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

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The LIM-HD gene *tailup* (*tup*; also known as *islet*) has been categorised as a prepattern 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, *araucan* (*ara*), *caupolican* (*caup*) and *mirror* (*mirr*), that are conserved from worms to vertebrates (reviewed by Cavodeassi et al., 2001; Gómez-Skarmeta et al., 1996; McNeill et al., 1997). Since the inactivity of Iro-C changes the developmental fate of cells within the presumptive notum territory towards wing hinge (Diez del Corral et al., 1999), the Iro-C genes are considered to have a 'pronotum' function and their domain of expression in the second instar disc defines the extent of the notum territory. However, the overexpression of Iro-C genes imposes a notum differentiation fate on wing cells only under a limited set of conditions (Aldaz et al., 2003; Wang et al., 2000). This suggests that genes other than Iro-C help to specify notum identity.

Dpp signalling is also important for notum development. In the second instar disc, it defines the distal limit of the notum by repressing Iro-C in the hinge territory (Cavodeassi et al., 2002). Later, Dpp signalling effects a medial (proximal) versus lateral subdivision of the notum. This involves activation of the GATA factor Pannier (Pnr) and the Friend of GATA factor U-shaped (Ush) (Cubadda et al., 1997; Ramain et al., 1993) in the medial notum territory (Sato and Saigo, 2000; Tomoyasu et al., 2000). Pnr, probably together with Ush (Haenlin et al., 1997), represses Iro-C in this region and permits its specification as medial notum (Calleja et al., 2000). An anterior/posterior subdivision is carried out by *eyegone* (*eyg*), a Pax-homeobox gene that is activated by Iro-C and Pnr and whose expression is confined to the anterior notum by the Dpp and Hedgehog pathways (Aldaz et al., 2003). In the absence of *eyg*, this territory does not develop. Forced coexpression of *eyg* and *ara* imposes an anterior notum developmental fate on posterior or lateral notum cells and even on wing cells (Aldaz et al., 2003).

tup encodes a LIM-homeodomain transcription factor that is implicated in axon pathfinding and neurotransmitter identity (Thor and Thomas, 1997). A vertebrate homologue of Tup, Isl1, is required for the proper development of the pancreas and heart, and the specification of several cell types, among them the pancreas islet cells and some motoneurons and interneurons (reviewed by Hobert and Westphal, 2000; Hunter and Rhodes, 2005). LIM-HD factors are capable of multiple protein-protein interactions (reviewed by Bach, 2000; Hobert and Westphal, 2000). In many contexts, a central co-factor is Chip (also known as NLI and Ldb), which homodimerises and assembles a 2LIM-HD–2Chip–2Ssdp hexamer (reviewed by Matthews and Visvader, 2003). The LIM-HD factor allows the complex to interact with DNA through its homeodomain, and transcriptional activation seems to be mediated by the Ssdp proteins (Nishioka et al., 2005). The organisation and regulatory properties of this hexamer have been mostly characterised for the LIM-HD Apterous (Ap) in the *Drosophila* wing (Chen et al., 2002; Fernández-Fúnez et al., 1998; Milán and Cohen, 1999; Rincón-Limas et al., 2000; van Meyel et al., 2003). In the third instar wing disc, *tup* is expressed in a posterior/central region of the notum territory that overlaps with the dorsocentral (DC) and scutellar proneural clusters of the *achaete-scute* genes (Biryukova and Heitzler, 2005; Cubas et al., 1991; Skeath and Carroll, 1991). Recent work (Biryukova and Heitzler, 2005) has shown that loss of function of *tup* promotes the formation of extra

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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 prepattern 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 Ssdp. *tup* and Iro-C, respectively, activated by the Dpp and EGFR signalling pathways, cooperate in accomplishing this commitment.

MATERIALS AND METHODS

Drosophila stocks

Most *Drosophila* stocks are described in FlyBase (http://flybase.org/). tup^{1} (isl^{IIIB29}), tup^{2} (isl^{IIIE16}) and tup^{isl-1} (isl^{E41}) 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^{ex4}) between the FRT-bearing insertions WHf04735 and XPd03613 (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^{IR} transgene constructed (Nagel et al., 2002) using an 810 nucleotide fragment of *tup* cDNA AF145674 (interval 96-906). *y*, *w* embryos were transformed (Rubin and Spradling, 1982) using pUChsπΔ2-3 as a transposase source.

Mosaic analyses

To generate clones of cells mutant for *tup*, *y, w, hs-FLP1.22; tup, FRT40A/CyO* males were crossed with either *y, w, hs-FLP1.22; ubi-GFP, FRT40A/CyO* or *y, w, hs-FLP1.22; P{y+}25F,* ck^{13}*, FRT40A/CyO* or *f, hs-FLP1.22; P{f+}30, ck, FRT40A/CyO* females. Homozygous *tup* clones were induced at different developmental stages by heat treatment at 37°C for either 30 or 60 minutes or by activating a *UAS-FLP* transgene with pnr^{MD237}*-Gal4* (Calleja et al., 2000), *MS248-Gal4* (Cavodeassi et al., 2002; Sánchez et al., 1997) or $Ubx\text{-}Gal4^{LDN}$ (de Navas et al., 2006). Clones null for members of the EGFR pathway were prepared by incubating at 37°C for 60 minutes *y, hs-FLP9F,* f^{36a}*; FRT82B, ubi-GFP, P{f+}87D, M(3)95A/FRT82B,* $Ras85D^{\Delta C40b}$ or *y, hs-FLP9F,* f^{36a}*; FRT82B, ubi-GFP, P{f+}87D, M(3)95A/FRT82B,* $pnt^{\Delta 88}$ or *y, w, hs-FLP1.22; FRT42D, arm-lacZ, M(2)l2/FRT42D,* $Egfr^{1K35}$ *(*$Egfr^{f2}$*)* larvae. The M^{+} genotype (Morata and Ripoll, 1975) of the clones was a requisite for their substantial growth. Clones mutant for *Chip* or *Ssdp* were obtained from *y, w, hs-FLP1.22; FRT42D, ubi-GFP/ FRT42D,* $Chi^{e5.5}$ or *y, hs-FLP9F,* f^{36a}*; FRT82B, ubi-GFP, P{f+}87D, M(3)95A/FRT82B,* $Ssdp^{neo48}$*, e* larvae which were treated at 37°C for 75 minutes.

Overexpression analyses

DC-lacZ/CyO; C765-Gal4 or dpp^{blk}*-Gal4/SM6a-TM6b/DC-lacZ* females (García-García et al., 1999; Gómez-Skarmeta et al., 1996; Staehling-Hampton et al., 1994) were crossed to either *UAS-ara* (Gómez-Skarmeta et al., 1996), *UAS-tup* (Thor and Thomas, 1997), *UAS-*$tup^{\Delta HD}$ (O'Keefe et al., 1998), *UAS-ara; UAS-tup* or *UAS-ara; UAS-* $tup^{\Delta HD}$ males, and the progeny raised at 25°C. One or two copies of *UAS-*tup^{IR} were overexpressed with the *MS248-Gal4* driver at 29°C. To overexpress *Mkp3* or *Dad* during notum specification, males homozygous for either the UAS-bearing P-GS insertion $Mkp3^{M76}$ (Ruiz-Gómez et al., 2005) or the *UAS-Dad* transgene (Tsuneizumi et al., 1997) were crossed with $ptc^{559.1}$*-Gal4, UAS-GFP/SM6a-TM6b/tub-*$Gal80^{ts}$ females (McGuire et al., 2003; Speicher et al., 1994). Progeny were raised at 17°C until mid- or late-second instar, then switched to 29°C for at least 24 hours and dissected. Clones of cells overexpressing diverse *UAS-X* transgenes were generated by incubating at 34°C for 15 minutes *y, w, hs-FLP1.22; Act>y+>Gal4, UAS-GFP/+ UAS-X/+* larvae. Other UAS-activated transgenes were: *UAS-Chip* (Milán and Cohen, 1999), *UAS-*$Chip^{\Delta DD}$ (van Meyel et al., 1999), *UAS-*tkv^{QD} (Das et al., 1998), *UAS-*$Ras1^{V12}$ (Karim and Rubin, 1998), *UAS-*Raf^{DN} (Baek et al., 1996) and *UAS-argos* (Howes et al., 1998).

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 (Cappel), rat anti-Ara/Caup (Diez 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 Axiophot 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^{ex4} and the previously described alleles tup^{1}, tup^{2} and tup^{isl-1}. 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^{ex4} 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 trichoidea (see Fig. S1B in the supplementary material). Seemingly parallel transformations occurred on the metathorax, 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

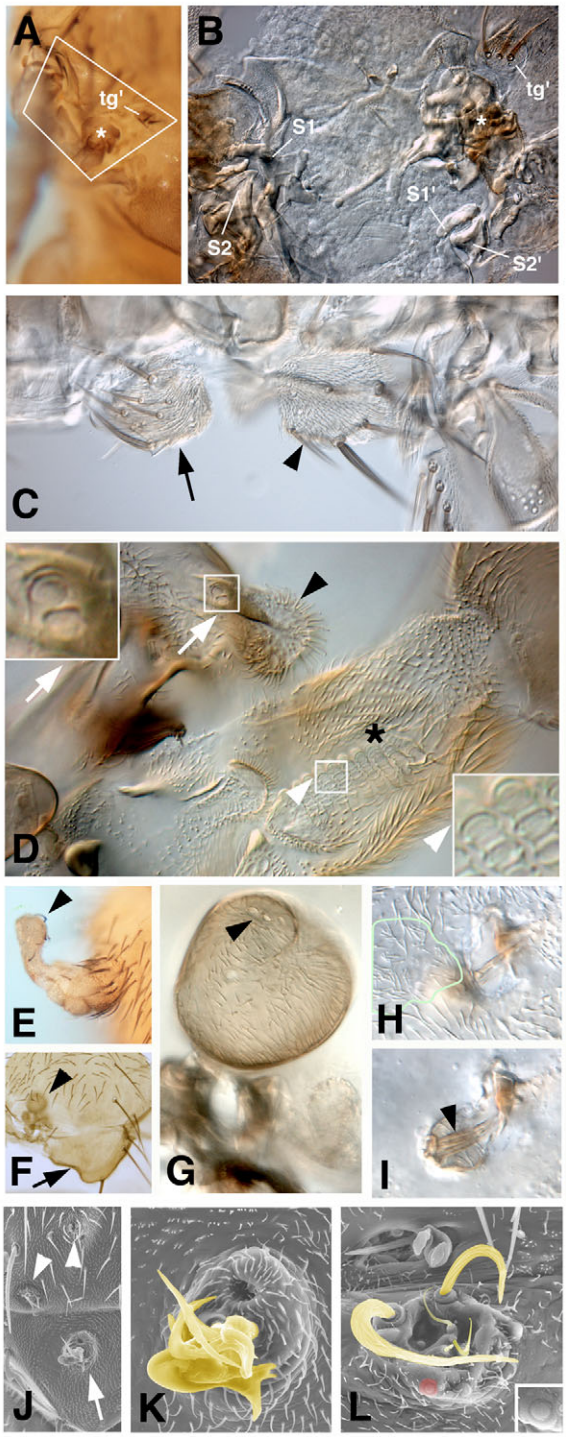

Fig. 1. Cuticular phenotypes associated with *tup* clones. (**A**) View of the lateral-posterior region of a notum displaying ectopic structures in a fly bearing *f tup*^{2} clones. The framed area is shown at high magnification in B, after mounting of the cuticle. (**B**) S1 and S2, extant first and second axilary sclerites; S1′ and S2′, recognisable ectopic sclerites; asterisk and tg′, mass of sclerotised tissue and an ectopic tegula-like structure, respectively, bearing macrochaetae and sensilla trichoidea. (**C**) Ectopic tegula (arrow) with *y tup*^{ex4} bristles, and extant tegula (arrowhead). (**D**) Pedicel of a haltere (asterisk) showing rows of sensilla campaniformia (white arrowheads). Black arrowhead, ectopic structure on the metanotum bearing at its base pedicel-like sensilla campaniformia (arrows). Insets show boxed regions at high magnification. (**E**) Protuberance on the anterior lateral notum with *f tup*^{2} microchaetae (arrowhead). (**F**) Notum showing loss of tissue and of bristles in the scutellum (arrow) and a vesicular invagination (arrowhead). (**G**) High magnification of the vesicle shown in F. Arrowhead, group of sensilla campaniformia. (**H**,**I**) Two focal planes of an invagination with *y tup*^{ex4} microchaetae arising from its interior (arrowhead). Green line, contour of a *crinkled* (*ck*)-marked twin-spot which covers the invagination. (**J**) Cuticular defects (arrow and arrowheads) on the scutum and scutellum of a fly with *f tup*$^{isl\text{-}1}$ clones. (**K**) Protuberance/invagination (indicated by the arrow in J) at high magnification. Note the apical hole of the invagination, and the abutting *ck* twin-spot tissue in the top-left corner of the panel. (**L**) Malformation with a central depression and *f tup*^{2} macro and microchaetae. Red, sensillum campaniforme, as shown at higher magnification in the inset. In K and L, some of the mutant *f tup* bristles are coloured yellow.

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 adhesive 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).

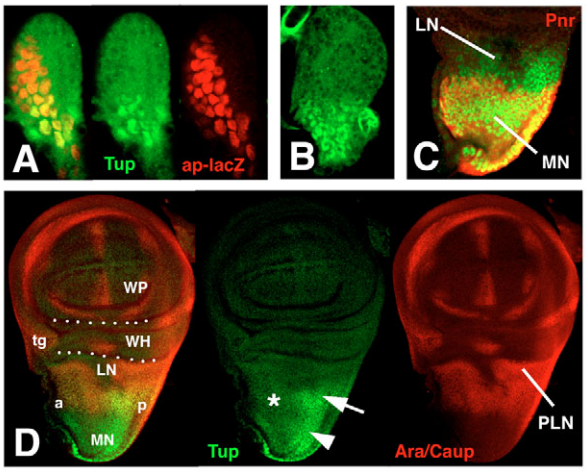

Fig. 2. Expression of *tup* in the imaginal wing disc. (**A**) Early second instar disc. Green, Tup; red, *ap^{rK568}-lacZ*, a marker for the dorsal compartment. (**B**) Late second instar disc. (**C**) Notum region of a mid-third instar disc. Red, *pnr-Gal4 UAS-lacZ*. (**D**) Late third instar disc. 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 (Diez 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.

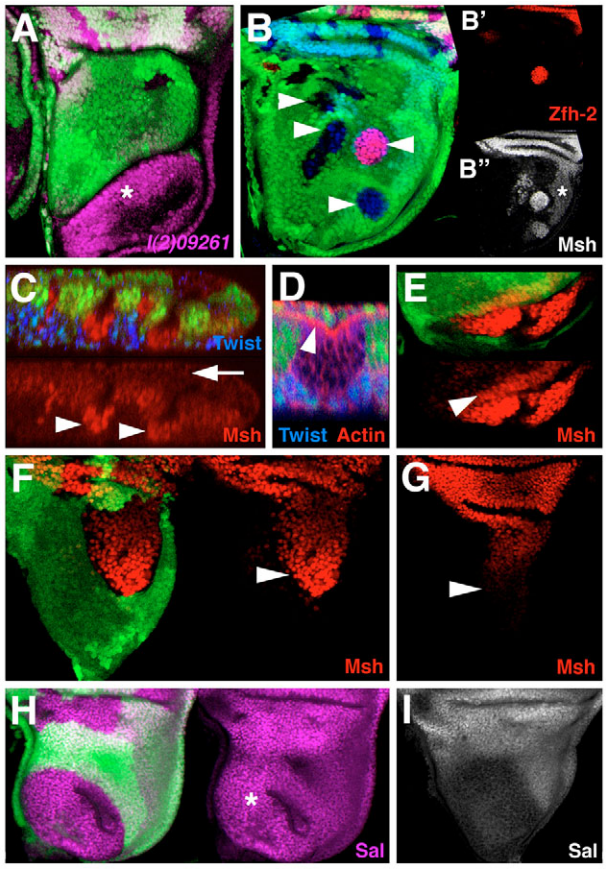

Fig. 3. Expression of wing-hinge markers in *tup* clones located in the notum region. *tup* clones are identified by the absence of GFP (green) expression. (**A**) First instar-induced *tup^{isl-1}* clone (asterisk). The *lacZ* insertion line *l(2)09261* is derepressed. (**B-B″**) *tup^{2}* clones (arrowheads) derepressing *zfh2* (B′) and/or *msh$^{\Delta 89}$-lacZ* (msh, B″). Asterisk, endogenous *msh* expression in the notum. (**C**) Optical *z*-axis section through *tup^{ex4}* clones. Red, *msh$^{\Delta 89}$-lacZ*; blue, Twist, a marker for adepithelial cells; arrowheads, nuclei of *tup^{ex4}* cells; arrow, peripodial membrane. (**D**) Extruding *tup^{ex4}* clone stained with phalloidin (red). Arrowhead, apical actin accumulation. (**E**) *tup^{2}* clone inducing *msh-lacZ* expression autonomously and non-autonomously (arrowhead). (**F**) First instar-induced *tup^{2}* clone with enhanced expression of *msh-lacZ*. Compare (arrowheads) with the wild-type disc (**G**). (**H**) Derepression of *sal* (purple) within a *tup^{ex4}* clone (asterisk) and in cells surrounding it. Compare with wild-type *sal* expression (**I**).

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

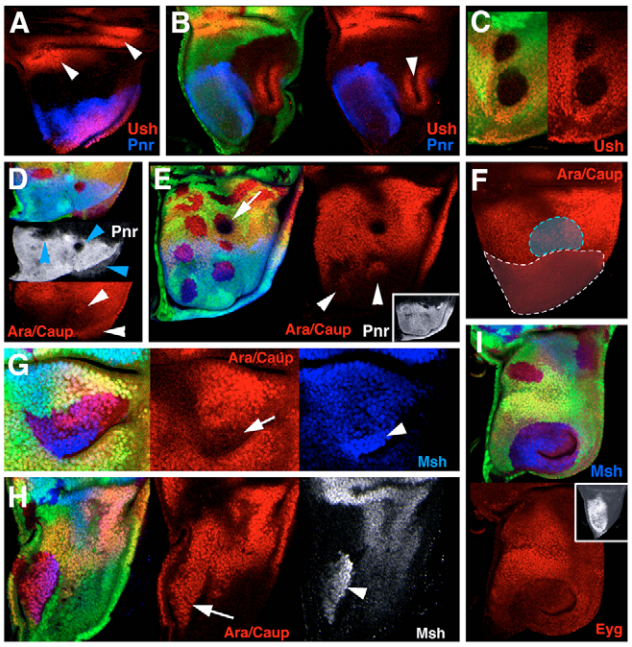

Fig. 4. Effect of *tup* clones on notum genes. *tup* clones are identified by the absence of GFP (green) expression. (**A**) Wild-type expression of *ush* and *pnr-Gal4* in the medial notum. Arrowheads, *ush* expression in the lateral notum and hinge regions. (**B**) First instar-induced tup^{ex4} clone. *pnr-Gal4* expression is lost. The Ush accumulation pattern (arrowhead) is similar to that in the hinge area. (**C**) Second instar-induced tup^{ex4} clones. *ush* is repressed. (**D**) tup^{2} clones (blue arrowheads) repress *pnr-Gal4* (blue or white) and two of the tup^{2} clones upregulate *ara/caup* (white arrowheads). (**E**) tup^{2} clones. Only that clone in the central notum (arrow) represses *ara/caup* (red). Proximal clones upregulate *ara/caup* (arrowheads). Inset shows that *pnr-Gal4* expression (white or blue) persists in most clones. (**F**) Regions are outlined where *tup* clones lose (blue, mapped with 13 clones overlapping the area) or gain (white, mapped with 28 clones) *ara/caup* expression. (**G**) tup^{isl-1} clone. *msh-lacZ* is upregulated (arrowhead) and *ara/caup* downregulated (arrow) in the same cells. (**H**) Anterior tup^{2} clone. *ara/caup* (arrow) and *msh-lacZ* (arrowhead) are both upregulated in some cells of the clone. (**I**) First instar-induced tup^{ex4} clone showing derepression of *msh-lacZ* and inhibition of *eyg*. Inset shows wild-type expression of *eyg*.

the disc that is transversed by several folds (Fig. 4A). *eyg* expression (Fig. 4I, inset) was lost from first instar-induced *tup* clones (Fig. 4I), but not from later-induced clones.

Notum-to-hinge transformations are also associated with the loss of Iro-C activity (Diez del Corral et al., 1999). We examined whether Iro-C products were lost in *tup* clones. Loss of Ara/Caup occurred only in a small area of the central notum (Fig. 4E-G), a region different from that where hinge structures most often arise (the lateral notum, Fig. 1A-C and see Fig. S1C in the supplementary material). Moreover, in the medial notum, *tup* clones frequently activated *ara/caup* (Fig. 4D,E), an effect probably resulting from the loss of Pnr (Calleja et al., 2000) and/or Ush, as the heterodimer Pnr-Ush (Haenlin et al., 1997) appears to be a repressor of Iro-C (Letizia et al., 2007).

Iro-C downregulates *msh* in the notum territory (Villa-Cuesta and Modolell, 2005), so the stimulation of *msh* in *tup* cells that did not express Iro-C (Fig. 4G) was expected. However, *msh* could also be upregulated in clones in which *ara/caup* were expressed (Fig. 4H). Thus, in some instances, *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 $Chip^{e5.5}$ clones located in the presumptive notum showed derepression of *zfh2* and downregulation of *eyg* (Fig. 5A), which indicated a notum-to-hinge transformation. Moreover, *msh* was also derepressed in part of the clones, but only in a non-autonomous manner (Fig. 5B). [Chip is required for *msh* expression in the hinge (Villa-Cuesta and Modolell, 2005), so the absence of *msh* activation within the clones was expected.] Some of the flies bearing *Chip* clones survived to adulthood and showed cuticular defects similar to those associated with early-induced *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 *Ssdp* affected notum specification. We used the hypomorphic $Ssdp^{neo48}$ allele, as clones null for *Ssdp* are not recovered in adults (van Meyel et al., 2003) and hardly grow in imaginal discs even in a *Minute* heterozygous background (data not shown). Forty per cent of $Ssdp^{neo48}$ clones lost *eyg* expression and gained *zfh2* expression (Fig. 5C), and adult flies bearing these clones showed cuticular defects similar to those harbouring *tup* or *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 *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 *UAS-Chip* or $UAS\text{-}Chip^{\Delta DD}$ (which lacks the dimerisation domain) in the posterior and proximal notum showed loss of *eyg* expression and acquired expression of *zfh2* (Fig. 5D and data not shown).

Overexpression of *tup* and *ara* synergistically promote notum development

We compared the ability of *tup* and the Iro-C gene *ara*, overexpressed either singly or together, to impose notum development on cells normally fated to differentiate into other structures. Ubiquitous, relatively late overexpression of *UAS-tup* (*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 *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 *UAS-ara* and *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-

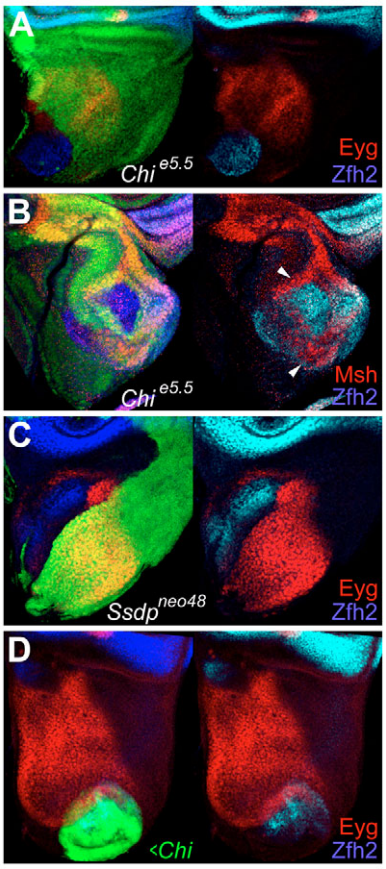

Fig. 5. *Chip* and *Ssdp* are required for notum specification. (**A**,**B**) *Chip*$^{e5.5}$ clones (absence of green) lose Eyg (red in A), accumulate Zfh2 (blue), and non-autonomously upregulate *msh* (red in B, arrowheads). (**C**,**D**) Clones of either *M*$^{+}$ *Ssdp*^{neo48} (C, absence of green) or *UAS-Chip*-expressing (D, green) cells lose Eyg (red) and accumulate Zfh2 (blue).

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*$^{\Delta HD}$).

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. 6J). The adults displayed notum-like structures near the coxa (Fig. 6I), which indicated a transformation of this ventral region of the body wall towards notum. This transformation was not 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*^{1K35} allele suppressed expression of *ara/caup* as expected, but not that of *tup* (Fig. 7A). Similar results were obtained with *Ras85D*$^{C\Delta 40b}$ (Fig. 7B) or *pnt*$^{\Delta 88}$ clones, or by overexpressing *UAS-argos* 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-Ras1*^{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 *Mkp3*, 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 *tkv*^{a12} 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-tkv*^{QD}, 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 categorised as a prepattern 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)09261*. 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

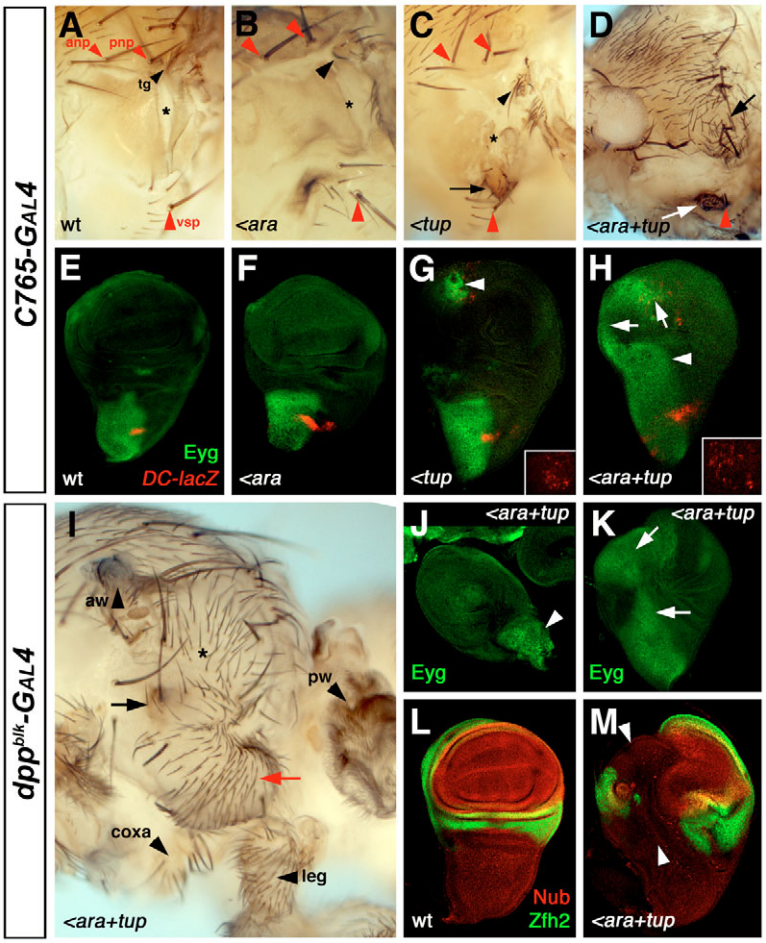

Fig. 6. Overexpression of *tup* and *ara* synergistically promote transformation towards notum. (**A-D**) Mesothoracic pleurae. Red arrowheads indicate anterior (anp) and posterior (pnp) notopleural, and ventral sternopleural (vsp) bristles; black arrowheads, tegulae (tg); asterisks, vertical clefts. (A) Wild type. (B) *UAS-ara; C765-Gal4*. (C) *UAS-tup/C765-Gal4*. Arrow, notum-like outgrowth on the vertical cleft. (D) *UAS-ara; UAS-tup/C765-Gal4*. Flies were grown at 17°C. Black arrow, notum expansion towards the pleura; wing is missing. White arrow, notum-like structure adjacent to sternopleural bristles (red arrowhead). (**E-H**) Wing discs showing expression of *eyg* (green) and *DC-lacZ* (red). Genotypes in E-H correspond with those shown in A-D, respectively. Arrowhead (G) indicates ectopic expression in the prospective pleura (this location was verified in an optical *z*-section). (H) *eyg* is expressed at the dorsal hinge (arrowhead) and the wing and pleura territories (arrows). Insets show red channel images of the pleura and wing pouch areas. (**I**) *UAS-ara; UAS-tup/dpp^{blk}-Gal4* fly. Notum-like structures form on the central wing (asterisk), pleura (black arrow) and sternopleurite (red arrow). aw and pw, anterior and posterior parts of the wing, respectively. (**J**) *UAS-ara; UAS-tup/dpp^{blk}-Gal4* mesothoracic leg disc. Arrowhead, ectopic *eyg* expression. (**K-M**) Wild-type (L) and *UAS-ara; UAS-tup/dpp^{blk}-Gal4* (K,M) wing discs showing either *eyg* (K) or *nub* and *zfh2* (L,M) expression. Arrows (K) indicate that *eyg* expression is expanded to the driver territory. Arrowheads (M) indicate that *nub* and *zfh2* expression is lost from the driver territory.

reflect 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 regions of mutant cuticle have not been recovered. The clones might give rise to flies lacking part or most of a heminotum. The dynamic expression pattern of *tup* fits well with the spatial distribution of these phenotypes and the early requirement for *tup* function for the development of the notum. Indeed, *tup* is expressed very early in the wing disc, when it has less than 100 cells, and the expression occurs within the region that will form the notum. We conclude that, similar to other LIM-HD factors such as Ap and the vertebrate Tup homologue Isl1 (reviewed by Hobert and Westphal, 2000; Hunter and Rhodes, 2005), Tup is required for the proper specification of not only cell types (Biryukova and Heitzler, 2005; Thor and Thomas, 1997), but also developing territories.

Tup associates with Chip and Ssdp for notum specification

Tup is known to bind the co-factor Chip (Biryukova and Heitzler, 2005; van Meyel et al., 1999). Since, in dorsal compartment specification, Chip functions in a 2Ap-2Chip-2Sspd hexamer, we asked whether a similar 2Tup-2Chip-2Sspd complex might mediate Tup function in notum specification. Our results support this interpretation. The loss of either Chip or Ssdp upregulated hinge genes (*zfh2*, *msh*), repressed a notum marker (*eyg*), and induced cuticular defects similar to those associated with *tup* clones. 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-

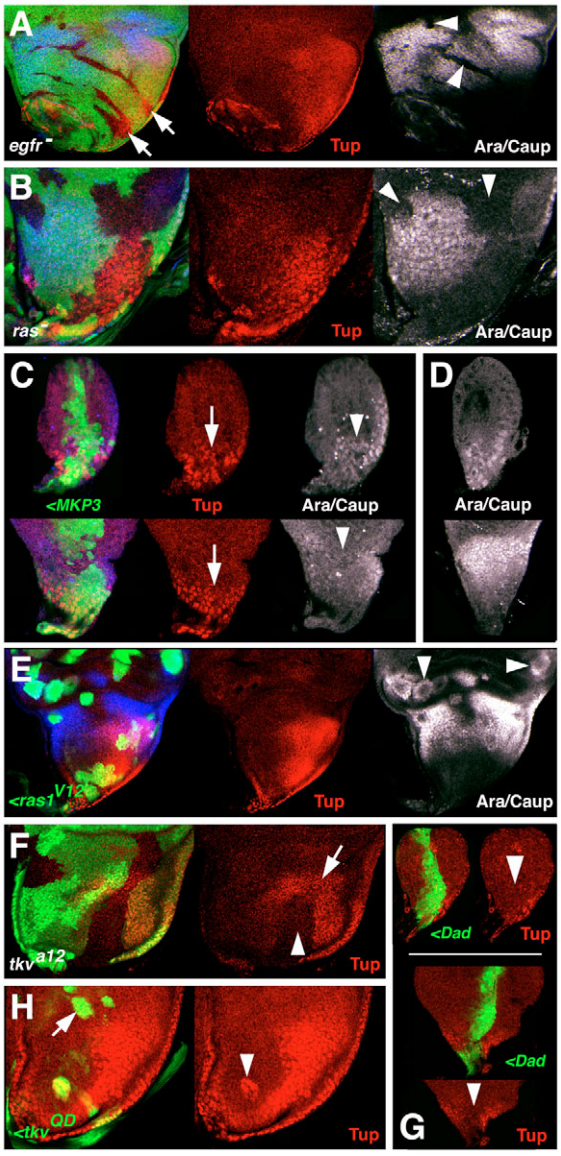

Fig. 7. Regulation of *tup* in the wing disc. Red, Tup; blue or white, Ara/Caup. (**A**) M^+ $Egfr^{1K35}$ clones (absence of green) remove *ara/caup* expression (arrowheads) but do not inhibit *tup* (arrows). (**B**) M^+ $Ras85D^{\Delta C40b}$ clones (absence of green) inhibit *ara/caup* (arrowheads), 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-*$Ras1^{V12}$ (green) activate *ara/caup* (arrowheads) in the wing hinge. *tup* is not activated, or only so at very low levels. (**F**) A tkv^{a12} 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-*tkv^{QD} (green) activate *tup* in the medial (arrowhead), but not in the lateral (arrow) notum.

interacting domain of Chip ($Chip^E$) severely reduces its ability to interact with Tup and suppresses the negative regulation by Tup of bristle formation (Biryukova and Heitzler, 2005). However, homozygous $Chip^E$ flies have no defects in notum specification (Ramain et al., 2000). This suggests that a residual interaction between $Chip^E$ and Tup might persist, as additionally suggested by the suppression of the extra bristles present in $Chip^E$ 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 tup^{d03613}, a strong hypomorphic allele (as substantiated by its embryonic lethality over the null tup^{ex4}; 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*, *Scr+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 (Couso 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.

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-Axin* or *UAS-dTCF^{DN}* (*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 attest 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

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