uam.es)
scutellar and DC macrochaetae, whereas overexpression of *tup* suppresses bristle development. Tup can physically interact with Pnr and with Chip (Biryukova and Heitzler, 2005; van Meyel et al., 1999), both positive regulators of achaete-scute expression in the DC proneural cluster (García-García et al., 1999; Ramain et al., 2000). Accordingly, *tup* has been considered a member of the prepatter genes that control achaete-scute expression (Biryukova and Heitzler, 2005). Here we show that, similarly to Iro-C, *tup* has an earlier ‘pronotum’ function that is essential to commit cells to notum development. For this function, *tup* most likely forms a complex with Chip and Sdpl.

**MATERIALS AND METHODS**

**Drosophila stocks**

Most *Drosophila* stocks are described in FlyBase (http://flybase.org/). *tup* (is*Δ*238*Δ*48), *tup* (is*Δ*238*Δ*48) and *tup* (is*Δ*238*Δ*48) were freed of associated lethal mutations, recombined with the FRT40A and characterised at the molecular level. This characterisation agreed with Biryukova and Heitzler (Biryukova and Heitzler, 2005). We obtained (see Parks et al., 2004) a deletion (*tup*Δ*654*) between the FRT-bearing insertions WH04735 and XP03613 (Thibault et al., 2004) that removes the entire ORF of *tup* (deletion of the interval 18.856.481-18.877.346 of chromosome 2L, version 4.2 of the annotated D. melanogaster genome). *tup*-specific RNAi was produced with a *UAS-*tup*Δ* transgene constructed (Nagel et al., 2002) using an 810 nucleotide fragment of *tup* cDNA AF145674 (interval 96-906).

**Mosaic analyses**

To generate clones of cells mutant for *tup*, *w, hs-FLP1.22; tup*, FRT40A/CyoO or *w, hs-FLP1.22; P{f+}87D, M(3)95A/FRT82B, Ssdp neo48, ey/H9004* larvae. Other *UAS-FLP1.22; Act>y+>Gal4, UAS-GFP/+ UAS-X/+* larvae. The *tup*Δ*654* allele was produced with a *UAS-Chip* activated transgene using an 810 nucleotide fragment of *tup* cDNA AF145674 (interval 96-906). *tup*Δ*654* males, and the progeny (Morata and Heitzler, 2005). Here we show that, similarly to Iro-C, *tup* has an earlier ‘pronotum’ function that is essential to commit cells to notum development. For this function, *tup* most likely forms a complex with Chip and Sdpl.

**Antibody staining**

Imaginal discs were fixed and stained as described previously (Cubas et al., 1991). Antibodies were: mouse anti-Tup (mAb 40.3A4, DSHB), rabbit anti-β-galactosidase (Cappell), rat anti-Ara/Caup (Díez del Corral et al., 1999), rabbit anti-Msh (McDonald et al., 1998) (provided by C. Doe), rabbit anti-Tsh (Ng et al., 1996), rat anti-Zfh2 (Whitworth and Russell, 2003), rabbit anti-Ush (Fossett et al., 2001), guinea pig anti-Eyg (Aldaz et al., 2003), mouse anti-Nub (Averof and Cohen, 1997), rabbit anti-Sal (de Celis et al., 1999). Secondary antibodies and rhodamine phalloidin were obtained from Molecular Probes or Jackson ImmunoResearch.

**Image acquisition**

Adult unmounted flies were photographed with a Zeiss Axioim phot microscope. Images of different focal planes were combined using Photoshop (Adobe). Fluorescence images were captured using a confocal system.

**RESULTS**

**tup** is necessary for notum development

Adult *tup* phenotypes were examined in mitotic recombination clones homozygous for the newly generated null deletion allele *tup*Δ*654* and the previously described alleles *tup*Δ*654*, *tup*Δ*654* and *tup*Δ*654*. We focused on the notum because in third instar wing discs *tup* is exclusively expressed in the notum rudiment (Biryukova and Heitzler, 2005; Butler et al., 2003). A quantitative summary of this phenotypic analysis, comprising over 1600 homozygous *tup*Δ*654* clones, is presented in Table S1 (see Table S1 in the supplementary material). Similar phenotypes were observed with the other *tup* alleles.

Clones were associated with a variety of phenotypes whose nature and frequency depended on the position of the clone (see Fig. S1C in the supplementary material) and on the developmental time of its induction (see Table S1 in the supplementary material). They ranged from partial or complete loss of a heminotum (see Fig. S1A in the supplementary material), to formation on the notum of ectopic wing-hinge structures, malformations of the notum cuticle (Fig. 1) and modifications to the bristle pattern. This latter phenotype will not be described, as effects of *tup* mutations on this pattern have already been reported (Biryukova and Heitzler, 2005). The ectopic hinge structures were tegulae (Fig. 1C) or tegula-like structures (Fig. 1A,B), recognisable sclerites (Fig. 1B) and hinge-like sensilla campaniformia (Fig. 1G,L) or trichoidia (see Fig. S1B in the supplementary material). Seemingly parallel transformations occurred on the metastar, a derivative of the haltere disc, in which *tup* is also expressed during larval development (data not shown). Sensilla campaniformia similar to those found in the basal part of the haltere were present in the metanotum (Fig. 1D), a region that does not harbour sensilla in the wild type.

Other malformations of the notum cuticle consisted of invaginations (Fig. 1F-I) or protrusions (Fig. 1E). Some invaginations gave rise to vesicles that displayed trichomes and hinge-like sensilla campaniformia (Fig. 1G). At late clone-induction times, a proportion of the vesicles were separated from the notum cuticle, lacked any kind of sensillum, but conserved trichomes (data not shown). Additional morphologically distinct malformations consisted of small, tubercle-like disruptions of the cuticle, with a corrugated appearance and roundish contour (Fig. 1J-L). At their centre, they could have shallow depressions (Fig. 1L) or deep and narrow invaginations (Fig. 1K). The presence of macro- and/or microchaetae indicated that the malformations still developed a
notum-like cuticle (Fig. 1E,H,I,K), although occasionally we observed sensilla campaniformia (Fig. 1L) or trichoidea (see Fig. S1 in the supplementary material). The invaginations, projections, tubercles, and attached and detached vesicles probably form a related group of lesions caused by a tendency of *tup* clones to detach from the notum epidermis, an indication of differential cellular adhesion properties. In summary, a proportion of *tup* clones give rise to structures indicative of notum-to-hinge transformations, whereas other clones induce malformations suggestive of modified cell-cell adhesion properties, but maintaining a notum-like identity.

**Expression of *tup* in the wing disc**

As early as late first/early second instar, *tup* expression was seen to be confined to the most proximal region of the disc (Fig. 2A,B), which corresponds to at least part of the prospective notum territory. During the second and part of the third instar, *tup* is expressed in all the medial notum territory (this being defined by the *pnr*-Gal4 marker) (Calleja et al., 2000) and was seen to extend into the lateral notum (Fig. 2C). In the mid-late third instar, strong expression was maintained in the posterior medial (arrow) and part of the lateral (arrowhead) notum (Fig. 2D). Weak residual activity might be present in the anterior notum (Fig. 2D, asterisk). Comparison with *ara/caup*, which at these stages are expressed in the lateral notum, indicates that the most lateral region of the posterior notum is essentially free of Tup (Fig. 2D) (see also Biryukova and Heitzler, 2005; Butler et al., 2003).

**tup clones show differential affinity in wing discs**

We examined the morphology of *tup* clones in the notum region of third instar wing discs. Clones induced at the first instar were generally large and with a smooth border, which at times was associated with an ectopic fold of the notum epithelium (Fig. 3A). Smaller, later-induced clones, could have either smooth and roundish, or wiggly borders (Fig. 3B). The smooth clones were more prevalent in the posterior notum, which is the region of strong *tup* expression (Fig. 2D). Smooth contours suggest a differential affinity between two cell populations, as these tend to minimise contacts. In addition, many roundish *tup* clones partially extruded themselves towards the subjacent adepithelial cells (Fig. 3C,D). This behaviour might correlate with the invaginations associated with the adult *tup* mutant epidermis. Still, at these stages, clone cells did not lose their apical connections with the neighbouring wild-type cells, as revealed by the continuous band of apical actin accumulation (Fig. 3D).
Fig. 2. Expression of tup in the imaginal wing disc. (A) Early second instar disc. Green, Tup; red, ap<sup>α558</sup>-lacZ, a marker for the dorsal compartment. (B) Late second instar disc. (C) Notum region of a mid-third instar disc. Red, pnr-Gal4 UAS-lacZ. (D) Late third instar disc. Red, Ara/CauP. Dotted lines indicate position of the LN/WH and WH/WP borders. Asterisk, region of possible low accumulation of Tup. a, anterior; p, posterior; MN, medial notum; LN, lateral notum; PLN, posterior lateral-most notum; WH, wing hinge; WP, wing pouch; tg, tegula.

Notum tup clones express hinge markers

Next, we analysed the expression of hinge markers in discs harbouring tup clones. msh (also known as Drop – Flybase), which is expressed strongly at the dorsal hinge and weakly in part of the posterior notum (D’Alessio and Frasch, 1996; Villa-Cuesta and Modolell, 2005) (Fig. 3B”, asterisk; Fig. 3G), was always upregulated in first instar-induced clones located at the medial and central notum (Fig. 3F), in some cases even in the neighbouring wild-type tissue (Fig. 3E). However, many clones located at the lateral-most notum failed to upregulate msh. In later-induced clones, derepression was generally limited to clones at or near the expression domain of tup. Moreover, the levels of expression were different from clone to clone (Fig. 3B,B”) and at times even among cells of the same clone (Fig. 3B”). Qualitatively similar observations were made with zfh2, which is expressed almost exclusively in the distal hinge (Whitworth and Russell, 2003) (Fig. 6L), spalt (sal; also known as salm – Flybase), which is expressed at high levels in the hinge and lateral notum territories and at a lower level in the posterior notum (de Celis et al., 1999) (Fig. 3I), and the lacZ insertion line l(2)09261, which is expressed in the hinge and wing pouch territories (Díez del Corral et al., 1999). As examples, we show early-induced clones in which l(2)09261 and sal were respectively upregulated (Fig. 3A,H), and one clone out of several expressing msh that also expressed zfh2 (Fig. 3B,B’).

In summary, the requirement of notum cells for tup is strongest in the first/second instar and decreases with the age of the disc. This is consistent with the incomplete transformation towards hinge exhibited by many clones in the adult. We should stress that large, early-induced clones (Fig. 3A,F,H), which invariably showed strong derepression of hinge markers, did not survive to adulthood as we never observed territories of tup cuticle of the corresponding large size. The infrequent adults that displayed strong defects in the fusion of the heminota or had most of a heminotum missing (see Table S1 in the supplementary material) might have harboured such clones.

tup clones lose notum markers

Next, we examined the effect of tup clones on genes important for notum development. pnr expression was removed in all first instar-induced clones (Fig. 4B), and also in most later-induced clones (~85%; Fig. 4E shows exceptions), especially in those located at the more distal part of the pnr domain (Fig. 4D). Ush, which accumulates in a region nested within the pnr domain (Fig. 4A), was removed in first and second instar-induced tup clones (Fig. 4C and not data shown), and was partially lost in third instar-induced clones. However, in large first instar-induced clones, ush was often expressed in a subregion of the clone. This subregion coexpressed msh (data not shown) and usually displayed a fold of the epithelium (Fig. 4B; see also 4I). These characteristics indicate a transformation towards hinge, as ush is normally expressed in the hinge region of
upregulated in clones in which \textit{ara/caup} were expressed (Fig. 4H). Thus, in some instances, \textit{tup} cells simultaneously expressed hinge and notum genes.

**Chip and Ssdp are co-factors of Tup for notum specification**

Since Tup can physically interact with Chip (Biryukova and Heitzler, 2005; van Meyel et al., 1999), we examined whether this co-factor was involved in the ‘pronotum’ function of Tup. This seemed to be the case. First instar-induced \textit{Chip^{5.5}} clones located in the presumptive notum showed derepression of \textit{zh2} and downregulation of \textit{eyg} (Fig. 5A), which indicated a notum-to-hinge transformation. Moreover, \textit{msh} was also derepressed in part of the clones, but only in a non-autonomous manner (Fig. 5B). [Chip is required for \textit{msh} expression in the hinge (Villa-Cuesta and Modolell, 2005), so the absence of \textit{msh} activation within the clones was expected.] Some of the flies bearing \textit{Chip} clones survived to adulthood and showed cuticular defects similar to those associated with early-induced \textit{tup} clones, including ectopic tegulae and sensilla trichoidea (see Fig. S2B in the supplementary material).

As the above results indicate that Tup and Chip are both positive effectors of notum specification, and given that they can physically interact (Biryukova and Heitzler, 2005; van Meyel et al., 1999), we asked whether they might function as an hexameric complex with Ssdp, similar to the 2Ap-2Chip-2Ssdp complex (reviewed by Matthews and Visvader, 2003). We tested whether \textit{Ssdp} affected notum specification. We used the hypomorphic \textit{Ssdp^{neo48}} allele, as clones null for \textit{Ssdp} are not recovered in adults (van Meyel et al., 2003) and hardly grow in imaginal discs even in a \textit{Minute} heterozygous background (data not shown). Forty per cent of \textit{Ssdp^{neo48}} clones lost \textit{eyg} expression and gained \textit{zh2} expression (Fig. 5C), and adult flies bearing these clones showed cuticular defects similar to those harbouring \textit{tup} or \textit{Chip} clones (see Fig. S2C in the supplementary material) and, in one example, showed an outgrowth composed of proximal costa tissue (see Fig. S2D,E in the supplementary material).

In the wing, an experimental excess of Chip titrates Ap and Ssdp, prevents formation of the hexameric complex, and phenotypically mimics the loss-of-function of \textit{Chip} (Fernández-Fúnez et al., 1998; Milán and Cohen, 1999; Rincón-Limas et al., 2000). Accordingly, we checked whether an excess of Chip also interfered with notum specification. First instar-induced clones overexpressing either \textit{UAS-Chip} or \textit{UAS-Chip^{5.5}} (which lacks the dimerisation domain) in the posterior and proximal notum showed loss of \textit{eyg} expression and acquired expression of \textit{zh2} (Fig. 5D and data not shown).

**Overexpression of \textit{tup} and \textit{ara} synergistically promote notum development**

We compared the ability of \textit{tup} and the Iro-C gene \textit{ara}, overexpressed either singly or together, to impose notum development on cells normally fated to differentiate into other structures. Ubiquitous, relatively late overexpression of \textit{UAS-tup} (\textit{C765-Gal4} driver) (Gómez-Skarmeta et al., 1996) induced formation of notum-like tissue in the mesopleura (Fig. 6A,C) and extra notum-like bristles on the tegula (Fig. 6C). By contrast, overexpression of \textit{UAS-ara} under the same conditions did not induce notum-like structures (Fig. 6B), although it reduced the size of the wing (see Gómez-Skarmeta et al., 1996). Overexpression of both \textit{UAS-ara} and \textit{UAS-tup} had a more drastic effect: the wing and wing hinge were replaced by a large structure of notum-like tissue (Fig. 6D). The notum-like structure was also present on the mesopleura, a territory where Iro-C is expressed in the wild type (Gómez-
observed when UAS-ara or UAS-tup were overexpressed singly. Taken together, these results suggest a synergism of Iro-C and tup in promoting notum development.

**tup and Iro-C are differently regulated**

In the notum territory, Iro-C is activated by the EGFR signalling pathway (Wang et al., 2000; Zecca and Struhl, 2002a). This led us to examine whether tup was also controlled by EGFR. Clones homozygous for the null Egfr$^{K55}$ allele suppressed expression of ara/caup as expected, but not that of tup (Fig. 7A). Similar results were obtained with Ras85D$f^{D280B}$ (Fig. 7B) or pnr$^{AS88}$ clones, or by overexpressing UAS-args or UAS-Raf$^{DN}$ (Raf is also known as phl – Flybase) (data not shown), all of which constitute milder conditions for inhibiting the EGFR pathway. Moreover, constitutive activation of the EGFR pathway by overexpressing UAS-Ras$^{V12}$, clearly activated ara/caup in the hinge territory, but not so tup (Fig. 7E). Similar clones in the notum did not modify tup expression. The independence of tup from the EGFR pathway was also verified at developmental times close to those of notum specification (Wang et al., 2000). In second and early third instar wing discs, overexpression of Mkp$^3$, a strong inhibitor of the pathway (Ruiz-Gómez et al., 2005), reduced notum growth and clearly inhibited ara/caup, whereas tup remained almost unaffected (Fig. 7C,D). Together, these data strongly argue against any control of tup by EGFR.

Dpp signalling negatively regulates Iro-C and restricts its expression to the lateral notum (Cavodeassi et al., 2002). By contrast, removal of Dpp signalling in ikv$^{AS12}$ clones suppressed tup expression (Fig. 7F), except in some of the clones located in the lateral-most region. Moreover, overexpression of Dad, a strong inhibitor of the Dpp pathway (Tsuneizumi et al., 1997), turned off tup in second and early third instar discs (Fig. 7G). Conversely, activation of the Dpp pathway by the overexpression of UAS-tup$^{G9261}$, upregulated tup in the medial notum, although not so in the lateral notum (Fig. 7H). We conclude that Dpp signalling is a principal positive regulator of tup, although additional regulators probably exist and should account for the expression of tup in the Dpp-insensitive regions. Hence, Iro-C and tup appear to be differently regulated in this disc.

**DISCUSSION**

**tup is required for dorsal mesothorax formation**

Tup has been categorized as a prepatterning factor that controls the expression of the proneural achaete-scute genes in the third instar wing disc (Biryukova and Heitzler, 2005). Here we show that tup functions earlier in the development of the dorsal mesothorax. Loss of tup causes a range of phenotypes, which taken together indicate interference with the assignment of cells to form notum. Thus, depending on the time of induction of the clones and their location, we observe the formation of notum-like cuticle with altered cell-cell adhesion properties, the generation of ectopic wing-hinge structures including tegulae, sclerites or sensilla typical of the proximal wing, or even the loss of the entire heminotum. Consistent with these adult phenotypes, in third instar wing discs tup mutant cells can upregulate genes typically expressed at high levels in the wing-hinge territory of the disc, such as zfh2, msh, sal and the lacZ insertion line l(2)J9261. Concomitantly, notum-expressed genes such as eyg, ush and pnr are generally repressed, although in some cases tup cells may abnormally express notum and hinge genes together. These data indicate that notum tup cells undergo transformation towards either an altered notum fate or a hinge fate. Moreover, the activation of hinge markers in wild-type cells surrounding some tup clones might

Skarmeta et al., 1996). None of these effects were observed (data not shown) upon overexpression of a truncated Tup protein lacking the homeodomain (UAS-tup$^{M91}$).

These transformations were verified in third instar wing discs. UAS-tup, but not UAS-ara, activated eyg in part of the mesopleura territory and the DC-lacZ transgene in some of the mesopleura cells (Fig. 6E-G). (DC-lacZ harbours the notum-specific DC enhancer of the AS-C) (García-García et al., 1999). Coexpression of UAS-tup and UAS-ara greatly expanded the area of expression of eyg to parts of the dorsal hinge, the ventral hinge, pleura and wing pouch (Fig. 6H), consistent with the formation of large, notum-like structures.

Overexpression of both UAS-ara and UAS-tup with dpp-Gal4, which drives expression in a central stripe of the wing pouch (Staehling-Hampton et al., 1994), transformed the central part of the wing to notum-like tissue (Fig. 6I), whereas the anterior and posterior parts developed as wing tissue. Consistently in this phenotype, eyg was upregulated in the overexpression territory (Fig. 6K), whereas Zfh2 and the wing pouch marker Nub (Ng et al., 1995) were lost (Fig. 6L,M, arrowheads). Moreover, this driver also directs expression in leg discs, and eyg was derepressed in the sternopleural region (Fig. 6I). The adults displayed notum-like structures near the coxa (Fig. 6J), which indicated a transformation of this ventral region of the body wall towards notum. This transformation was not
reflected the presence of ectopic notum/hinge borders, which are known to promote non-autonomous effects (Diez del Corral et al., 1999; Villa-Cuesta and Modolell, 2005).

Unequivocal notum-to-hinge transformations are consistently observed in clones induced during the first larval instar. In later-induced clones, this phenotype becomes less manifest and the modified notum cuticle phenotype becomes prevalent. Accordingly, the upregulation of hinge marker genes and the converse downregulation of notum genes in the notum territory are most consistently observed in first instar-induced clones. This suggests that the requirement for the ‘pronotum’ function of tup progressively decreases as development advances. Lesions associated with tup clones can appear anywhere within the notum, although each particular phenotype shows a degree of topographic specificity. Interestingly, the activation of hinge genes and the repression of notum genes are best shown in early-induced clones located in the presumptive medial notum. Probably, these clones, which are normally large, do not yield adult structures as the expected large presumptive medial notum. Moreover, an excess of Chip would be expected to titrate Tup and/or Ssdp in incomplete complexes and mimic the loss-of-function phenotype of notum-to-hinge transformation, as was experimentally observed.

By contrast, during the later process of sensory organ formation, Tup appears to act by sequestering both Chip and Pnr, thus preventing activation of the proneural genes achaete-scute (Biryukova and Heitzler, 2005). This negative function of Tup does not seem relevant for notum specification, where both Tup and Chip work as positive effectors. Moreover, the Tup homeodomain is dispensable for titrating Chip and Pnr (Biryukova and Heitzler, 2005), but this is not the case for its ‘pronotum’ function (J.deN., unpublished). Interestingly, a missense mutation within the LIM-
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interacting domain of Chip (Chip⁵) severely reduces its ability to interact with Tup and suppresses the negative regulation by Tup of bristle formation (Biryukova and Heitzler, 2005). However, homozygous Chip⁵ flies have no defects in notum specification (Ramain et al., 2000). This suggests that a residual interaction between Chip⁵ and Tup might persist, as additionally suggested by the suppression of the extra bristles present in Chip⁵ individuals by UAS-tup overexpression (Biryukova and Heitzler, 2005). A weak interaction between Tup and Chip, which might only permit the formation of low levels of hexameric complex, might still allow proper notum specification. This suggestion agrees with the fact that tup¹⁰³⁶¹, a strong hypomorphic allele (as substantiated by its embryonic lethality over the null tup¹⁰, J.deN., unpublished), allows proper notum formation in homozygosis (Biryukova and Heitzler, 2005).

tup and Iro-C cooperate in notum development

Similarly to tup, Iro-C also has a ‘pronotum’ function. However, their roles are not entirely equivalent. Anywhere within the notum territory, loss of Iro-C during first or second instar induces a clear switch to hinge fate (Diez del Corral et al., 1999). By contrast, loss of tup causes an assortment of different combinations of derepressed hinge genes and repressed notum genes. Moreover, many tup clones induced during the second larval instar, and even some induced in the first, can develop recognisable notum cuticle. Thus, we propose that tup reinforces/stabilises the commitment of cells to develop as notum, a commitment imposed mainly by Iro-C. This reinforcement or stabilisation might be most necessary in the proximal part of the disc, where expression of ara/caup ceases after the second instar, but that of tup persists. This might account for the derepression of hinge genes being most manifest in this region. Depending on the location and time of Tup deprival, its loss may be inconsequential or lead to a partial or even a complete loss of notum commitment. Such diversity of consequences led us to explore whether tup might act on target genes by affecting chromatin remodelling. However, no genetic interactions have been found with Polycomb (Pc, Scp+Pcl+esc) or trithorax (trx, osa, brm, Trl, lawc) group genes (J.deN., unpublished).

In contrast to the absolute requirement for Iro-C for notum specification, overexpression of UAS-ara can impose a notum fate only on the wing anlage, and only when provided early in the development of the disc (Aldaz et al., 2003; Wang et al., 2000) (R. Diez del Corral, PhD thesis, Universidad Autónoma de Madrid, 1998). An extra notum with mirror-image disposition versus the extant notum is generated at the expense of the wing, a phenotype identical to that resulting from early deprivation of Wg function (Cousso et al., 1993; Morata and Lawrence, 1977; Ng et al., 1996; Sharma and Chopra, 1976). As UAS-ara overexpression can interfere with Wg expression (R. Diez del Corral, PhD thesis, Universidad Autónoma de Madrid, 1998), Wg deprival probably explains the formation of the extra notum. Thus, by itself, overexpression of UAS-ara probably lacks a genuine potential for imposing the notum fate. Similar notum duplications arise upon early and strong overexpression of UAS-tup (MD638, dpp-Gal4 and ptc-Gal4 drivers) and, again, they probably result from inhibition of Wg activity (J.deN., unpublished). Consistent with this interpretation, weaker and later expression of either UAS-tup or UAS-ara (C765 driver) (Gómez-Skarmeta et al., 1996) has little or no capacity to promote notum fate. However, when coexpressed, these transgenes are effective in imposing the notum fate and this should not be attributed to Wg depletion. Indeed, the transformation consists of an expansion of the notum tissue (Fig. 6D), rather than a notum duplication (Morata and Lawrence, 1977). Moreover, as detected by the onset of the ectopic expression of notum markers (eyg, DC-lacZ), the transformation occurs in late third instar discs (J.deN., unpublished) that have a nearly wild-type morphology and a distinguishable wing pouch (Fig.

Fig. 7. Regulation of tup in the wing disc. Red, Tup; blue or white, Ara/Caup. (A) M⁰ Egrf¹²⁵ clones (absence of green) remove ara/caup expression (arrowheads) but do not inhibit tup (arrow). (B) M⁰ Ras85D¹⁴⁴⁸ clones (absence of green) inhibit ara/caup (arrowheads), but not tup expression. (C) Second (top row) and early third (bottom row) instar discs. Overexpression of Mkp3 (green) inhibits ara/caup (arrowhead), but not tup (arrow). (D) Expression of ara/caup in wild-type discs of similar age to those shown in C. (E) Clones expressing UAS-Ras¹³¹² (green) activate ara/caup (arrowheads) in the wing hinge. tup is not activated, or only so at very low levels. (F) A tkv¹² clone (absence of green) removes tup expression in the medial (arrowhead), but not in the lateral (arrow) notum. (G) Second (top) and early third (bottom) instar discs. Overexpression of UAS-Dad (green) blocks Tup accumulation (arrowheads). Compare with Fig. 2B, C. (H) Clones expressing UAS-tkv¹² (green) activate tup in the medial (arrowhead), but not in the lateral (arrow) notum.
6H). This indicates that these markers are activated in territories previously specified as wing, hinge or pleura, and subsequently forced to acquire notum identity. Moreover, overexpression of the Wg pathway antagonists UAS-Asx or UAS- dTFCM (dTCF) is also known as pan – Flybase) with the same driver failed to transform wing towards notum (J.deN., unpublished). Finally, the activation of eyg and the formation of notum tissue in the sternopleurite, a derivative of the leg disc, also attests to the capacity of tup plus ara to commit cells to develop as notum.

EGFR and Dpp signalling pathways collaborate in notum specification

It is well established that signalling by the EGFR pathway is essential for notum development. Its inhibition prevents activation of Iro-C and the growth of the notum territory (Simcox et al., 1996; Wang et al., 2000; Zecca and Struhl, 2002b). By contrast, Dpp negatively regulates Iro-C and restricts its domain of expression at both its distal and proximal borders (Cavodeassi et al., 2002). Our data indicate a novel function of Dpp in notum development; namely, the activation or maintenance of tup expression in second and third instar discs. In the notum region of the early disc, Dpp signalling occurs at low levels (Cavodeassi et al., 2002), but our results suggest that these are sufficient for activating tup. Expression of tup is largely independent on EGFR signalling. Thus, EGFR and Dpp signalling seem to cooperate in specifying notum identity to the cells of the proximal part of the disc by activating their respective ‘pronotum’ downstream genes, Iro-C and tup.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/9/1779/DC1

References


mirror encodes a novel PBX-class homeoprotein that functions in the definition of the dorso-ventral border of the Drosophila eye. Genes Dev. 11, 1073-1082.


Table S1. Frequencies* of different phenotypes associated with tup** clones†

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<tr>
<th>Time of induction (hours AEL)</th>
<th>Number of clones examined</th>
<th>Twinspots lacking a mutant clone</th>
<th>No mutant phenotype</th>
<th>Cuticle spheres inside the notum</th>
<th>Cuticular lesions bearing sensory organs</th>
<th>Ectopic tegulae</th>
<th>Clones affecting microchaetae‡</th>
<th>Clones affecting macrochaetae§</th>
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ND, Not determined.

*Figures indicate the number of clones displaying the indicated phenotype and, in parentheses, the percentage of clones displaying that phenotype.

†Clones were induced by treatment at 37°C for 30 minutes and were recognized by the y marker. The twinspots were marked with ck**. The phenotypes whose frequencies are reported in this table result from this treatment. However, when either heat treatment was increased to 60 minutes, or clones were produced by Gal4 driver-induced Flp expression or by overexpression of a UAS-tupIR transgene, the mutant tup territories displayed additional phenotypes. Under these conditions, quantification was not carried out owing to the presence of too high a number of clones or to the impossibility of accurately recognizing the extension of the Tup-depleted territory. Thus, a qualitative description is reported. The phenotypes were: (1) thorax closure defects; (2) absence of a whole heminotum; (3) non-everted discs (which developed inside the thorax and abdomen); (4) protrusions of the cuticle which may bear sensilla trichoidea and/or campaniformia in the metathorax; (5) formation of ectopic tegulae outside the notopleural region (see¶); and (6) formation of ectopic sclerites. Some of these phenotypes are described in the main text. Of these phenotypes, the last two occurred rarely, whereas the others were relatively frequent (loss of heminotum, failure of disc eversion, protrusions in the metathorax) or appeared in most flies examined (defect of thorax closure).

‡The defects observed included patches of high density of microchaetae, shafts displaying reversed polarity, and large regions of the anterior notum presenting bristles (both tup** and wild type) arranged in swirls.

§tup mutant clones could affect all notum macrochaetae, although they had stronger and more frequent effects on the posterior scutum and scutellum. The defects consisted of the appearance of extra bristles, both in an autonomous and non-autonomous manner. The APA was an exception, for it was always removed when a clone occurred at this position. DC and PPA bristles were also absent in certain clones comprising their respective areas.

¶Ectopic tegulae, induced by treatment at 37°C for 30 minutes, occurred in only the notopleural region; so, only clones located in this region were scored.

**Cuticular spheres were also present in the lateral region, but were scored as either belonging to the anterior or posterior notum subregions.

††Drawing representing the extent of the different notum regions as used in this phenotypic analysis: