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Prepatterning the *Drosophila* notum: The three genes of the *iroquois* complex play intrinsically distinct roles

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Abstract

The *Drosophila* thorax exhibits 11 pairs of large sensory organs (macrochaetes) identified by their unique position. Remarkably precise, this pattern provides an excellent model system to study the genetic basis of pattern formation. In imaginal wing discs, the *achaete–scute* proneural genes are expressed in clusters of cells that prefigure the positions of each macrochaete. The activities of prepatterning genes provide positional cues controlling this expression pattern. The three homeobox genes clustered in the *iroquois* complex (*araucan*, *caupolican* and *mirror*) are such prepattern genes. *mirror* is generally characterized as performing functions predominantly different from the other *iroquois* genes. Conversely, *araucan* and *caupolican* are described in previous studies as performing redundant functions in most if not all processes in which they are involved. We have addressed the question of the specific role of each *iroquois* gene in the prepattern of the notum and we clearly demonstrate that they are intrinsically different in their contribution to this process: *caupolican* and *mirror*, but not *araucan*, are required for the neural patterning of the lateral notum. However, when *caupolican* and/or *mirror* expression is reduced, *araucan* loss of function has an effect on thoracic bristles development. Moreover, the overexpression of *araucan* is able to rescue *caupolican* loss of function. We conclude that, although retaining some common functionalities, the *Drosophila iroquois* genes are in the process of diversification. In addition, *caupolican* and *mirror* are required for *stripe* expression and, therefore, to specify the muscular attachment sites prepattern. Thus, *caupolican* and *mirror* may act as common prepattern genes for all structures in the lateral notum.

Keywords: Drosophila notum; Prepattern; *iroquois complex*; *stripe*; Redundancy

Introduction

The *Drosophila* notum exhibits two types of sensory organs: large bristles (macrochaetes) and small bristles (microchaetes). Many microchaetes are distributed in spaced rows in wide areas of the notum while macrochaetes develop in fixed numbers (22) at constant positions. This precise positioning results from the building up of a multi-step process in which positional information is gradually refined (Gomez-Skarmeta et al., 2003). A most-studied step of this process is the expression of the proneural genes *achaete* (*ac*) and *scute* (*sc*) in distinct clusters of cells in the prospective notum region of the wing imaginal discs (Calleja et al., 2002). These proneural clusters are arranged in a disposition prefiguring the distribution of the future notal macrochaetes (Cubas et al., 1991; Romani et al., 1989; Skeath and Carroll, 1991). The activity of the genes of the *ac–sc* complex, which encode proteins containing a basic Helix–Loop–Helix domain (Campuzano et al., 1985; Villares and Cabrera, 1987) allows cells to adopt a neural fate. During third larval to early pupal stages, one (sometimes two) sensory organ precursors (SOPs) is singled out from each proneural cluster (Simpson, 1990).

The complex expression pattern of *ac* and *sc* is controlled through the action of *cis*-regulatory enhancer sequences (Gomez-Skarmeta et al., 1995; Ruiz-Gomez and Modolell, 1987) responding to local positional cues provided by the activities of prepattern genes, like *pannier* (*pnr*) and the *iroquois* complex (*iro-C*) genes (Gomez-Skarmeta et al., 2003). Together, the expression domains of these genes cover the entire notum and subdivide it into two major regions, the medial and the

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lateral notum (Calleja et al., 2000). Calleja et al. (2002) have proposed that there is a hierarchy of activity amongst prepattern genes, with *pnr* and the *iro-C* at its top. Other factors either act downstream of *pnr* and the *iro-C* (Aldaz et al., 2003) or modify the transcriptional activity of their products (Cubadda et al., 1997; Haenlin et al., 1997; Ramain et al., 2000).

Encoding a member of the GATA family of transcription factors, *pnr* is expressed in the medial notum and regulates development of bristles in this region (Calleja et al., 2000; Haenlin et al., 1997). Pnr directly activates transcription of *ac–sc* through binding to target sequences in the dorso-central enhancer element, which drives expression in one proneural cluster from which the two dorso-central bristles arise (Garcia-Garcia et al., 1999; Gomez-Skarmeta et al., 1995).

The *Drosophila iro-C* comprises three homeobox genes, *araucan* (*ara*), *caupolican* (*caup*) and *mirror* (*mirr*) (Gomez-Skarmeta et al., 1996; McNeill et al., 1997; Netter et al., 1998). They encode evolutionary conserved transcription factors present in all animal species where they were sought for (Feijoo et al., 2004; Gomez-Skarmeta and Modolell, 2002; Perovic et al., 2003). Previous studies, based mainly on a deficiency of the whole complex, have lead to the conclusion that the *iro-C* genes specify the identity of diverse territories and their subsequent patterning during development (Cavodeassi et al., 2001). Early in the in the wing disc development, the three *iro-C* genes are expressed in all the prospective notum cells and appear required to specify the identity of the entire notum (Diez del Corral et al., 1999). Later on, Pnr, probably when heterodimerized with U-shaped (Letizia et al., 2007), restricts the *iro-C* genes expression to the prospective lateral notum (Calleja et al., 2000) where they are required for *ac–sc* expression. Flies harboring rearrangements involving the *iro-C* lack all bristles in the lateral notum (Dambly-Chaudiere and Leyns, 1992) due to the loss of *sc* expression resulting in the failure of SOP formation (Leyns et al., 1996). This phenotype was named the Iroquois phenotype, after the haircut of the Iroquois American Indians, and gave its name to the complex of genes, and later to the family of paralogs (Burglin, 1997). The capacity of Ara (and by correlation Caup) to bind an *ac–sc* enhancer led to the conclusion that Iro proteins directly activate *ac–sc* expression (Gomez-Skarmeta et al., 1996), although this was challenged by Bilioni et al. (2005).

The *iro-C* genes *ara* and *caup* probably arose from the most recent duplication event in the complex. They share ~44% identity in their protein-coding sequences and display similar mRNAs expression patterns during development (Gomez-Skarmeta et al., 1996). Mirr is more divergent in sequence (Ara-Mirr: 34% identity, Caup-Mirr: 33% identity) as well as in expression pattern. *mirr* is generally described in the literature as performing functions largely different from the other *iro-C* genes (Bilioni et al., 2005). Conversely, *ara* and *caup* are presented in previous studies as performing redundant functions in most if not all processes in which they are involved (Cavodeassi et al., 2001), particularly in the establishment of the neural prepattern of the lateral notum (Gomez-Skarmeta et al., 1996). This is essentially based on the similarity of their expression patterns, on misexpression studies and on the phenotypic analysis of rearrangements affecting the *iro-C*. These include deficiencies deleting the whole complex, and inversions or transposable elements (TE) insertions affecting it. Each of them is susceptible to disrupt more than one gene of the complex. Although there are reports of mutations affecting only *mirr* (Jordan et al., 2000; Kehl et al., 1998; McNeill et al., 1997; Netter et al., 1998; Sun et al., 1995), there is no clear description of loss-of-function (LoF) mutations affecting only *ara* or only *caup*. In addition, *UAS-ara* and *UAS-caup* transgenes are generally used indistinctly (sometimes under the denomination "*UAS-iro*") in misexpression studies as if they performed strictly identical functions (de Navascues and Modolell, 2007; Singh et al., 2004; Villa-Cuesta and Modolell, 2005).

We have thus addressed the question of the specific role of each *iro-C* gene in the patterning of the notum. We have obtained and characterized LoF mutations affecting *ara* or *caup*. The analysis of their effects and a functional replacement approach using the *Gal4–UAS* system to exchange the expression domains of the *iro-C* genes allowed us to demonstrate that the *iro-C* genes products are intrinsically different in their contribution to the patterning of the notal bristles. In normal conditions, *ara* is not required for the establishment of this neural prepattern which requires *caup* and *mirr*. However, *ara* LoF elicits the loss of some thoracic macrochaetes when *caup* and/or *mirr* expression is reduced. Moreover, the overexpression in the *caup* domain of expression of *ara* but not of *mirr* is able to rescue the Iroquois phenotype elicited by *caup* LoF. In addition, we show that *caup* and *mirr*, but not *ara*, are necessary for the expression of *stripe* (*sr*), which specifies flight muscle attachment sites ("muscular prepattern"). Thus, the three *iro-C* genes appear to play distinct biological roles: the prepatterning of the prospective lateral notum is essentially controlled by the activity of Caup and Mirr. We assume that the *iro-C* genes are probably in the process of diversification by subfunctionalization, although some overlap in their function may be retained by selection because of its contribution to genetic robustness.

Materials and methods

Fly stocks

The positions of all TE insertions and breakpoints in the *iro-C* used here are described in Fig. 1. ara^{rF209} (Gomez-Skarmeta et al., 1996), ara^{T}, $ara^{B6.8}$, $caup^{Sc2}$ and $mirr^{re3}$ (Netter et al., 1998) are *P*[*lacZ*] enhancer trap lines. $caup^{BG01626}$ (abbreviated hereafter $caup^{BG}$) is a *P*[*GT1*] insertion (Bellen et al., 2004). $mirr^{f01086}$, $mirr^{c04837}$ and $caup^{f3450}$ are insertions of the *piggyBac* vector *WH* described in Thibault et al. (2004). ara^{G3}, ara^{G4}, $caup^{G1}$, $caup^{G2}$, $caup^{G3}$, $mirr^{G1}$ and $mirr^{G8}$ are *P*[*Gal4*] drivers that we obtained by conversion of *P*[*lacZ*] insertions (A. Ikmi and D. Coen, unpublished results).

Tp(3;3)iro-1 (abbreviated hereafter *iro1*) is a multiple rearrangement with one breakpoint in *caup* first intron (Gomez-Skarmeta et al., 1996; Leyns et al., 1996). We generated the $Df(3L)iro^{BSI}$ (abbreviated *Df-BSI*) by *P* secondary mutagenesis on $ara^{B6.8}$. It has one of its breakpoints in the second intron of *ara* and the other 50 bp 5′ of the *mirr* transcription start site (A. Ikmi and D. Coen, unpublished). This deficiency lacks all *iro-C* genes function. The *In(3L)TSI* (abbreviated *TSI*) was generated by *P* secondary mutagenesis on ara^{T} and has its proximal breakpoint in *ara* first intron and its distal breakpoint in 70F (A. Ikmi and D. Coen, unpublished). $mirr^{e48}$ is a deletion (1 kb) of the *mirr* promoter (McNeill et al., 1997).

We used sr^{lacZ} or the combination sr^{Gal4};*UAS-GFP* to visualize *sr* expression (Usui et al., 2004). $neur^{A101}$, an enhancer trap line at the *neuralized* locus, was used to mark SOPs (Huang et al., 1991).

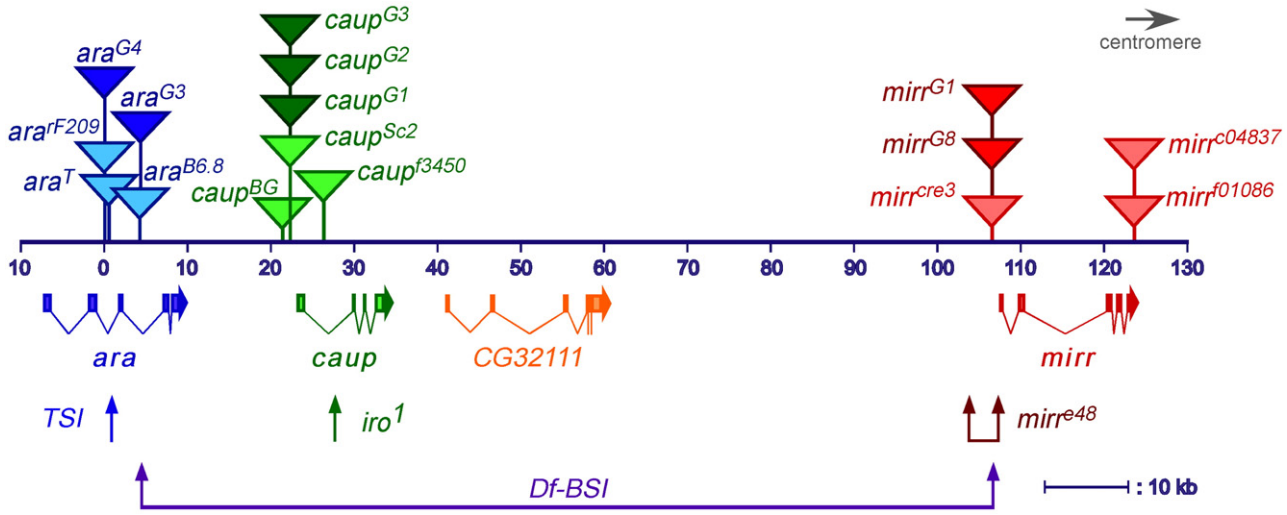

Fig. 1. Physical map of the *iro-C*. Genomic DNA is shown as a blue bar. Transcription units are shown as horizontal arrows under the DNA line. *CG32111* is a transcription unit identified by the *Drosophila* Genome Project. It has been described as a possible non-coding RNA (FlyBase). Vertical arrows indicate positions of chromosomal breakpoints associated with *Tp(3;3)iro-1* (*iro1*) and *In(3L)TSI* (*TSI*) in the *iro-C*. Chromosomal deletions $mirr^{e48}$ and *Df-BSI* are shown as a horizontal bar and vertical arrows indicate their breakpoints. Triangles represent insertions. ara^{rF209}, ara^{T}, $ara^{B6.8}$, $caup^{Sc2}$, and $mirr^{cre3}$ are *P*[*lacZ*] enhancer trap insertions. $caup^{BG}$ is a *P* insertion. $caup^{f3540}$, $mirr^{f01086}$ *and* $mirr^{c04837}$ are *piggyBack* insertions (see Materials and methods for more details). ara^{G3}, ara^{G4}, $caup^{G1}$, $caup^{G2}$, $caup^{G3}$, $mirr^{G1}$ and $mirr^{G8}$ are *P*[*Gal4*] enhancer trap insertions.

The *iro-Gal4* lines described above and in the text were used to visualize gene expression with a *UAS-GFP* (Bloomington Stock Center) or to drive the expression of *UAS-ara* (Gomez-Skarmeta et al., 1996), *UAS-caup* (Diez del Corral et al., 1999), or *UAS-mirr* (McNeill et al., 1997). $tub\text{-}gal80^{ts}$ was used to reduce the activity of the Gal4 protein (McGuire et al., 2004).

Quantitative RT-PCR

Total RNA was isolated from third instar larvae using the RNeasy Mini RNA isolation Kit (Qiagen). Quality assessment of isolated RNA was performed with a 2100 Bioanalyser. One microgram of total RNA was used in 20 µl reverse transcription reaction with 500 ng of oligo-dT, in the presence of the Superscript II (Invitrogen). Quantitative RT-PCR were then conducted on ABI prism 7900 HT using SYBR green PCR Master Mix according to manufacturer's protocol. The primer sequences used were:

ara-exon4-forward 5′-GCCTTGGTGTCGGACGAT-3′
ara-exon5-reverse 5′-CGGCGCGCAGTATGTTC-3′
caup-exon4-forward 5′-GGTGGGAAACTCTCTACAGAAGCT-3′
caup-exon5-reverse 5′-TCCCGGTCCAGATCCAGTT-3′
mirr-exon2-forward 5′-CCTACCTCTCCGGGATTGC-3′
mirr-exon3/4-reverse 5′-TGCCATAGCTGTTGAACGGATA-3′
rp49-forward 5′-GGCCCAAGATCGTGAAGAAG-3′
rp49-reverse 5′-CCGATGTTGGGCATCAGATAC-3′.

The *rp49* gene was used as the reference gene for relative quantification experiments using standard conditions of PCR. Each reaction was run in triplicate. The level of expression of each target gene was then calculated as $2^{-\Delta\Delta Ct}$ as described by Livak and Schmittgen (2001).

Immunohistochemistry

Third instar larval wing discs were processed following standard protocols. For β-Galactosidase immunostaining, we used a 1:200 dilution of mouse primary antibody (Promega Z3781), and a 1:1000 dilution of secondary antibody conjugated to Alexa-594 (Molecular probes A-21044). For GFP immunostaining, we used a 1:1500 dilution of rabbit primary antibody (Molecular Probes A-11122), and a 1:1000 dilution of secondary antibody conjugated to Alexa-488 (Molecular Probes A-11012). The artificial colors blue, green and red are respectively associated to the *ara*, *caup* and *mirr* enhancer trap reporters.

In situ hybridization

Third instar larval wing discs were processed following standard protocols. *In situ* hybridization reactions were performed with gene-specific digoxigenin-RNA probes detected with a 1:2000 dilution of an α-Dig mouse monoclonal antibody conjugated to AP (Roche, Switzerland). The gene-specific probes for each *iro-C* gene were designed from cDNA regions where they have very low if any similarity between each other. These regions correspond to the 3′ UTR of each *iro-C* gene.

Counting of thoracic macrochaetes

Unless otherwise mentioned a minimum of 10 flies was scored for each genotype tested. For each fly, the number of missing bristles was examined for the 11 pairs of notal macrochaetes, which were identified by their unique positions. At each position, the number of present bristles per heminotum was used to represent in the figures the effects of the mutations affecting the *iro-C* on macrochaetes patterning. The actual data are presented in Table S1 in the supplementary material.

Results

iro-C genes present similar but distinct patterns of expression in the prospective notum

In order to investigate the functional specificity of each *iro-C* gene during the patterning of the notum, we wanted first to compare as precisely as possible their expression pattern in third instar (L3) larvae wing discs.

To do so, we have used a combination of a *P*[*lacZ*] enhancer trap insertion in one *iro-C* gene and a *P*[*Gal4*] insertion in another *iro-C* gene (Fig. 1) driving an *UAS-GFP* transgene. This experimental design allowed us to visualize simultaneously the expression pattern of two *iro-C genes* reporters and to compare them directly (Fig. 2).

The expression pattern of these reporter genes is very similar to the expression profile of the corresponding *iro-C* gene as

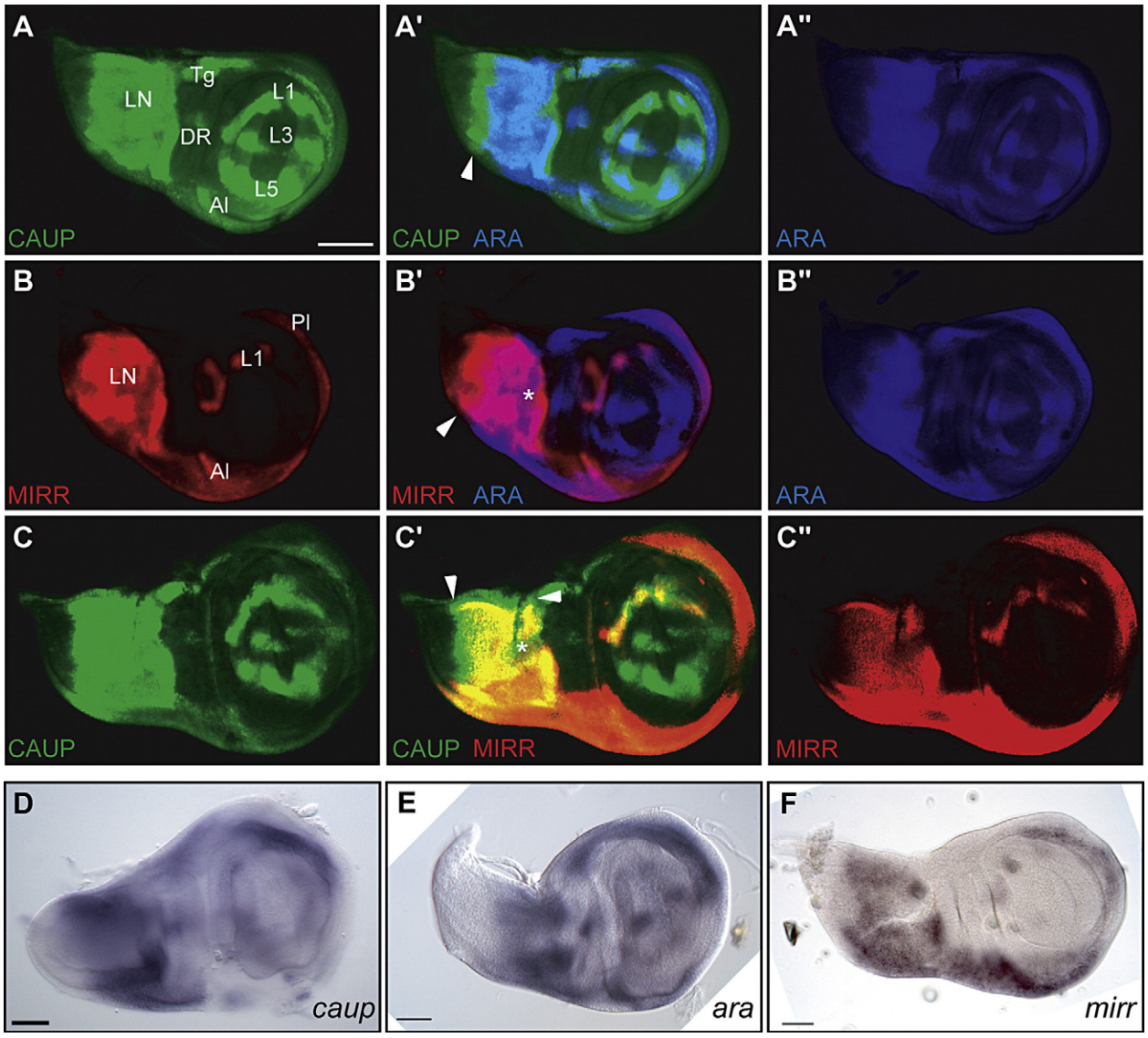

Fig. 2. Comparison of the expression patterns of the *iro-C* genes in L3 larvae wing disc. Wing discs from: (A–A″) = ara^{rF209}/+, +/$caup^{G3}$, +/*UAS–GFP*; (B–B″) = ara^{rF209}/+, +/$mirr^{G8}$, +/*UAS–GFP*; (C–C″) = +/$caup^{G3}$, $mirr^{cre3}$/+, *UAS–GFP*/+. All discs are stained with anti-β-Galactosidase (A″, B″, C″) and anti-GFP (A, B, C) antibodies. (A′, B′, C′) merged image. $caup^{G3}$ (A, C; green) and ara^{rF209} (A″,B″; blue) are expressed in the precursors for lateral notum (LN), alula (Al), dorsal radius (DR), longitudinal veins L1, L3 and L5, pleura (Pl) and tegula (Tg). $caup^{G3}$ is more widely expressed than ara^{rF209} in the prospective notum (A′, arrowhead) and in the precursors for L1 and L5 veins. In the prospective dorsal radius and pleura, ara^{rF209} is more broadly expressed than $caup^{G3}$. *mirr* (B, C″; red) is expressed in the prospective lateral notum, alula, pleura and longitudinal veins L1, where *ara* and *caup* are also expressed. However, in the prospective alula, *mirr* expression domain is larger than *ara* and *caup* ones. The arrowheads and asterisks point to major differences between the spatial expression domains of *iro-C* enhancer trap lines in the prospective notum. (D, E, F) *In situ* hybridization with a specific probe for each *iro-C* gene on a L3 wing disc from *wt* larvae. The scale bar is 40 μm.

detected by *in situ* hybridization (Kehl et al., 1998; Gomez-Skarmeta et al., 1996; our unpublished results). The expression pattern of *caup* reporters ($caup^{G1/2/3}$ and $caup^{Sc2}$) and *mirr* reporters ($mirr^{G1/8}$ and $mirr^{cre3}$) mimics the endogenous expression profile of *caup* and *mirr*, respectively, as detected by *in situ* hybridization (Fig. 2: compare A,C to D and B, C″ to F; our unpublished results). Among all *ara* reporters, only ara^{rF209} reproduces completely the endogenous expression pattern of *ara* (Fig. 2: compare A″, B″ to E; our unpublished results).

caup and *ara* reporters are for the most part expressed in the same regions of the wing disc (Figs. 2A, A′, A″). However they show some specific expression domains: particularly, in the prospective notum, *caup* domain is larger and spreads more medially than *ara* (Fig. 2A′, arrowhead). The *mirr* expression domain overlaps only partly the *ara* and *caup* domains (Figs. 2B′, C′). However, they do not exactly coincide within these regions: notably, *mirr* domain is reduced in the most lateral region of the prospective notum (Figs. 2B′, C′, asterisks). In the prospective notum, *caup* expression domain is more extended medially than *mirr* domain, which in turn is expressed more broadly than *ara* (Figs. 2B′, C′, arrowheads). This confirms and strengthens the differences detected by *in situ* hybridization (Figs. 2D, E, F).

In conclusion, the expression pattern of the reporters for each *iro-C* gene shows some specificity (Fig. 2). Although there could be some discrepancy between the reported pattern and the actual domain of expression of the corresponding gene, this suggests that the three *iro-C* genes present distinct expression patterns, albeit similar, in the prospective notum. These differences may be responsible for different roles of each *iro-C* gene during the patterning of the notum.

caup is required for the formation of all lateral bristles on the notum

We have generated a new deletion of the *iro-C*, *Df-BSI* by *P* element secondary mutagenesis on $ara^{B6.8}$ (Fig. 1). Although *Df-BSI* is homozygous lethal, *iro1*/*Df-BSI* flies are viable and display the Iroquois phenotype (Figs. 3A, B). This confirms that the *iro-C* is required for the formation of all sensory organs (macrochaetes and microchaetes) in the lateral notum. We tried thus to identify the *iro-C* gene(s) responsible for the Iroquois phenotype. As *iro1* has a breakpoint in the first *caup* intron (Fig. 1), we examined first the effects on macrochaetes formation of six TE insertions in *caup* or 5′ to its transcription start site (Fig. 1). All six insertions are homozygous viable and all but one ($caup^{BG}$) lead to a partial loss of macrochaetes in the lateral notum that we call a mild Iroquois phenotype (Fig. 3C). This phenotype is generally enhanced in heterozygotes over *Df-BSI* indicating that these insertions behave like hypomorphic mutations. This enhancement is even stronger when these insertions were made heterozygous over *iro*1: in $caup^{G1/2/3}$/*iro1*, all the seven lateral macrochaetes were absent. Thus, all insertions or rearrangements affecting *caup* elicit the Iroquois phenotype, at least to some extent. This suggests that *caup* LoF is viable and causes the loss of most if not all macrochaetes on the lateral notum. To confirm the role of *caup* in the patterning of these

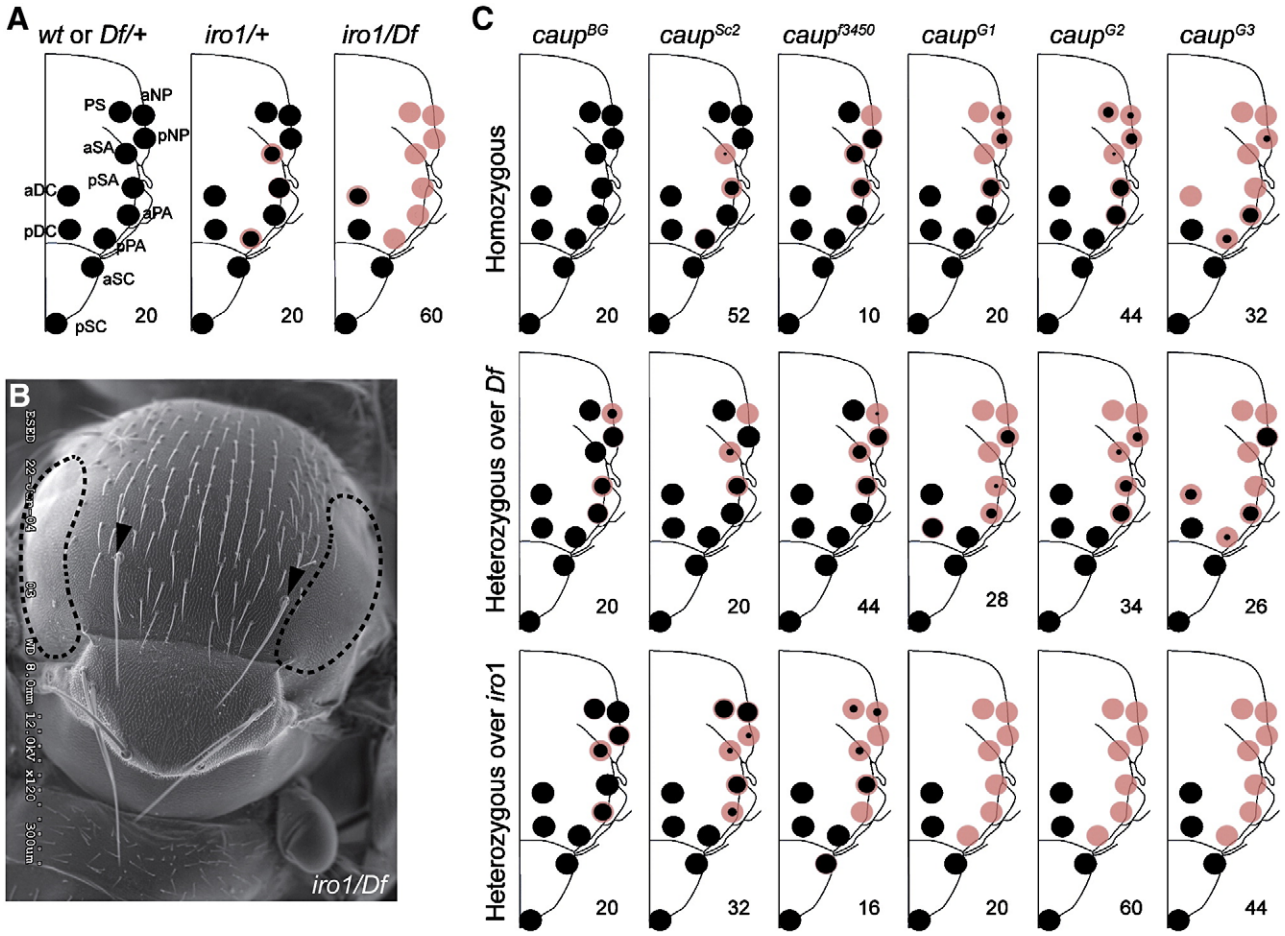

Fig. 3. Effects of insertions and rearrangements affecting *caup* on the patterning of notal macrochaetes. (A) Schematic drawing of a heminotum showing the frequencies of the 11 macrochaetes (dots) in *wt*, *Df*/+, *iro1*/+ and *iro1*/*Df* flies. The area of the black dot is proportional to the observed occurrence frequency of the corresponding macrochaetes. The area of the pink dot corresponds to 1.00, the expected frequency when the macrochaetes pattern is wt. The number of scored heminota is indicated for each genotype. aSA and pPA macrochaetes are affected in *iro1*/+ heterozygotes. Heterozygous *iro1*/*Df* flies displays the Iroquois phenotype: PS, aNP, pNP, aSA, pSA, aPA and pPA are completely missing. In rare cases, dorsocentral macrochaetes are also affected. The *Df* used here is *Df-BSI* (B) Dorsal view of scanning electron micrograph of the thorax of an *iro1*/*Df* adult. All lateral bristles (macrochaetes and microchaetes) are missing (dotted areas) and only two of the four dorsocentral macrochaetes are present (arrowheads). Anterior is to the top. (C) Schematization of the macrochaetes frequencies per heminotum displayed by homozygotes, and heterozygotes over *Df* or over *iro1* for each insertion in *caup*. It should be noted that, in all cases, the microchaetes are never totally missing. All flies were raised at 25 °C. Abbreviations: DC, dorsocentral; NP, notopleural; PA, postalar; PS, presutural; SA, supraalar; SC, scutellar. The prefixes “a” and “p” indicate anterior and posterior, respectively.

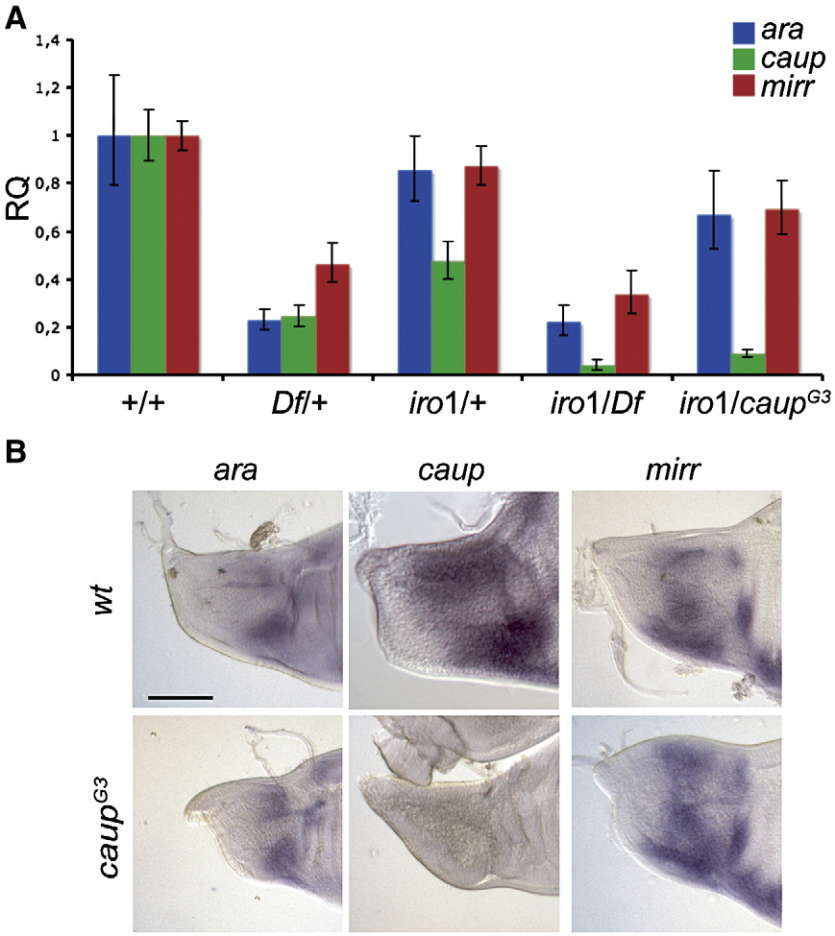

Fig. 4. The Iroquois phenotype is associated with *caup* loss of function. (A) Quantitative RT-PCR analysis performed on mRNA extracted from L3 larvae of these five genotypes: *wt*, *Df*/+, *iro1*/+, *iro1*/*Df* and *iro1*/*caup*^{G3}. *Df* corresponds to *Df-BSI*. RQ: Relative quantity of mRNA for each *iro-C* gene (wt=1). (B) *In situ* hybridization with a specific probe for each *iro-C* gene on a L3 wing disc from *wt* and homozygous *caup*^{G3} larvae. The scale bar in B is 40 µm.

macrochaetes, we carried out a quantitative RT-PCR analysis on mRNAs from L3 larvae of five genotypes (Fig. 4A). The only significant difference between *Df-BSI*/+ and *iro1*/*Df-BSI* individuals is the absence or drastic reduction of *caup* mRNA quantity. Conversely, *ara* and *mirr* levels are not significantly different between larvae of these two genotypes. Therefore *caup* LoF is likely to be responsible for the phenotypic difference between the *Df-BSI*/+ (wild type, wt) and *Df-BSI*/*iro1* (Iroquois) individuals. In *caup*^{G3}/*iro1* heterozygotes, which display a total loss of the lateral macrochaetes, the expression level of *caup* is also severely reduced. *ara* and *mirr* levels may be slightly reduced in comparison to *wt* or *iro1*/+ larvae but they remain largely higher than the levels observed in *Df-BSI*/+ individuals. In *caup*^{G3} homozygotes, spatial expression of *ara* and *mirr* appears indistinguishable from wt whereas *caup* mRNA can not be detected in any larval disc or tissue (Fig. 4B and data not shown). Hence *caup*^{G3} is a strong hypomorphic allele of *caup* that affects significantly neither expression level nor spatial expression of *ara* and *mirr* but severely impairs the macrochaetes patterning. Altogether we can conclude that *caup* is required for macrochaetes formation in the lateral notum.

*iro*1 dominantly affects the macrochaetes pattern (Fig. 3A). In *iro*1/+ heterozygotes, only *caup* expression is significantly reduced. As it is still superior to the level observed in *Df-BSI*/+ (Fig. 4A), the dominant effect of *iro*1 cannot be attributed to *caup* haploinsufficiency. *iro*1 breaks into *caup* first intron (Fig. 1). It may produce a fusion or truncated protein causing *iro1* to behave like a dominant negative allele of Caup.

We also observed a slight difference in microchaetes pattern between *iro1*/*Df-BSI* and *iro1*/*caup*^{G3} flies. Conversely to *iro1*/*Df-BSI*, the lateral microchaetes of *iro1*/*caup*^{G3} adults are not completely missing (data not shown). The difference may be explained by a residual *caup* expression in *caup*^{G3} and/or by the decrease of both *ara* and *mirr* levels in *iro1*/*Df-BSI*

Table 1
Phenotypic consequences of insertions and rearrangements affecting *ara*

Allele	*ara*^{T}	*ara*$^{B6.8}$	*ara*^{G3}	*ara*^{rF209}	*ara*^{G4}	*TSI*
Homozygote	+*	±* (ho)	±* (ho)	+* (ho)	±* (ho)	−
Heterozygous over *Df-BSI*	+*	±* (ho)	±* (ho)	−	−	−
Heterozygous over *TSI*	+*	±* (ho)	±* (ho)	−	−	na
Heterozygous over *iro1*	+*	+*	+	+*	+*	+
Heterozygous over *mirr*^{e48}	+	+	+	+	+	+

+, Viable. ±, semi-viable. −, lethal at the pupal stage. ho: heldout wings (hinge and alula are wt). na: not applicable. *: the macrochaete pattern is described in Fig. 5.

larvae in comparison to *iro1*/*caup*^{G3} larvae (Fig. 4A). The dorsocentral macrochaetes are also affected in *caup* mutant flies (see *caup*^{G3}/*caup*^{G3}, *caup*^{G3}/*Df-BSI* and *iro1*/*Df-BSI*, Fig. 3). *caup* expression domain includes the SOPs for dorsocentral macrochaetes (Fig. S1 in the supplementary material) and may thus contribute to their patterning.

The formation of all the lateral bristles is independent from the ara function

The analysis of the effects of one inversion and five *P* element insertions in the *ara* gene (Fig. 1) is presented in Table 1 and Fig. 5. *TSI* has one breakpoint in the second intron

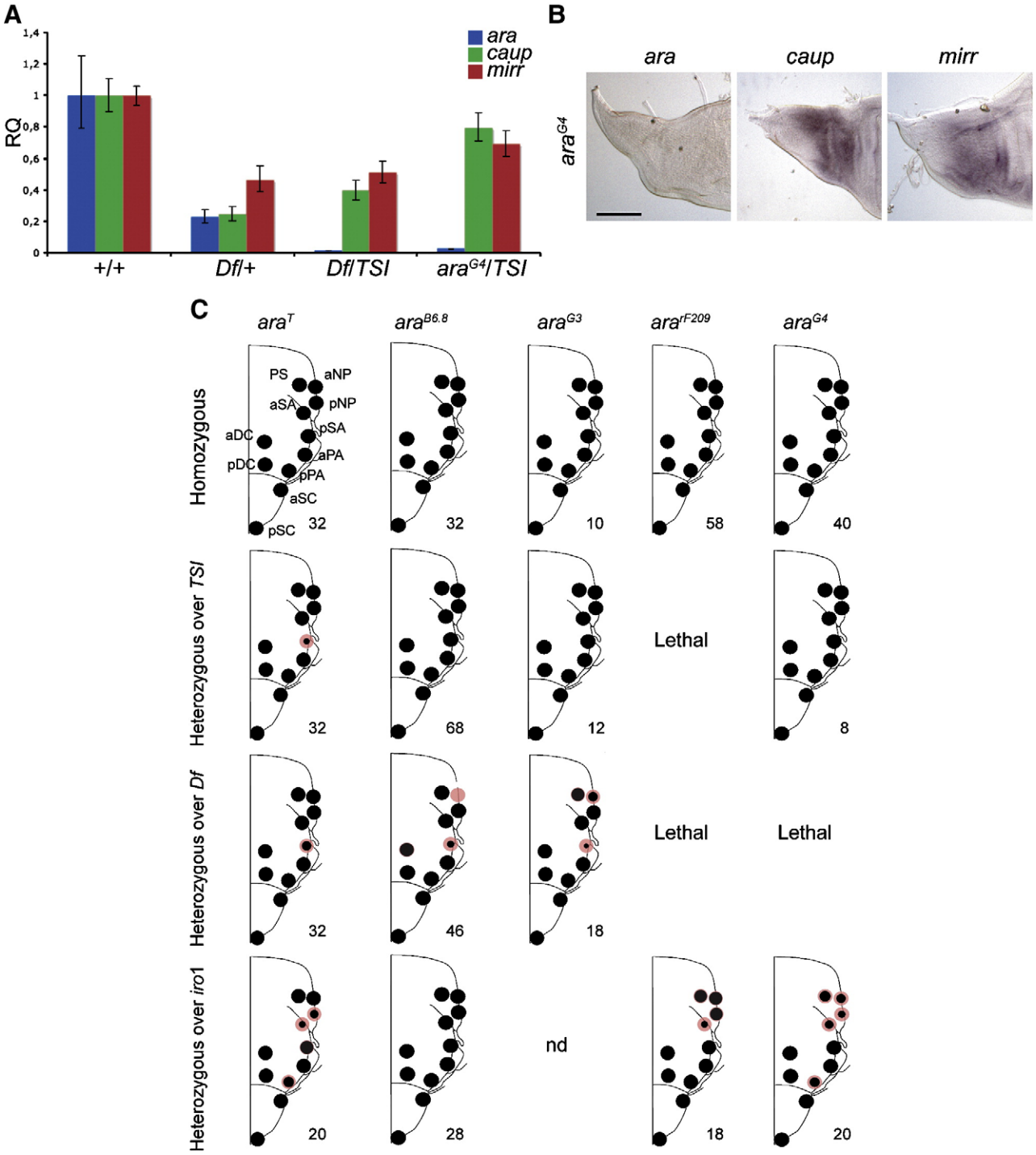

Fig. 5. Effects of insertions and rearrangements affecting *ara*. (A) Quantitative RT-PCR analysis performed on mRNA extracted from L3 larvae of these genotypes: *wt*, *Df*/+, *Df*/*TSI* and *ara*^{G4}/*TSI*. RQ: Relative quantity of mRNA for each *iro-C* gene (wt=1). (B) *In situ* hybridization with a specific probe for each *iro-C* gene on an *ara*^{G4} L3 wing disc. (C) Schematization of the macrochaetes frequencies per heminotum displayed by homozygotes, and heterozygotes over *TSI* or over *Df* or *iro1* for each insertion in *ara*. nd: not determined. Other legends as in Fig. 3. All flies were raised at 25 °C except *ara*^{G4}/*TSI* heterozygotes which were raised at 18 °C. The scale bar in B is 40 μm.

of *ara*, presumably resulting in a complete loss of *ara*. *TSI* is lethal at the pupal stage both when homozygous and when heterozygous over *Df-BSI*, indicating that *ara* is required for viability. Four insertions behave like hypomorphic mutations affecting most probably *ara*. They are homozygous viable (ara^{rF209}) or semi-viable ($ara^{B6.8}$, ara^{G3} and ara^{G4}) and the adults display a heldout wing phenotype. The heterozygosity over *Df-BSI* or over *TSI* leads to the same phenotype ($ara^{B6.8}$ and ara^{G3}) or to pupal lethality (ara^{rF209} and ara^{G4}). *TSI* and all *ara* insertions complement *mirr* LoF for viability and all the transheterozygote adults eclosed are wt (data not shown).

In *Df-BSI*/*TSI* larvae, which die as pupae, *ara* mRNA level is null or radically reduced when compared with *Df-BSI*/+, while *caup* and *mirr* levels are similar in these two genetic backgrounds (Fig. 5A). Similarly *TSI*/ara^{G4} heterozygotes die at pupal stage and show an extreme decrease of *ara* expression. Although slightly reduced in comparison to *wt*, *caup* and *mirr* levels are still superior to *Df-BSI*/+ levels. Moreover, in ara^{G4} homozygotes, *ara* mRNA is neither detected in imaginal discs nor in other larval tissues (Fig. 5B and data not shown) while spatial expression of *caup* and *mirr* appears normal (compare Fig. 4B to Fig. 5B). In conclusion, *ara* LoF is lethal. *TSI* and ara^{G4} are respectively a null and a strong hypomorphic alleles of *ara* having no or minor effects on *caup* and *mirr*.

For all insertions, bristles pattern on the notum is wt in homozygous adults (Fig. 5C). The same is true for heterozygotes over *TSI*, the only exception being ara^{T}/*TSI* where the pSA is often missing. Particularly, at 18 °C, rare *TSI*/ara^{G4} escapers adults eclose (4/95) and display a normal thoracic bristle pattern (Fig. 5C). In addition, *TSI*/*iro1* individuals are fully viable and wt, noticeably in their bristle pattern. Altogether, we can conclude that *ara* is dispensable for macrochaetes formation while required for viability.

However, when heterozygous over *Df-BSI*, all *ara* hypomorphic mutants display a partial loss of some lateral macrochaetes (Fig. 5C). Therefore, solely in this sensitized genetic context with only one functional copy of *caup* and *mirr*, *ara* LoF elicits an effect on bristle development. This suggests that *ara* may contribute to the patterning of bristles when *caup* and/or *mirr* levels are reduced.

mirr is required for the patterning of a subset of macrochaetes

mirr LoF leads to embryonic lethality (McNeill et al., 1997). Clonal LoF of *mirr* in the notum induces notum to hinge transformation (Diez del Corral et al., 1999), precluding the analysis of the *mirr* role in the bristle patterning of the notum by this method. Kehl et al. (1998) reported that $mirr^{LoF}$ viable combinations affect four of the seven lateral macrochaetes (PS, pSA, aPA and pPA). This is correlated with *mirr* expression in the corresponding SOPs.

Here we have examined the effects on thoracic macrochaetes formation of four TE insertions in *mirr* in genotypic conditions compatible, at least to some extent, with the viability of adults. $mirr^{f01086}$ and $mirr^{c04837}$ are homozygous viable (Fig. 1) and display a wt bristles pattern when homozygous. However, these insertions elicit a partial loss of some lateral macrochaetes when heterozygous over *Df-BSI* or $mirr^{e48}$ (Fig. 6A). $mirr^{G1}$ and $mirr^{G8}$ are homozygous lethal and do not complement *mirr* LoF alleles for viability. At 18 °C, rare $mirr^{G1}/mirr^{e48}$ escaper adults eclose (4/200). They display a total or partial loss of PS, pSA, aPA, pPA and aDC macrochaetes and have a reduced alulae (data not shown). In addition, a similar phenotype is observed in $mirr^{G8}$/+ heterozygotes (Fig. 6B). Therefore, $mirr^{G8}$ is a dominant allele of *mirr*. Altogether, we can conclude that *mirr* is required for the patterning of a subset of macrochaetes in the lateral notum. In addition, as dorsocentral macrochaetes are strongly affected in $mirr^{G1}/mirr^{e48}$ *and* $mirr^{G8}$/+ flies, *mirr* contribute to the patterning of dorsocentral macrochaetes, in agreement with the inclusion of the corresponding SOPs within the *mirr* expression domain (Figs. 6C, D).

Ara, but not Mirr, is efficient in replacing the loss of caup function

P[*Gal4*] elements inserted in *caup* reproduce its expression pattern and elicit a total loss of the lateral macrochaetes when heterozygous over *iro1*. This allows to test the capacity of $caup^{Gal4}$ driven *caup* overexpression to rescue this phenotype. However *caup* overexpression is deleterious, as mentioned by Cavodeassi et al. (2001). Indeed, we found that $caup^{G1}$/+;*UAS-caup* and $caup^{G3}$/+;*UAS-caup* combinations are lethal, whereas the $caup^{G2}$/+;*UAS-caup* combination is semi-viable. Hence $caup^{G1/3}$ are stronger drivers than $caup^{G2}$ consistently with their more efficient activation of *UAS-GFP* (data not shown). $caup^{G2}$ was thus used in all further experiments.

At 25 °C, *UAS-caup*;$caup^{G2}$/*iro1* flies partially recovered some lateral macrochaetes, with mostly normal morphology and positions (Fig. 7A). The lack of complete rescue could be due to *caup* overexpression detrimental effects as observed on $caup^{G2}$/+;*UAS-caup* individuals (Fig. 7A). As the *UAS*/*Gal4* system is thermosensitive (Duffy, 2002), we repeated both experiments at 18 °C. At this temperature, the *caup* overexpression in a *wt* background had actually milder effects and, in a *caup* LoF background, all the lateral macrochaetes were rescued to some extent (Fig. 7A). Thus, while requiring *caup* function, macrochaetes development is inhibited by too high levels of Caup. Indeed, we observed a more than 10-fold increase of *caup* expression in *UAS-caup*;$caup^{G2}$/*iro1* larvae as compared to *wt* (Fig. 7B). A precise dosage of Caup is thus required for the correct formation of macrochaetes.

We were now able to test the capacity of $caup^{G2}$ driven *ara* overexpression to rescue the *caup* LoF macrochaetes phenotype. At 25 °C and 18 °C, *UAS-ara*;$caup^{G2}$/*iro1* flies show a similar macrochaetes pattern as *UAS-caup*;$caup^{G2}$/*iro1* flies (Fig. 7A). *ara* and *caup* are overexpressed at similar levels in *UAS-ara*;$caup^{G2}$/*iro1* and *UAS-caup*;$caup^{G2}$/*iro1* larvae respectively (Fig. 7B). Thus *ara*, when overexpressed in a *caup* LoF background, is able to replace the loss of *caup* function. In addition, similarly to $caup^{G2}$/+;*UAS-caup* flies, $caup^{G2}$/+;*UAS-ara* flies are semi-viable and display a partial loss of some macrochaetes.

In contrast, the *UAS-mirr*;$caup^{G2}$ combination is lethal at all temperatures. We used then the $GAL80^{ts}$ system (McGuire et

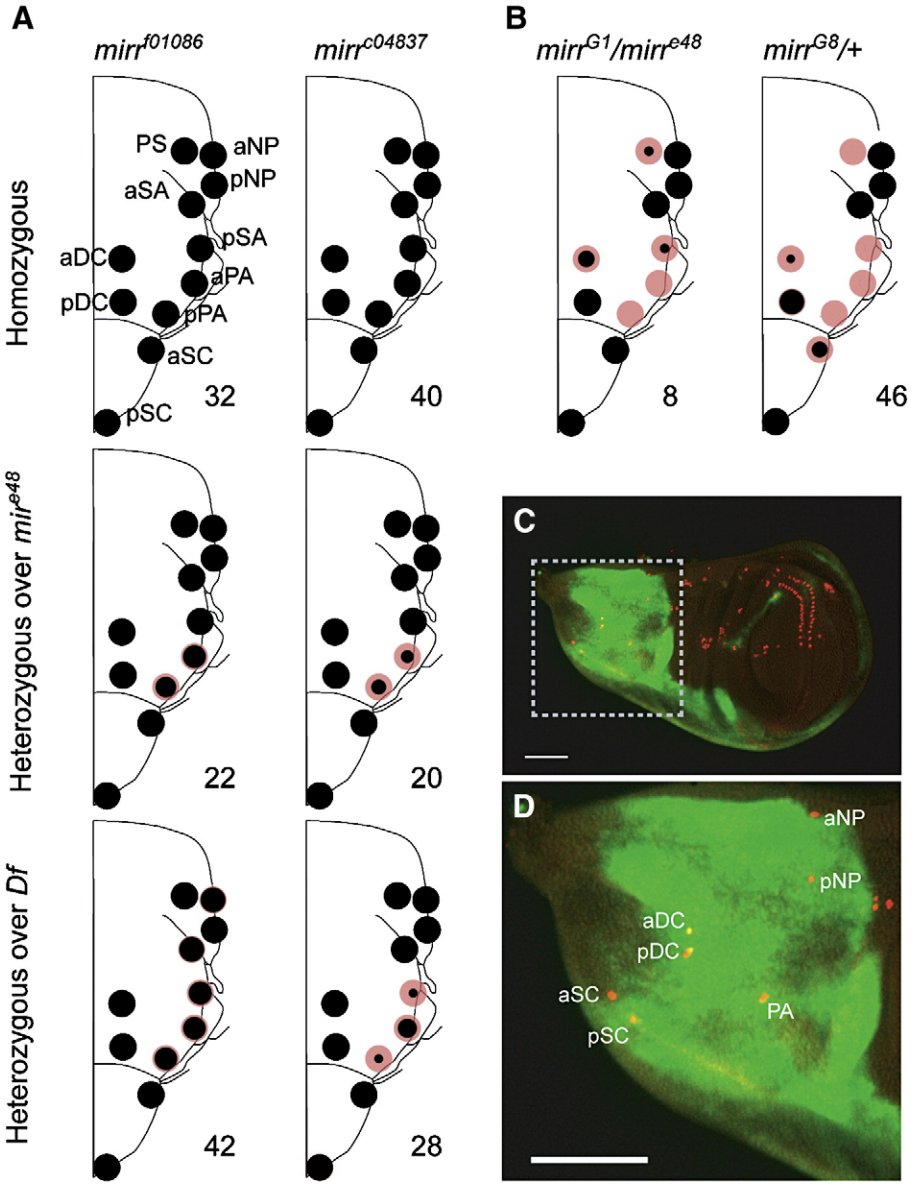

Fig. 6. Effects of insertions affecting *mirr* on macrochaetes patterning. (A) Schematization of the macrochaetes frequencies per heminotum displayed by homozygotes, and heterozygotes over *Df* or over $mirr^{e48}$ for $mirr^{f01086}$ *and* $mirr^{c04837}$ insertions. (B) Schematization of the macrochaetes distribution per heminotum in $mirr^{G1}/mirr^{e48}$ escapers and $mirr^{G8}$/+ heterozygotes. Legends as in Fig. 3. In the lateral notum, $mirr^{G1}/mirr^{e48}$ and $mirr^{G8}$/+ flies display the loss of four of the seven macrochaetes: PS, pSA, aPA and pPA. The formation of the DC macrochaetes is also affected in these flies. We have observed that the DC macrochaete occupy a position which is neither the aDC position nor the pDC one when only one DC bristle per heminotum is present. In this case, we have considered this DC macrochaete as a pDC. The scutellum of heterozygous $mirr^{G8}$/+ flies is frequently deformed making impossible to assess an identity to the bristle when only one SC macrochaete is present per hemiscutellum: we have then counted this SC macrochaete as a pSC. (C) The expression of *mirr* in the L3 wing disc is visualized with the combination of $mirr^{G8}$ and *UAS-GFP* (green). SOPs are labeled with $neur^{A101}$*-lacZ* (red) and immunoassayed with an anti-β-Galactosidase antibody. (D) High magnification view of (C). $mirr^{G8}$ and $neur^{A101}$ are co-expressed in the SOPs for DC, pSC, PA and pNP. The SOP for aNP resides outside $mirr^{G8}$ domain of expression. The scale bars are 40 μm.

al., 2004) to reduce *mirr* overexpression to levels compatible with viability. *UAS-mirr/Gal80^{ts};caup^{G2}/iro1* flies are indeed viable at 25 °C but, although *mirr* expression is more than three-fold increased in *UAS-mirr/Gal80^{ts};caup^{G2}/iro1* larvae (Fig. 7B), they show only a very weak rescue of some lateral macrochaetes (Fig. 7A). This cannot be interpreted as a dominant effect of *mirr* overexpression: *UAS-mirr/Gal80^{ts}; caup^{G2}*/+ have a wild-type bristle pattern at 25 °C. Thus *mirr*, when overexpressed at levels compatible with viability, is not efficient in replacing the loss of *caup* function. Reciprocally, neither *caup* nor *ara* is able to rescue the lethality associated with *mirr* LoF when expressed in the *mirr* domain of expression under the control of $mirr^{G1}$, while the expression of *mirr* is able to do so (our unpublished results). These results support the conclusion that the Mirr protein is intrinsically different from both Caup and Ara.

In the rescue experiments, the overexpression of one of the three *iro-C* genes does not modify the expression of the two others (Fig. 7B), suggesting that there is no trans-regulation between each *iro-C* gene at this stage of development.

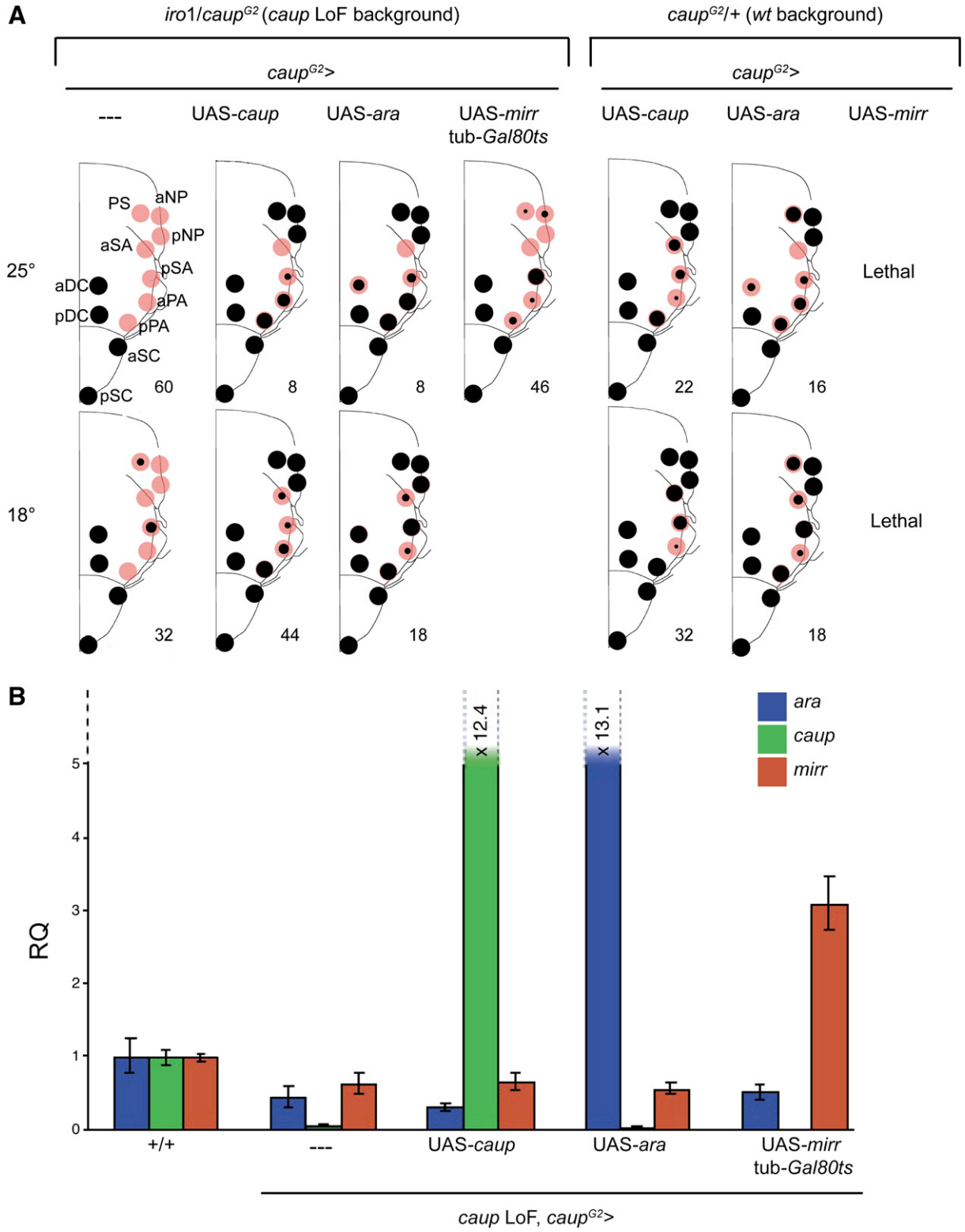

Fig. 7. Effects of the *iro-C* genes overexpression on macrochaetes patterning. (A) Schematization of bristle phenotypes of flies overexpressing *Iro-C* genes under the control of the $caup^{G2}$ driver in a *caup* LoF ($iro1/caup^{G2}$) and a *wt* ($caup^{G2}$/+) genetic backgrounds. Legends as in Fig. 3. Crosses were made at 25 °C and 18 °C. Rescue experiments were performed in $caup^{G2}$/*iro*1 heterozygotes harboring an *UAS-ara*, *UAS-caup* transgene or the *UAS-mirr, tub-Gal80ts* combination. Dotted line (- - -) means that no UAS transgene was used. Overexpression experiments were performed in $caup^{G2}$/+ heterozygotes flies carrying *UAS-ara*, *UAS-caup* or *UAS-mirr*. (B) Quantitative RT-PCR analysis performed, at 25 °C, on mRNA extracted from L3 larvae of the following genotypes: *wt*, $caup^{G2}/iro1$ (*caup* LoF), *UAS-caup;* $caup^{G2}/iro1$, *UAS-ara;*$caup^{G2}/iro1$, *UAS-mirr*/tub-Gal80ts;$caup^{G2}/iro1$. RQ: Relative quantity of mRNA for each *iro-C* gene (wt=1).

sr is a target for caup and mirr, but not for ara

A common set of prepattern genes and signaling molecules regulates the positions of bristle precursors and tendon precursors (Calleja et al., 2002; Ghazi et al., 2003). The *sr* gene expression is required to specify tendon cell precursors, prefiguring indirect flight muscle attachment sites (Nabel-Rosen et al., 1999) and specifying a "muscular prepattern". In L3 larvae, *sr* is expressed in four domains in the prospective notum (Fernandes et al., 1996; a, b, c and d in Figs. 8A, B). In the medial notum, *pnr* is not only required for *ac–sc* expression (neural prepattern), but also for *sr* expression in the "a" domain (muscular prepattern; Calleja et al., 2000; Ghazi et al., 2003). Less is known about the regulation of *sr* in the prospective lateral notum. The *iro-C* genes are good candidates to regulate *sr* expression in this domain. *caup* expression domain includes the lateral *sr* domains (b, c and d in Fig. 8A′), whereas the medial domain (a) is positioned at its border. In *ara* LoF wing discs (ara^{G4} homozygote), *sr* expression is wt (Fig. 8C). Thus *ara* is not necessary for *sr* expression in the notum.

Conversely, two lateral domains of *sr* (b and d) are absent in *caup* LoF wing discs ($caup^{G3}$/*iro1*) (Fig. 8D). Therefore, *caup* is required for normal *sr* expression and for the muscular prepatterning of the notum.

In $mirr^{G8}$/+ heterozygotes, "b" and "d" domains are also absent and the "a" and "c" domains are partially reduced (Fig. 8E). Thus, $mirr^{G8}$ dominantly affects *sr* expression showing the involvement of *mirr* in the muscular prepatterning of the notum.

Discussion

caup and mirr but not ara are required for the neural patterning of the lateral notum

In earlier works, the *iro-C* genes functions were mainly assessed from studies of mutations affecting solely *mirr* (Diez del Corral et al., 1999; Jordan et al., 2000; Kehl et al., 1998; McNeill et al., 1997; Netter et al., 1998; Zhao et al., 2000), of deficiencies deleting the whole complex or of rearrangements susceptible to affect several genes, and on misexpression experiments (Cavodeassi et al., 2000; Diez del Corral et al., 1999; Gomez-Skarmeta et al., 1996; Pichaud and Casares, 2000). In order to unravel the respective roles of *ara*, *caup* and *mirr* and to analyze their possible functional redundancy in the neural prepatterning of the notum, we have combined the analysis of LoF mutations of these genes with a functional replacement approach.

mirr appears to be required for the formation of four out of the seven lateral macrochaetes (PS, pSA, aPA and pPA): their loss is elicited by LoF alleles of *mirr* (Fig. 6B; Kehl et al., 1998) and by a dominant allele ($mirr^{G8}$, Fig. 6B). The proneural clusters as well as the SOPs corresponding to the macrochaetes unaffected by *mirr* mutations may reside outside (aNP) or inside (pNP) the *mirr* domain of expression (Kehl et al., 1998; Fig. 6D). Therefore the requirement or dispensability of *mirr* for the patterning of the bristles in the lateral notum appear to depend partly but not only on its domain of expression. All the phenotypes observed here were elicited by mild perturbations of the *mirr* function, compatible with viability of adults. Therefore,

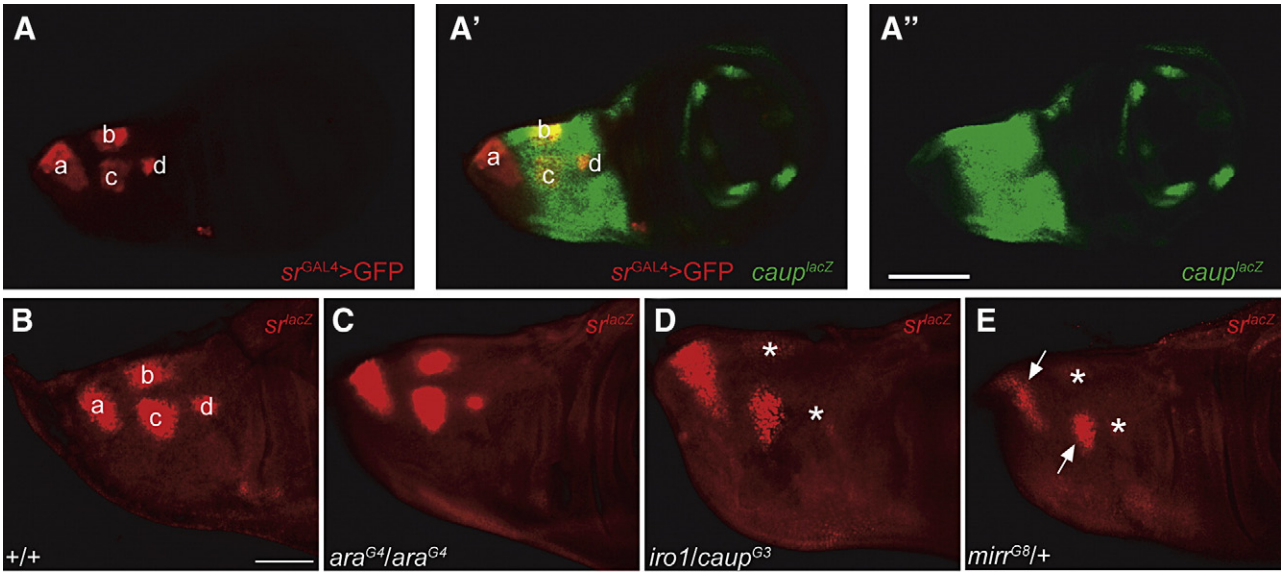

Fig. 8. Effects of *iro-C* gene loss of function on *sr* expression. (A–A″) Wing disc from $caup^{Sc2}$/+, +/sr^{GAL4}, +/*UAS–GFP* L3 larvae labeled with anti-β-Galactosidase antibody to visualize *caup* (green: A″) and observed for GFP fluorescence to visualize *sr* (red: A). (A′) Merged image. *caup* expression covers the lateral *sr* expression domains b, c and d. (B–E) L3 wing discs labeled with anti-β-Galactosidase antibody to visualize expression *sr-lacZ* (red). (B) Wild type *sr* expression. (C) ara^{G4}/ara^{G4}, +/sr^{LacZ} wing disc: *ara* loss of function does not affect *sr* expression. (D) $iro1/caup^{G3}$, +/sr^{LacZ} wing disc: *caup* loss of function abolishes *sr* expression in the b and d domains (asterisks). (E) $mirr^{G8}$/+, +/sr^{lacZ} wing disc: *sr* expression is abolished in the b and d domains (asterisks), and partially reduced in the a and c domains (arrows). The scale bars are 40 μm and 30 μm in A–A″ and B–E, respectively.

a role of *mirr* in the patterning of the other lateral macrochaetes cannot be completely excluded.

In the prospective lateral notum, *ara* and *caup* appear to have mostly identical domains of expression, which, at least, enclose all the SOPs for lateral macrochaetes (Fig. 2A′; Gomez-Skarmeta et al., 1996). These authors have suggested that there is a functional redundancy of these two genes on the grounds of their similar protein-coding sequences and patterns of expression, and this is generally admitted (Mann and Morata, 2000). However no evidence of this has been reported to date. Notably no LoF mutations affecting only one of these two genes have been described previously. We have characterized mutations abolishing (or drastically reducing) *caup* expression without an appreciable effect on *ara* and *mirr* (e.g. *iro1* or $caup^{G3}$) and null (or strong hypomorph) mutations of *ara* that do not affect significantly *caup* or *mirr* (e.g. *TSI* or ara^{G4}).

We have analyzed six TE insertions in *caup* and we have shown that they behave like an allelic serie of *caup* hypomorphic mutations: flies homozygous for these insertions are viable and display phenotypes ranging from wt or the loss of only a few macrochaetes to the loss or a strong frequency decrease of all the seven lateral macrochaetes (strong Iroquois phenotype). These phenotypes are aggravated when these TE insertions are heterozygous over an *iro-C* deficiency and even more over the *iro1* rearrangement, which has a breakpoint in the first *caup* intron. Heterozygous *iro1/Df-BSI* L3 larvae display no *caup* expression (or an extremely low level) although *ara* and *mirr* levels are similar to the control conditions. Similarly, the strongest *P* allele of *caup* ($caup^{G3}$) causes a drastic reduction or an absence of *caup* expression without affecting notably *ara* and *mirr*, neither in expression level nor in expression domain in the notum. Therefore, we can assume that *caup* is required for the patterning of all lateral macrochaetes and, presumably, for the direct or indirect activation of *sc* expression in the lateral notum (Leyns et al., 1996).

Besides inactivating *caup* expression, the *P*[Gal4] insertions in the *caup* gene reproduce its expression pattern. By combining such a *Gal4* driver with an *UAS-caup* transgene, we show that *caup* overexpression in its normal domain of expression is able to rescue the *caup* LoF phenotype to an almost wt situation. The frequency of occurrence of all the seven lateral macrochaetes is either normal or at least elevated as compared to the mutant situation. Taken together, loss and gain of function approaches demonstrate that *caup* is required for the patterning of all the lateral macrochaetes of the notum.

We applied the same approach to study the loss of function of *ara* and analyzed the effects of five insertions and an inversion (*TSI*) in the *ara* gene. In ara^{G4}/*TSI* and *TSI/Df-BSI* L3 larvae, *ara* expression is absent or strongly reduced while *caup* and *mirr* expression are not significantly affected as compared to the control situations. These larvae die as pupae, showing the requirement of *ara* for viability. The very rare adult escapers do not present any bristle defects. The same is true for the four other *ara* hypomorphic mutants studied. In summary, the sole LoF of *ara* is never associated with a loss of macrochaetes. Consequently, conversely to *caup*, *ara* is dispensable for the patterning of all the lateral bristles of the notum.

In conclusion, the Iro proteins are different for their contribution to the neural patterning of the lateral notum. Only Caup and Mirr are required for the patterning of the lateral macrochaetes and therefore to control the activation of expression of *ac* and *sc* in the corresponding proneural clusters (Leyns et al., 1996).

Ara is able to perform the Caup function in the neural patterning of the notum

When it is strongly overexpressed in the *caup* domain of expression, Ara, although not required, is sufficient to rescue the lack of lateral macrochaetes phenotype elicited by *caup* LoF. Therefore, unexpectedly considering the LoF results, when overexpressed Ara can carry out the function of Caup in the neural patterning of the notum. This property may play a biological role, as can be seen in the exacerbation of the phenotypic defects elicited by *ara* LoF in sensitized conditions (reduction of *caup* and/or *mirr* function). Although to a much less extent, Mirr is also able to perform in part the Caup function, as seen from the limited rescue of PS, aNP and aSA by *mirr* overexpression in a *caup* LoF background. This functional difference between Ara and Mirr correlates well to their sequence divergence with Caup. A possible explanation for the difference observed between the LoF of *ara* and *caup* is that Ara, in physiological conditions, has a lower activity than Caup to promote proneural genes expression. The increase of the amount of the Ara protein to non-physiological levels probably increases the expression of proneural genes sufficiently to compensate for the loss of Caup activity. This is in good agreement with the findings of Bilioni et al. (2005) that *Drosophila* Iro proteins bind *in vitro* the same sequence but differ in their strength of binding to this site. Consequently, owing essentially to the proteins intrinsic properties, the Iro transcription factors do not regulate *in vivo* the same target genes.

stripe, a new target for Caup and Mirr

Calleja et al. (2002) have suggested that a common network of prepattern genes regulates all structures of the notum. Indeed, in the prospective medial notum, *pnr* is required for both *ac–sc* and *sr* expression, which respectively specify the development of bristles and tendon precursors. Preliminary observations indicate that *pnr* also regulates pigment patterns in medial notum (Calleja et al., 2002).

In the wing discs of larvae mutant for *caup* or for *mirr*, two (b and d) out of the three domains of *sr* expression located in the prospective lateral notum are missing. Furthermore, a *mirr* mutant partially affects *sr* expression in the two remaining domains (a and c). In addition, adult flies lacking either *caup* or *mirr* display falling wings, suggesting an abnormal attachment of indirect flight muscles. Therefore, *caup* and *mirr* activate *sr* expression and in consequence participate to the specification of lateral flight muscle attachment sites. It had been shown that Iro proteins can form homodimers and heterodimers that bind *in vitro* the same palindromic site called Iro Binding Site (IBS; Bilioni et al., 2005). Thus, it is possible that the activity of a

Caup-Mirr heterodimer controls the regulation of *sr* expression in regions (b) and (d). The *sr* expression pattern is normal in the wing discs of larvae lacking the *ara* function. Hence *ara* is not required for the expression of *sr* in the prospective notum. Nonetheless, a reduction of *ara* function, as seen in hypomorphic mutants or escapers to the lethality, leads to heldout wings. This phenotype is often observed in flies carrying mutations that affect direct flight muscles (Kozopas and Nusse, 2002). It is thus possible that *ara* regulates the specification of direct flight muscle attachment sites. In summary, here again, the *iro-C* genes products are not functionally equivalent in their contribution to the muscular attachment sites prepattern.

Although there are no strong evidences that *iro-C* regulates pigment patterns, preliminary reports suggest that this is the case (Calleja et al., 2000). From all these data, the *iro-C* genes *caup* and *mirr* appear as common prepattern genes for the specification of all the structures in the lateral notum, similarly to *pnr* in the medial notum.

In addition, *pnr* and *iro-C* domains partially overlap each other at the virtual border between the medial and the lateral notum (Sato et al., 1999). We have observed that the DC macrochaetes and the "a" *sr* domain can be affected in *caup* and *mirr* mutants. These structures are dependant on the *pnr* function (Calleja et al., 2002). Therefore, both *pnr* and the *iro-C* appear to prepattern a region of the notum, at the intersection of their expression domains, which could correspond to the medial–lateral band of *wg* expression overlapping these domains (Phillips and Whittle, 1993; Simpson, 1996).

The iro-C genes exert both distinct and overlapping functions in development

mirr LoF leads to embryonic lethality (McNeill et al., 1997). Although the targets of *ara* are yet to be identified, we show here that *ara* LoF cause pupal lethality, whereas flies lacking *caup* function are viable and exhibit developmental defects. Thus, *ara*, *caup* and *mirr* play distinct biological functions during development. These different roles can be attributed in part to differences in expression pattern. For instance, *mirr* is the first *iro-C* gene that is detected during embryogenesis (Gomez-Skarmeta et al., 1996; McNeill et al., 1997) and it is the only *iro-C* gene that is expressed and plays a role in oogenesis (Jordan et al., 2000; Zhao et al., 2000). However, this diversity in expression domains cannot account for all the observed functional differences. The overexpression of these three proteins in the same domains (here with the same *caup-Gal4* driver) have different consequences: while overexpression of *caup* and *ara* to more than 10 times the normal level is compatible with viability, the overexpression of *mirr* to the same level is lethal. When expressed at levels compatible with viability, *mirr* is unable to rescue the *caup* LoF while *ara* is. Thus, the differences in the *iro-C* genes roles cannot be only attributed to differences in their patterns of expression but rather should also be due to differences in their coding sequences.

In mammals, there are two clusters of three *iroquois* genes (*Irx*A and *Irx*B; Gomez-Skarmeta and Modolell, 2002). Expression patterns, LoF and misexpression studies reveal a similar situation where the *Irx* genes may have redundant or non-redundant roles, depending on the gene and/or on the developmental process (Bruneau et al., 2001; Cheng et al., 2005; Lebel et al., 2003).

The roles of the *Drosophila iro-C* genes have been documented in numerous other developmental processes, for instance: eye dorsal–ventral patterning, wing veins patterning, wing-body wall boundary, dorsal–ventral axis formation in oogenesis (Cavodeassi et al., 2001). Here, we have put together the tools and conditions allowing to address the question of the specific roles and/or of the functional redundancy of the *iro-C* gene in these other developmental processes.

The iroquois genes appear to be in the process of diversification

The long-term fates for duplicated genes include inactivation, maintenance and diversification (Otto and Yong, 2002). Maintenance of duplicated developmental genes can be the result of selective pressure exerted through dosage requirements and/or contribution to the genetic and developmental robustness necessary to reproducibly elaborate correct patterning of diverse territories (Gu, 2003; Krakauer and Nowak, 1999). Alternatively, the subfunctionalization model proposes that, after a duplication of genes, each copy may sustain deleterious mutations in different structural and/or regulatory elements. Eventually, the ancestral functions are partitioned between the copies that are both retained (Force et al., 1999; Lynch and Conery, 2000).

An interesting example is the *ac–sc* complex. Similarly to *ara* and *caup*, *ac* and *sc* arose from the most recent duplication event in this complex (Skeath and Carroll, 1991). Marcellini et al. (2005) have shown that *ac*, but not *sc*, is dispensable for the development of the sensory bristles. They propose two hypotheses (and favor the second) for the reason why evolution has retained *ac*: first, an as yet undiscovered function; second, a contribution to genetic robustness. However the situations of the *ac–sc* and *iro* complexes are not identical: Marcellini et al. (2005) have been unable to find any phenotypic consequence of the *ac* LoF in an otherwise *wt* background while we observed that *ara* LoF cause lethality. The reasons why the three *Drosophila iro-C* genes are maintained appear thus different and may proceed from the two (non-exclusive) hypotheses mentioned above. *mirror* has clearly evolved to perform different functions from *ara* and *caup*. This can be seen in expression pattern as well as in LoF phenotypes and in the functional properties of the protein. We provide here evidences for the functional divergence between *ara* and *caup*: first, the LoF of *ara* is lethal while the LoF of *caup* is not; second, *caup* is required for the neural and the muscular prepatterning of the prospective notum, while *ara* is dispensable. Additionally, we show subtle differences in their expression pattern in the L3 wing disc. Other differences have yet to be characterized more precisely at other stages and in other tissues and their role investigated. The three *iro-C* genes appear in the process of diversification and subfunctionalization, both in their expression domains and in the functions of their encoded proteins. In

addition, the partial ability of *ara* to perform *caup* functions, at least in the neural patterning of the notum, may contribute to buffer this patterning against intrinsic and extrinsic perturbations. It could thus be retained by a selective pressure at work on fly populations surviving in the wild.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.12.034.

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