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Antagonistic and cooperative actions of the EGFR and Dpp pathways on the *iroquois* genes regulate *Drosophila* mesothorax specification and patterning

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In *Drosophila,* restricted expression of the Iroquois complex (Iro-C) genes in the proximal region of the wing imaginal disc contributes to its territorial subdivision, specifying first the development of the notum versus the wing hinge, and subsequently, that of the lateral versus medial notum. Iro-C expression is under the control of the EGFR and Dpp signalling pathways. To analyze how both pathways cooperate in the regulation of Iro-C, we isolated several wing disc-specific cis-regulatory elements of the complex. One of these (IroRE^{2}) integrates competing inputs of the EGFR and Dpp pathways, mediated by the transcription factors Pointed (downstream of EGFR pathway) and Pannier/U-shaped and Mothers against Dpp (Mad), in the case of Dpp. By contrast, a second element (IroRE^{1}) mediates activation by both the EGFR and Dpp pathways, thus promoting expression of Iro-C in a region of elevated levels of Dpp signalling, the prospective lateral notum near the anterior-posterior compartment boundary. These results help define the molecular mechanisms of the interplay between the EGFR and Dpp pathways in the specification and patterning of the notum.

KEY WORDS: Iroquois complex, Imaginal wing disc, *Drosophila*, Notum development, Dpp signalling, EGFR signalling

INTRODUCTION

Drosophila imaginal discs provide an excellent model system to investigate how genetic information specifies the different body regions of an organism and how these regions are patterned. A pair of wing imaginal discs gives rise to the notum, pleura, wings and wing hinges of the insect, structures that display characteristic patterns of sensory organs and veins. Morphologically, cells from third instar imaginal discs are essentially indistinguishable one from another. However, classical genetic analyses and molecular data have uncovered the existence of a plethora of genes expressed in the discs with specific spatial and temporal patterns whose products provide a vast amount of positional information (Mann and Morata, 2000). The genes that actually carry out the specification of the different body regions and fates, for instance the specification of body wall versus appendage or the implementation of neural versus epidermal fate, decode this positional information. Their cis-regulatory elements (REs) act as integrating devices receiving inputs present in the cells, which consist of combinations of transcription factors, and eliciting responses in the form of the restricted patterns of expression of the genes they are controlling (Modolell and Campuzano, 1998).

The *araucan* (*ara*), *caupolican* (*caup*) and *mirror* (*mirr*) genes of the Iro-C (Gomez-Skarmeta et al., 1996; McNeill et al., 1997) belong to the class of genes whose expression specifies territories. Thus, the homeoproteins they encode are essential for, among other functions (reviewed by Cavodeassi et al., 2001), the specification of the notum territory and its subsequent patterning (Diez del Corral et al., 1999; Gomez-Skarmeta et al., 1996). These diverse functions rely on their spatially and temporally restricted expression patterns. Thus, at the second larval instar, these genes are expressed in the most proximal region of the wing disc. There, they confer on cells the ability to form notum since, in their absence, these cells give rise to structures of the dorsal wing hinge (Diez del Corral et al., 1999). Furthermore, the confrontation of Iro-C-expressing and non-expressing cells generates an organizing border similar to those found between compartments (Diez del Corral et al., 1999; Villa-Cuesta and Modolell, 2005). Afterwards, expression of Iro-C is restricted to the prospective lateral notum where the Iro proteins function as components of the prepattern that governs the expression of the achaete-scute complex (AS-C) genes and, accordingly, help define the pattern of adult sensory organs (Gomez-Skarmeta et al., 1996).

To understand the development of the notum it is thus necessary to clarify how expression of the Iro-C is controlled. Previous reports have shown that, in the prospective notum region of the wing disc, Iro-C is under the control of the Epidermal growth factor receptor (EGFR) and Decapentaplegic (Dpp) signalling pathways. During the second larval instar, EGFR signalling is necessary for notum development and expression of Iro-C (Wang et al., 2000; Zecca and Struhl, 2002a; Zecca and Struhl, 2002b), whereas the Dpp pathway contributes to the confinement of the expression of Iro-C to the notum region (Cavodeassi et al., 2002). In the early third larval instar, this pathway further restricts expression of the Iro-C genes to the prospective lateral notum (Cavodeassi et al., 2002). How both pathways converge on the regulation of Iro-C genes is still unknown. To address this point, we have characterized the regulatory sequences of the Iro-C, a gene complex that spans approximately 150 kb of DNA (Gomez-Skarmeta et al., 1996; McNeill et al., 1997). Expression of Iro-C genes is thought to be controlled by enhancer regulatory sequences that would act jointly on, at least, *ara* and *caup* (Gomez-Skarmeta et al., 1996). We have identified five partially redundant wing disc-specific cis-regulatory elements within the Iro-C (IroREs) and demonstrated the ability of some of them to mediate regulation by the EGFR and Dpp signalling pathways. We show that the transcription factor Pointed (Pnt) mediates the activation of Iro-

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C by the EGFR pathway and the involvement of Pannier (Pnr), U-shaped (Ush) and Mothers against Dpp (Mad) transcription factors in Dpp-dependent repression. In addition, we propose a mechanism for the coexistence of Iro-C expression and the activity of the Dpp pathway at the prospective lateral notum near the anterior-posterior (AP) compartment boundary. Our results help clarify how the territorial specification and patterning of the *Drosophila* thorax is effected by the antagonistic/cooperative action of EGFR and Dpp pathways on Iro-C expression.

MATERIALS AND METHODS

Fly stocks and mitotic recombination clones

All the mutant alleles and transgenes are described in FlyBase (http://flybase.org). Marked clones of mutant cells were generated by FLP-mediated mitotic recombination (Xu and Rubin, 1993; Lee and Luo, 1999) in larvae of the following genotypes:

y w hsFLP1.22; *ubi-nlsGFP FRT40A/ush^{VX22} FRT40A* (or *tkv^{a12} FRT40A*, or *yan^{443} FRT 40A* or *yan^{884} FRT 40A*); *IroRE-lacZ/+*

y w hsFLP1.22; *IroRE-lacZ/+; FRT82B ubi-nlsGFP/FRT82B grn^{7L12}* (or *FRT82B pnt$^{\Delta 88}$* or *FRT82B pnr^{VX6}*)

y w hsFLP1.22; *IroRE-lacZ/+; FRT82B M ubi-nlsGFP/FRT82B ttk^{1e11}*

yw hsFLP tubα1-Gal4, UAS-GFP; *tubα 1-Gal80 FRT40A/ush^{VX22} FRT40A*; *UAS-tkv^{QD}/IroRE-lacZ*

Second instar (48-72 hours after egg laying; AEL) or early third instar (72-96 hours AEL) larvae were heat shocked for 1 hour at 37°C.

Misexpression experiments

Clones of cells overexpressing different genes were obtained as described by Ito et al. (Ito et al., 1997). *Actin5C>yellow^{+}>Gal4, UAS-GFP; IroRE-lacZ* males were mated with females carrying *hsFLP1.22* and different UAS transgenes (*UAS-tkv^{QD}*, *UAS-Mad*, *UAS-pnr*, *UAS-ush*, *UAS-vein*, *UAS-Ras^{V12}*, *UAS-Raf^{DN}*, *UAS-Raf^{Act}*, *UAS-pntP1*). Larvae were treated 24-48 hours AEL or 72-96 hours AEL, for 10 minutes at 37°C. The clones were revealed by GFP expression.

Iro-C reporter constructs

*Eco*RI or *Hin*dIII fragments of genomic Iro-C DNA from the lambda phage and P1 walk described by Gomez-Skarmeta et al. (Gomez-Skarmeta et al., 1996), encompassing 110 kb of the DNA proximal to the *iro^{DFM2}* breakpoint, were subcloned in the C4PLZ enhancer tester plasmid that contains a weak P-element promoter (Wharton, Jr and Crews, 1993). Fragments encompassing IroRE^{1} and IroRE^{2} were ligated and subcloned into C4PLZ to obtain the transgenic flies IroRE^{1}-IroRE^{2}-*lacZ*. IroRE$^{2\text{-}B1}$ to IroRE$^{2\text{-}B5}$ fragments were obtained by PCR amplification using 1.6 kb IroRE$^{2\text{-}B}$ as a template and appropriate primer sets. (Primers sequences are available upon request.) PCR-amplified fragments cloned into pGEM Teasy vector (Promega), were excised by *Eco*RI digestion and subcloned into C4PLZ. The *lacZ* reporter plasmids were introduced into *y w^{1118}* embryos by standard P-element transformation (Ashburner, 1989). Three to six independent transgenic lines were established and examined for each construct.

Immunohistochemistry

Imaginal discs were dissected and stained as described previously (Gomez-Skarmeta et al., 1996). Primary antibodies were: rabbit and mouse anti-β-galactosidase (Cappel and Promega), rat anti-Caup, an antibody that recognizes both Ara and Caup (Diez del Corral et al., 1999) and mouse anti-Wingless (DSHB). Secondary antibodies were from Jackson Immunoresearch Laboratories and Molecular Probes. Confocal images were acquired using Bio-Rad Microradiance and Zeiss LSM510 Meta confocal microscopes. Images were imported and assembled using Adobe Photoshop 7.0 software.

Sequence analysis

Flyenhancer (http://flyenhancer.org/Main) (Markstein and Levine, 2002) and cis-analyst (http://rana.lbl.gov/cis-analyst/) (Berman et al., 2002) programs were used in the search of putative transcription factor binding sites. Cross-species sequence conservation was monitored using the VISTA program (http://genome.lbl.gov/vista/index.shtml) (Couronne et al., 2003).

Mutagenesis of GATA and ETS putative binding sites

Mutagenesis was performed by the site directed QuikChange system (Stratagene). To obtain the IroRE$^{2B\text{-}2}$-GATA-mut, IroRE$^{2B\text{-}2}$ DNA subcloned into pGEM Teasy, was used as template to mutate the putative GATA binding site GATAAG into CTGAAG with the following primers: forward 5′-GATGGCGATGGCAGCctgAAGCCCCATGATTTTG-3′ and reverse 5′-CAAAATCATGGGGCTTcagGCTGCCATCGCCATC-3′. Iro-RE$^{2B\text{-}2}$ DNA was similarly used to obtain the Iro-RE$^{2B\text{-}2}$-ETS-mut, with the putative conserved ETS binding site CGGGATG changed into CGCATTG, using the following primers: forward 5′-CCGGGGATTGGGAAATGGGTTC-GcatTGGCCAGTTTAGTCG-3′ and reverse 5′-CGACTAAACTGGC-CAatgCGAACCCATTTCCCAATCCCCGG-3′. Altered bases are shown in lowercase. Mutations were confirmed by sequencing.

RESULTS

Identification of transcriptional regulatory elements within the Iro-C

The Iro-C genes *ara* and *caup* show similar patterns of expression in the wing disc. In early second instar larvae, they are expressed in the whole prospective mesothorax region (Cavodeassi et al., 2002; Gomez-Skarmeta et al., 1996). Later, in the third instar, their expression is restricted to the lateral notum (Fig. 1A). In addition, at this developmental stage, novel domains of expression appear in the prospective regions of the L1, L3 and L5 veins, tegula, dorsal radius, dorsal and ventral pleura and alula (Diez del Corral et al., 1999; Gomez-Skarmeta et al., 1996). The expression of *mirr* is slightly different, being absent from the L3, L5 and tegula domains but present at the other domains (Kehl et al., 1998). The Iro-C harbours two additional transcription units, *lincoyan* (*linc*), whose pattern of expression at the notum is identical to that of *ara* and/or *caup* and *quilapan* (*quil*), which is ubiquitously expressed (R. Diez del Corral, PhD thesis, Universidad Autónoma de Madrid, Spain, 1998). Previous genetic analysis suggested the existence of enhancer-like REs that would drive the coincident expression of *ara* and *caup* in the wing disc (Gomez-Skarmeta et al., 1996). Thus, *In(3L)iro^{DFM2}*, associated with a breakpoint within the *ara* transcription unit (Fig. 1B), removes *ara* expression in the wing disc except in the L3 vein domain, in contrast to *caup* expression which is only lost from that domain (Gomez-Skarmeta et al., 1996). This suggests the existence of vein L3-specific RE(s) distal to the *In(3L)iro^{DFM2}* breakpoint and other RE(s), specific for the remaining domains of Iro-C expression, located proximal to such breakpoint. To identify notum-specific REs, we analyzed the regulatory potential of 31 different genomic fragments, spanning approximately 110 kb of genomic Iro-C DNA (Fig. 1B).

Only five of those fragments drove *lacZ* expression at specific regions of the imaginal wing disc (Fig. 1B). One of them, 3.3 kb in length and named Iro regulatory element^{2} (IroRE^{2}), reproduced most of the expression pattern of Iro-C in the prospective notum (Fig. 1D,D′,H,H′). Thus, IroRE^{2}-*lacZ* was expressed in the proximal region of early third instar wing discs (the presumptive notum region; Fig. 1H,H′) and at the presumptive lateral notum in third instar wing discs (Fig. 1D,D′). Note, however, that the pattern of IroRE^{2}-mediated *lacZ* expression did not exactly coincide with that of *ara/caup*. Thus, β-gal was not detected in a triangular area, located near the notum/hinge border and centred around the AP compartment boundary, where expression of *ara/caup* is enhanced (Fig. 1D, arrowhead, compare with D″). This is precisely the region where expression of *lacZ* was driven by another Iro-C genomic fragment of 3.9 kb, IroRE^{1} (Fig. 1C,C′). Accordingly, an IroRE^{1}-IroRE^{2} composite RE was found to drive *lacZ* expression in a pattern very similar, albeit not identical, to that of the endogenous *ara/caup* genes (see Fig. S1 in the supplementary material).

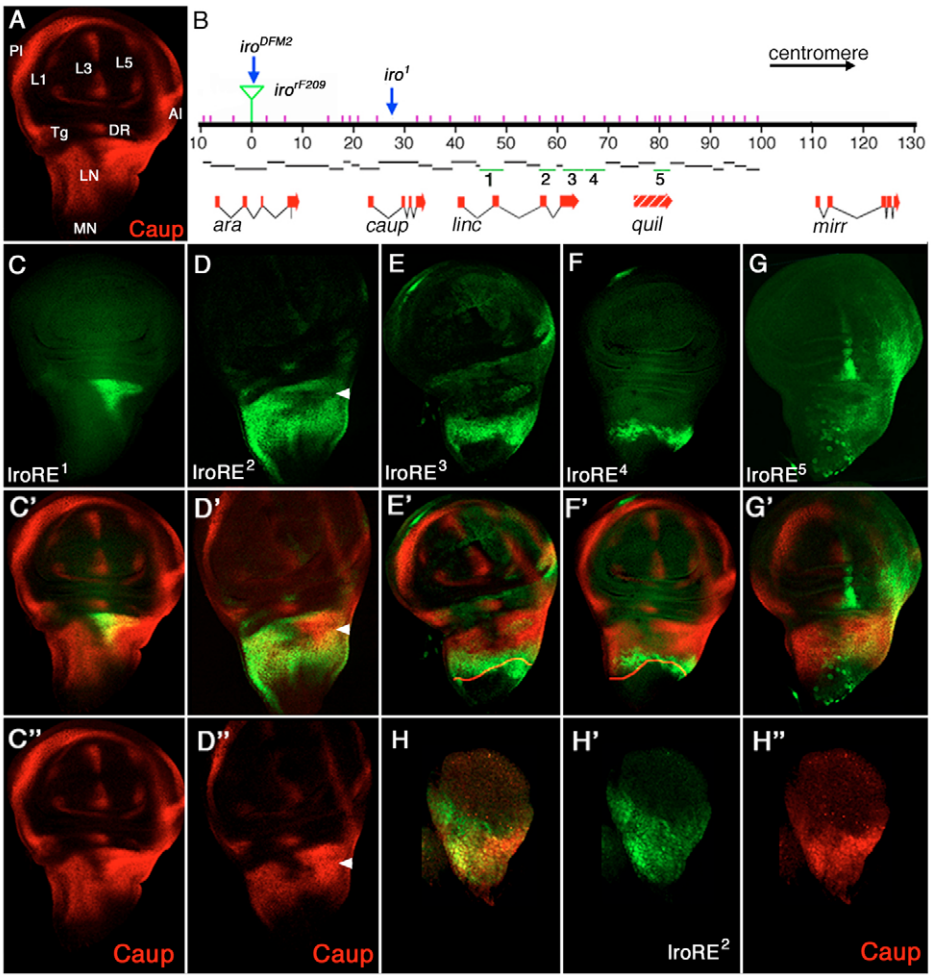

Fig. 1. Search for cis-regulatory elements in the Iro-C. (**A**) Expression of *ara/caup* in a third instar wing disc. Ara/Caup accumulated at the prospective alula (Al), dorsal radius (DR), lateral notum (LN), longitudinal veins L1, L3 and L5, pleura (Pl) and tegula (Tg) but not at the medial notum (MN). (**B**) Physical map of the Iro-C locus (Gomez-Skarmeta et al., 1996). Genomic DNA is shown as a thick black bar. Transcription units are shown as red arrows; location of *quil* is approximated. Blue arrows indicate positions of breakpoints associated with the iro^{DFM2} and iro^{1} mutations. The green triangle represents the P insertion iro^{rF209}. Small vertical magenta bars delimit fragments (horizontal black and green bars) tested for their enhancer ability. Fragments 1-5 (green bars) show enhancer activity. (**C-H″**) *lacZ* expression patterns (green) driven by the indicated REs and endogenous expression of *ara/caup* (red) in late third (C-G′) and early third (H-H″) instar wing discs. Differences in the expression domains of IroRE^2-*lacZ* and *ara/caup* are indicated by an arrowhead in D-D″. The wavy red lines in E′ and F′ mark the proximal limit of *caup* expression. Wing discs are oriented ventral side up and anterior to the left.

Two other genomic fragments, IroRE^3 and IroRE^4 (3.4 and 3.7 kb), adjacent to each other (Fig. 1B), drove *lacZ* expression in a stripe of cells located at the proximal region of the presumptive lateral notum, which partially overlapped with the *caup* expression domain (Fig. 1E-F′). Finally, IroRE^5 (2.8 kb) drove expression mainly in the prospective alula and peripodial membrane (Fig. 1G).

The identified REs also drove expression of *lacZ* in other imaginal discs and in the embryo, in patterns resembling that of the Iro-C genes (see Figs S2, S3 in the supplementary material). We did not identify any RE driving expression in the L1, L3 or L5 veins.

EGFR and Dpp signalling regulate transcription mediated by IroRE^2

In the proximal region of the wing disc, activation of EGFR by its ligand *vein* leads to the expression of the Iro-C genes (Wang et al., 2000; Zecca and Struhl, 2002b). We thus examined whether EGFR signalling similarly regulates expression driven by IroRE^2, by monitoring β-gal accumulation in clones of cells with altered EGFR signalling. Excess of signalling conditions (*UAS-Ras*^{V12} or *UAS-Raf*^{Act} overexpression clones), promoted upregulation of *lacZ* expression, similar to that observed with the endogenous *ara/caup* genes and with similar topological restrictions (Fig. 2A-B′) (Zecca and Struhl, 2002b). Namely, in clones induced at the second instar, *lacZ* activation occurred at the hinge and pleura domains (Fig. 2A,A′, arrows) but not at the central wing pouch or proximal notum (Fig. 2A,A′, arrowheads and not shown). In later induced clones, *lacZ* expression occurred within the wing pouch, albeit at lower levels than at the hinge (Fig. 2B,B′, insets), and in the anterior region of the medial notum (Fig. 2B,B′, arrow). Conversely, *lacZ* expression was eliminated in *UAS-Raf*^{DN} overexpressing clones (Fig. 2C,C′). As described for the endogenous *ara/caup* genes, overexpression of *vein* had no effect (Zecca and Struhl, 2002a). We conclude that EGFR signalling positively controls IroRE^2.

Dpp signalling represses Iro-C expression (Cavodeassi et al., 2002). Thus, we next investigated the ability of this pathway to modulate the expression of IroRE^2-*lacZ*. Inactivation of the pathway (*tkv*^{a12} clones) caused strong ectopic expression of *lacZ* in the proximal notum (Fig. 3A,A′) and a weaker one at the hinge (Fig. 3C,C′). Furthermore, *lacZ* expression was upregulated in the extant domain of IroRE^2 (Fig. 3B,B′). Conversely, IroRE^2-*lacZ* was repressed when the Dpp pathway was overactivated in *UAS-tkv*^{QD} (a constitutively activated form of Tkv) (Nellen et al., 1996) or *UAS-Mad* overexpressing clones (Fig. 3D,D′ and not shown). Thus, Dpp signalling negatively regulated IroRE^2. Taken together the above results indicate that the EGFR and Dpp pathways regulate *ara/caup* expression in the notum mainly through IroRE^2.

IroRE^2 contains putative binding sites for effectors of the EGFR and Dpp signalling pathways

We next whished to identify the sites of IroRE^2 that mediate its response to the EGFR and Dpp pathways. We reduced the IroRE^2 to a 1.6 kb subfragment (IroRE$^{2\text{-}B}$; Fig. 4B), which maintained enhancer activity in the notum (Fig. 4C) and was activated by EGFR

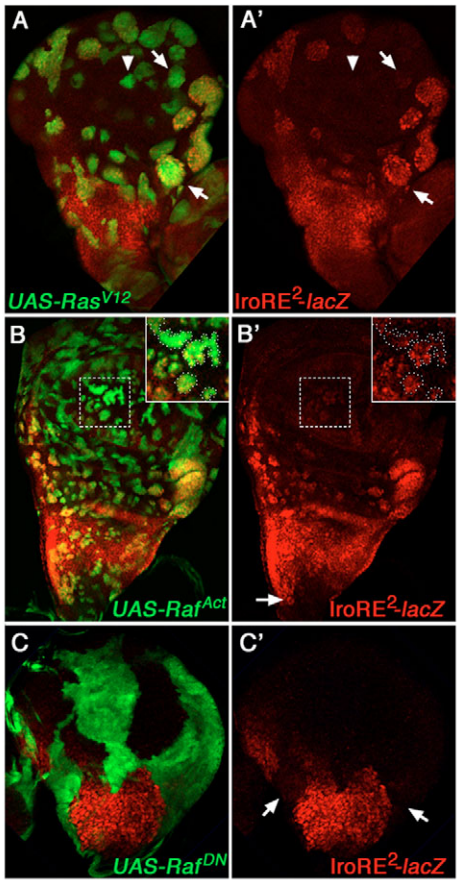

Fig. 2. EGFR signalling activates IroRE^2-*lacZ*. (**A-B′**) Expression of *lacZ* (red) is activated in clones of cells (green) expressing activated forms of Ras (A,A′) or Raf (B,B′) (arrows) except in the wing pouch (arrowheads). Insets in B,B′ show enlarged and enhanced pictures of the central wing pouch. Clones were induced at 48-72 (A,A′) or 72-96 hours AEL (B,B′). (**C**,**C′**) *lacZ* expression is lost (arrows) in clones of cells expressing a dominant negative form of Raf (green, induced at 24-48 hours AEL).

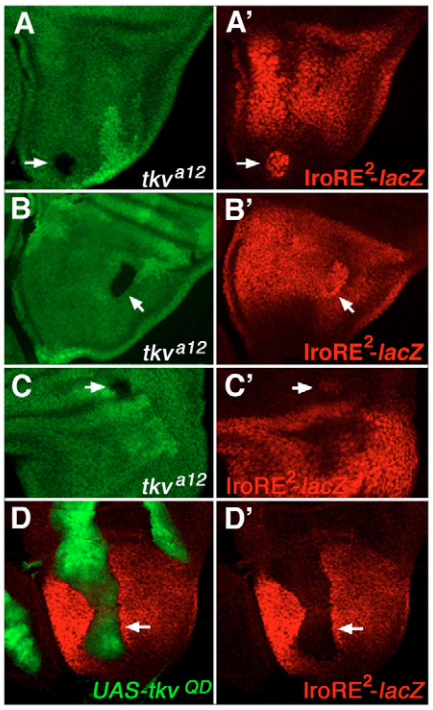

Fig. 3. Dpp signalling represses IroRE^2-*lacZ*. (**A-C′**) *tkv^{a12}* mutant cells (absence of green), upregulate IroRE^2-*lacZ* (red, arrows) in the prospective medial notum (A,A′), lateral notum (B,B′) and hinge (C,C′). (**D**,**D′**) Overexpression of *UAS-tkv^{QD}* in clones of cells (green) abolishes expression of IroRE^2-*lacZ* (arrows).

and repressed by Dpp signalling (not shown). Next, we examined its sequence for putative binding sites of effectors of these pathways using Flyenhancer (Markstein and Levine, 2002) and cis-analyst (Berman et al., 2002) programs.

Regulation of gene expression by the EGFR pathway is mediated by Pointed (Pnt; an ETS activator transcription factor) and/or by removal of repression by the ETS factor Yan and the zinc-finger factor Tramtrack (Ttk) (reviewed in Rebay, 2002). As shown in Fig. 4B (and see Fig. S4 in the supplementary material), IroRE$^{2\text{-B}}$ contains nine putative ETS binding sites (Xu et al., 2000) and five putative Ttk binding sites (Brown and Wu, 1993; Read et al., 1990). With respect to the Dpp pathway, heteroliogomers of Mad and Medea act as intracellular signal transducers (Affolter et al., 2001). In accordance with its Dpp-dependent regulation, we identified a putative binding site for Mad (Kim et al., 1997) within IroRE$^{2\text{-B}}$ (Fig. 4B; see Fig. S4 in the supplementary material).

Previous genetic evidence has shown repression of *ara/caup* expression in the prospective medial notum by the GATA transcription factor Pannier (Pnr) (Calleja et al., 2000), which is a target of Dpp signalling in the wing disc (Sato and Saigo, 2000; Tomoyasu et al., 2000). Consistent with a direct regulation, in IroRE$^{2\text{-B}}$ we also found nine putative GATA binding sites (Haenlin et al., 1997; Martin and Orkin, 1990) (Fig. 4B; see Fig. S4 in the supplementary material).

A high proportion of the putative binding sites found in our analysis were located within conserved regions between *D. melanogaster*, *D. pseudoobscura*, *D. virilis* and *D. mojavensis* as shown by comparison with the VISTA program (Couronne et al., 2003) (Fig. 4A; see Fig. S4 in the supplementary material).

Pnt is required for Iro-C expression

We identified putative binding sites for Pnt, Yan and Ttk in the IroRE$^{2\text{-B}}$ sequence. Accordingly, we investigated the function of these transcription factors in the regulation of IroRE^2-*lacZ* and *ara/caup* genes.

We first examined the function of *pnt*. Gain-of-function *pnt* clones (induced at 72-96 hours AEL) caused ectopic accumulation of β-gal in the wing pouch, wing hinge and proximal notum and enhanced accumulation (above the extant levels) at the lateral notum (Fig. 5A,A′, arrowheads). (The central hinge and wing pouch were somehow refractory to such activation when clones were induced at 48-72 hours AEL; not shown.) Consistently, loss of function of *pnt* (*pnt$^{\Delta 88}$* clones) reduced or totally removed *lacZ* expression driven by IroRE^2, especially in the central and posterior regions of the lateral notum (Fig. 5B,B′). Moreover, expression of *ara/caup* was similarly abolished in *pnt* clones (Fig. 5C,C′, arrowhead).

The expression of IroRE^2-*lacZ* and *ara/caup* genes was unaffected either in cells devoid of Yan (*yan^{443}* or *yan^{884}* clones) or Ttk (*ttk^{1e11}* clones) (not shown). This suggested that Yan and Ttk do

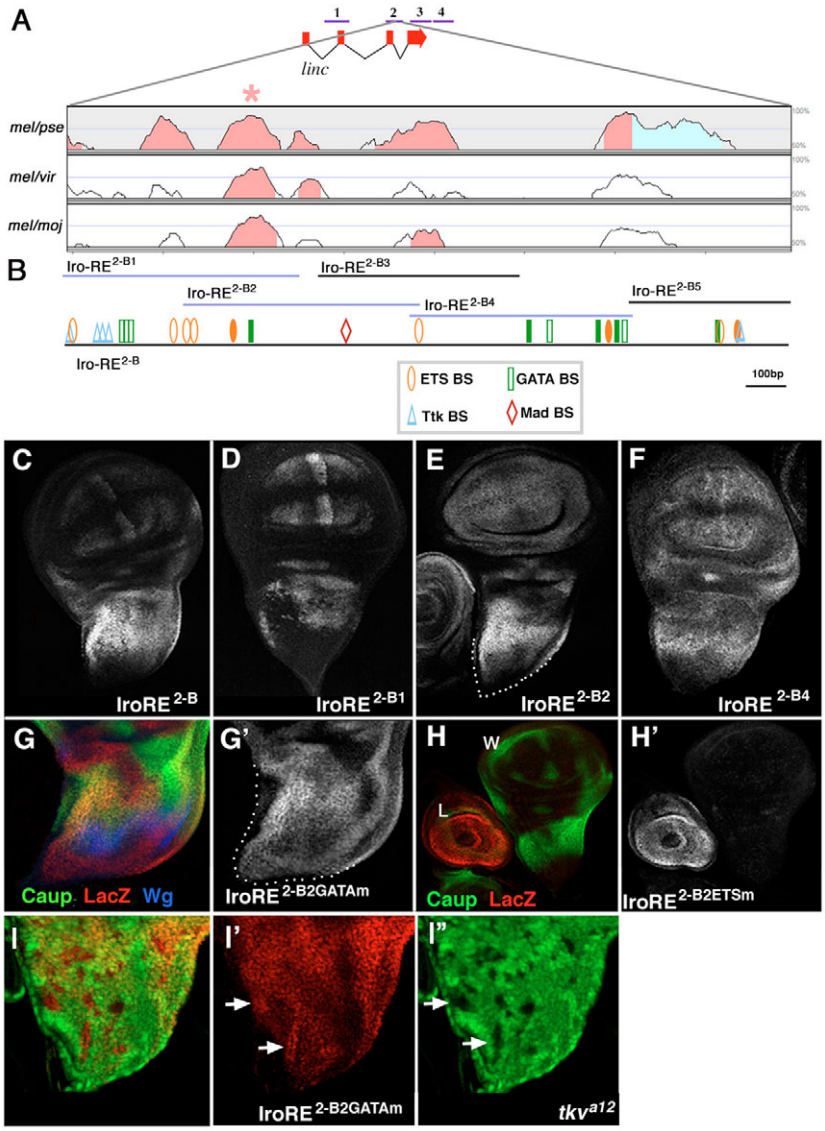

Fig. 4. Delimiting a minimal notum enhancer. (**A**) VISTA plot comparing the *D. melanogaster* IroRE$^{2\text{-}B}$ sequence with those of *D. pseudoobscura*, *D. virilis* and *D. mojavensis* (window size: 100 bp). Pink peaks represent regions with more than 70% of identity (the most conserved one is marked by an asterisk). The light blue peak indicates the putative *linc* coding region. (**B**) Subfragments of IroRE$^{2\text{-}B}$ tested for transcription enhancing activity. Only the blue regions activate transcription. Putative binding sites for ETS proteins, Ttk, GATA proteins and Mad are indicated. Filled symbols, binding sites matching the consensus sequence; open symbols, one or two mismatches compared to the consensus sequence. (**C-F**) *lacZ* expressions mediated by the indicated fragments. Note in C that IroRE^{2B} drives a weak expression in the prospective intervein regions, suggesting that shortening has removed some wing-pouch repressor elements. (**G**,**G′**) Expression mediated by IroRE$^{2\text{-}B2GATAm}$ extends into the prospective medial notum, proximal to the *wg* (blue) and *ara/caup* (green) domains. The dotted lines in E and G′ outline the proximal region of the discs. (**H**,**H′**) Mutation of the ETS binding site of IroRE$^{2\text{-}B2}$ that most closely matches the consensus strongly reduces enhancer activity in the wing disc. (**I-I″**) Up-regulation of IroRE$^{2\text{-}B2GATAm}$-*lacZ* expression in *tkv*^{a12} clones, some of which are indicated by arrows.

not regulate IroRE^{2}-*lacZ* or *ara/caup* expression. Thus, Pnt appears to be the main effector of the EGFR pathway in the regulation of Iro-C through IroRE^{2}.

Regulation of Iro-C expression by the Dpp pathway

Pnr is a GATA DNA binding protein that acts on its own as a transcriptional activator but switches to repressor when in a complex with the Friend of GATA (FOG) protein U-shaped (Ush) (Cubadda et al., 1997; Haenlin et al., 1997; Ramain et al., 1993). Since Pnr represses *ara/caup* in the proximal region of third instar wing discs (Calleja et al., 2000), we assayed the effect of *pnr* or *ush* loss-of-function on the transcription of IroRE^{2}-*lacZ*. *pnr*^{VX6} or *ush*^{VX22} mutant clones, located at the most proximal region of the disc, exhibited ectopic *lacZ* expression (Fig. 6A,A′ and Fig. 7A,A′, arrows), indicating a repressor function of these proteins on transcription mediated by IroRE^{2}. Similarly, lack of *pnr* or *ush* caused ectopic *ara/caup* expression (Fig. 7A,A″, arrows) (Calleja et al., 2000). Consistent with a repressor role for Pnr/Ush, *UAS-ush* was able to repress expression of IroRE^{2}-*lacZ* and *ara/caup* in the proximal most domain of the lateral notum (Fig. 7B-C′, arrows), most likely through the formation of Pnr/Ush heterodimers.

Surprisingly, the effect of lack of *pnr* on transcription mediated by IroRE^{2} differed along the proximodistal axis of the disc. Thus, β-gal was absent from *pnr*^{VX6} clones situated in a region marked by *wg* expression (Fig. 6B,B′, arrows). Since Pnr activates expression of *wg* whereas the heterodimer Pnr/Ush represses it (Calleja et al., 1996; Sato and Saigo, 2000; Tomoyasu et al., 2000), expression of *wg* reveals cells containing Pnr but devoid of Ush. These data suggested that Pnr activates expression of IroRE^{2}-*lacZ* in the absence of Ush and, indeed, activation of this reporter gene was observed in *pnr* overexpression clones (Fig. 6C,C′). On the contrary, overexpression of *pnr* inhibited *ara/caup* expression in the notum and did not ectopically activate it (not shown). Thus, regulation of *ara/caup* and IroRE^{2}-*lacZ* by Pnr appears to be slightly different, most likely due to the fact that additional regulatory elements, other than the IroRE^{2}, cooperate to establish the pattern of the endogenous *ara/caup* genes (see Discussion).

Considering that the expression of *pnr* and *ush* in partially overlapping domains in the medial region of the wing disc depends on Dpp activity (Sato and Saigo, 2000; Tomoyasu et al., 2000), the above results suggest that Pnr/Ush dimers may mediate Iro-C repression by the Dpp pathway at the medial notum through IroRE^{2}. However, these observations do not rule out a possible direct repression of IroRE^{2}-

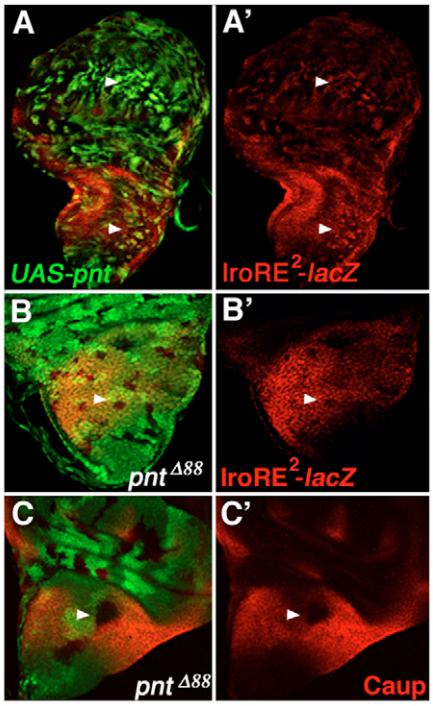

Fig. 5. Pnt activates IroRE^2-*lacZ* and *ara/caup* expression. (**A**,**A′**) Overexpression of *pnt* in clones of cells (green) activates *lacZ* (red, arrowheads). (**B**,**B′**) In *pnt*$^{\Delta 88}$ null clones (absence of green) IroRE^2-*lacZ* is downregulated (red, arrowhead). (**C**,**C′**) *ara/caup* expression (red) is similarly lost in *pnt*$^{\Delta 88}$clones (arrowhead).

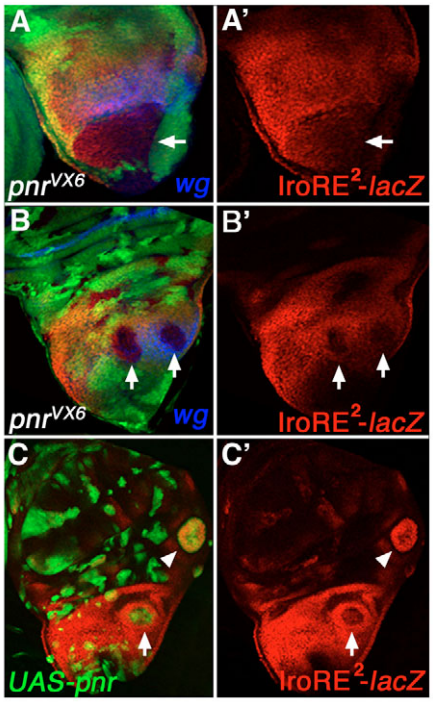

Fig. 6. Regulation of IroRE^2-*lacZ* by Pnr. (**A-B′**) *pnr*^{VX6} mutant cells (absence of green) show ectopic expression of IroRE^2-*lacZ* (red) when located in the medial notum (arrows in A,A′) but abolish expression in the *wg* notal domain (shown in blue, arrows in B,B′). In this region, expression of *pnr* (visualized in *pnrGal4/UAS-GFP* larvae) overlaps with that of IroRE^2-*lacZ* (not shown). (**C**,**C′**) Overexpression of *UAS-pnr* (green), activates IroRE^2-*lacZ* at the hinge and alula domains (arrowhead) and alter the disc epithelium of the prospective lateral notum forming a fold around some clones (arrows). *lacZ* ectopic activation was stronger at the hinge, alula and pleura than at the wing pouch, consistent with a weak activation of *ush* by overexpressed *pnr* at this domain (A.L. and S.C., unpublished).

lacZ by Mad binding to its putative binding sites present in this RE. To address this point, we made use of the MARCM technique (Lee and Luo, 1999) to overexpress *UAS-tkv*^{QD} in *ush*^{VX22} clones. We reasoned that if the repressor effect of *UAS-tkv*^{QD} was solely due to activation of *pnr* and *ush* (Sato and Saigo, 2000) and formation of the Pnr/Ush repressor heterodimer, repression should not occur in the absence of Ush. However, absence of *ush* did not relieve *lacZ* repression by the Dpp pathway (Fig. 7D). Reduced expression of IroRE^2-*lacZ* was also found in UAS-Mad, *ush*^{VX22} clones (not shown). These results suggest a role for Mad, in addition to Pnr/Ush, in Dpp-dependent repression through IroRE^2 in the medial notum (see below).

We also found that expression of IroRE^2-*lacZ* was upregulated in *tkv*^{a12} clones located at the wing hinge (Fig. 3C,C′). Interestingly, *ush* and the GATA family protein Grain (Grn) are expressed at the hinge region abutting the Iro-C expression domain (Brown and Castelli-Gair Hombria, 2000; Haenlin et al., 1997). This opened up the possibility that an Ush/Grn dimer might mediate the Dpp-dependent Iro-C repression at the hinge. However, there was no upregulation of *ara/caup* or IroRE^2-*lacZ* expression in *ush* or *grn* mutant clones at the hinge region (Fig. 7A-A″, arrowheads, and not shown), suggesting that this was not the case. Accordingly, elimination of Mad-dependent repression could account for the enhanced expression of *lacZ* found in *tkv*^{a12} clones in the wing hinge. A similar situation may apply to the lateral notum (Fig. 3B,B′), a region devoid of Pnr and Ush, where Mad could be the Dpp pathway effector.

Definition of a minimal notum enhancer

IroRE$^{2\text{-}B}$ contains putative binding sites for effectors of the EGFR and Dpp pathways. To determine the *in vivo* functionality of these binding sites, we first set to define the minimal functional length of this RE by assaying the enhancer potential of five overlapping 500 bp subfragments. Three of them (IroRE$^{2\text{-}B1}$, IroRE$^{2\text{-}B2}$ and IroRE$^{2\text{-}B4}$) drove *lacZ* expression in the wing disc in overlapping regions contained within the Iro-expressing domain but also outside of it (Fig. 4D-F). Interestingly, putative ETS and GATA binding sites are not scattered within the IroRE$^{2\text{-}B}$ sequence but appear clustered within these subfragments (Fig. 4B). Clustering of binding sites might contribute to synergistic regulation by the transcription factors (Arnosti et al., 1996). Removal of the 200 initial bp of the IroRE$^{2\text{-}B}$, which harbours several putative binding sites, did not affect its regulatory potential (not shown), indicating the dispensability of these sites. Furthermore, a *lacZ* construct containing exclusively the 200 initial bp of the IroRE$^{2\text{-}B}$ was not expressed at the wing disc (not shown). In sum, these data pointed to the 240 bp sequence shared by IroRE$^{2\text{-}B1}$ and IroRE$^{2\text{-}B2}$ and the region covered by IroRE$^{2\text{-}B4}$ as the most relevant for the control of *lacZ* expression. Note that the former region harbours a highly conserved sequence among the *Drosophila* species analyzed (Fig. 4A, asterisk) and putative ETS and GATA binding sites (Fig. 4B).

Neither the IroRE$^{2\text{-}B2}$ fragment, which contains a Mad binding site, nor the IroRE$^{2\text{-}B1}$ and IroRE$^{2\text{-}B4}$ fragment, devoid of such a site, drove expression in the prospective medial notum (Fig. 4D-F). Since the three fragments, on the contrary, contain GATA binding sites, this suggests that the GATA protein Pnr should be the main repressor

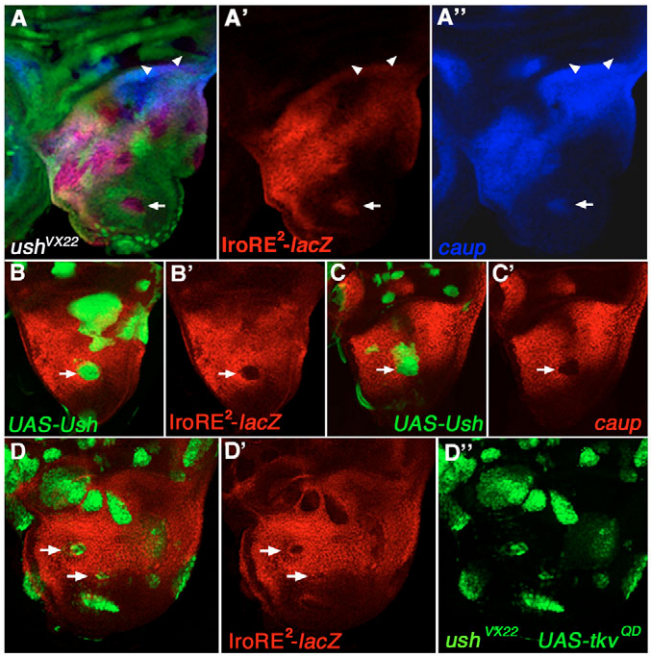

Fig. 7. IroRE^{2} mediates repression of *ara/caup* by Ush. (**A-A″**) In *ush*^{VX22} null clones (absence of green) IroRE^{2}-*lacZ* (red) and *ara/caup* (blue) (arrows) are derepressed. Loss of *ush* at the hinge has no effect (arrowheads). (**B-C′**) Overexpression of *ush* in clones (green) downregulates IroRE^{2}-*lacZ* (B,B′) and *ara/caup* (C,C′) expression at the proximal-most lateral notum (arrows). (**D-D″**) Downregulation of IroRE^{2}-*lacZ* (red) by overexpression of *UAS-tkv*^{QD} is still evident in clones of *ush*^{VX22} mutant cells (green) (arrows).

of Iro-C at the medial notum through the IroRE$^{2\text{-}B}$. A mutation was created in the unique GATA site of IroRE$^{2\text{-}B2}$ to assess the relative contribution of Pnr to the establishment of the proximal border of Iro-C expression. The resulting IroRE$^{2\text{-}B2GATAm}$-*lacZ* construct showed expansion of *lacZ* expression into the medial notum (Fig. 4G,G′), thus stressing the main contribution of GATA proteins. The potential contribution of Mad to IroRE$^{2\text{-}B2}$-*lacZ* regulation was assayed in *tkv*^{a12} clones generated in flies harbouring the IroRE$^{2\text{-}B2GATAm}$-*lacZ* construct. Interestingly, further upregulation of *lacZ* expression was found in the clones (Fig. 4I-I″). These results, in agreement with those of Fig. 7D-D″, indicate that Mad, in addition to Pnr/Ush, should contribute to Dpp-dependent repression in the medial notum.

It is noteworthy that removal of the ETS binding site of IroRE$^{2\text{-}B2}$ that most closely matches the consensus strongly reduced *lacZ* expression in the wing disc, although it did not affect leg and eye disc expression (Fig. 4H,H′ and not shown). This indicates that EGFR signalling activates IroRE$^{2\text{-}B2}$-mediated transcription in the wing disc by using the conserved Pnt binding site.

EGFR and Dpp signalling pathways activate transcription mediated by IroRE^{1}

Although the Dpp pathway represses Iro-C expression (Cavodeassi et al., 2002) and, that mediated by IroRE^{2}, this does not prevent the spatial coincidence, in the lateral notum of third instar discs, of Iro-C proteins and elevated levels of Dpp activity (Fig. 9C). Moreover, the overexpression of *UAS-tkv*^{QD} is unable to repress Iro-C expression in a domain of the lateral notum near the Dpp source (Cavodeassi et al., 2002). Interestingly, IroRE^{1} drives *lacZ* expression in that domain (Fig. 1C,C′). Thus, we examined the effect of the Dpp pathway on IroRE^{1}-*lacZ* regulation. Overexpression of *UAS-tkv*^{QD} did not repress *lacZ* expression but instead activated it, although exclusively in a domain restricted to the lateral notum (Fig. 8A,A′, arrows). Accordingly, loss of Dpp signalling (*tkv*^{a12} clones) significantly reduced β-gal accumulation (Fig. 8B,B′, arrows). IroRE^{1}-mediated transcription was also activated by the EGFR pathway, as shown by ectopic *lacZ* expression in *UAS-Raf*^{Act} clones (Fig. 8C,C′, arrows) and its converse reduction in *UAS-Raf*^{DN} clones (Fig. 8D,D′, arrow). Thus, Iro-RE^{1} may be instrumental in allowing expression of Iro-C genes in the posterior lateral notum despite the repressor effect of the Dpp pathway through other regulatory elements.

DISCUSSION

Antagonistic relationship between Dpp and EGFR signalling in the specification and patterning of the notum

A common theme in development is the convergence of different signalling pathways to implement a given developmental program. For instance during embryonic development, the antagonistic activity of the EGFR and Dpp pathways sets the limits between the neuroectoderm and the dorsal ectoderm (von Ohlen and Doe, 2000). A similar situation applies to the specification of prospective body regions within the wing imaginal disc. Here, during the early second instar, EGFR and Dpp pathways act antagonistically on the regulation of the Iro-C (Cavodeassi et al., 2002; Wang et al., 2000; Zecca and Struhl, 2002b) restricting its expression to the prospective notum region where it specifies notum development rather than hinge (Diez del Corral et al., 1999). Later, at the early third instar, again the concomitant activity of EGFR and Dpp signals (the latter now also emanating form the most proximal region of the wing disc) partition the prospective notum into two different subdomains, the medial and the lateral notum, the latter being specified by *ara/caup* expression (Cavodeassi et al., 2002; Diez del Corral et al., 1999). Thus, to understand how regionalization of the adult fly body is achieved it is important to elucidate the mechanisms responsible for the joint interpretation of both signalling pathways.

The opposing effects of the EGFR and Dpp pathways on Iro-C expression may have resulted from direct cross talk between these pathways. For instance, both in vertebrates and invertebrates the ability of EGFR to antagonize BMP signalling by phosphorylation and inhibition of Smad proteins has been reported (Kretzschmar et al., 1997; Kubota et al., 2000). Although not previously described, Dpp signalling may similarly interfere with the activity of the EGFR pathway. However, this appears not to be the case in the wing disc since expression of *argos*, a readout of the EGFR pathway (Rebay, 2002) can be detected in the proximal-most notum of the wing disc (not shown), a region with high levels of Dpp signal. Furthermore, overexpression of activated *tkv* does not repress expression of *kekkon*, another EGFR downstream gene (Rebay, 2002) (our unpublished results).

Here we show that the opposing effects of the EGFR and Dpp pathways on Iro-C expression result from the convergence of both pathways on at least two distinct Iro-C regulatory elements, IroRE^{1} and IroRE^{2}. These two REs drive gene expression in two complementary domains of the prospective notum region of the wing disc, and appear to mediate most of the regulation of the Iro-C genes by the Dpp and EGFR pathways in this region of the wing disc. Furthermore, IroRE^{1} provides a regulatory mechanism for the

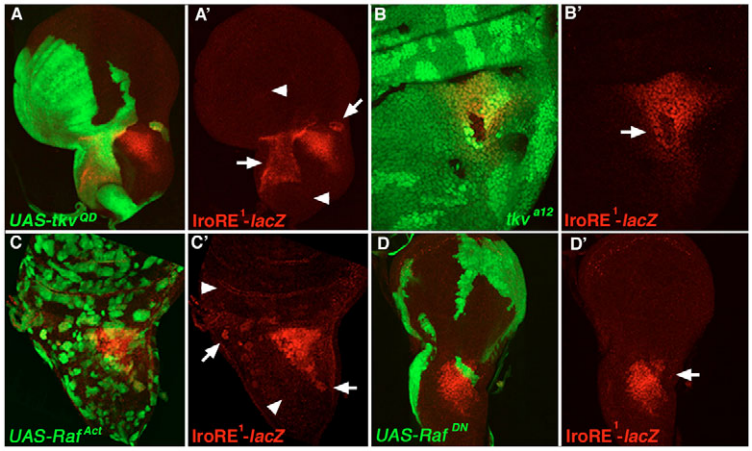

Fig. 8. Dpp and EGFR pathways activate IroRE^1-*lacZ* expression. Cells expressing activated forms of Tkv (**A**,**A′**) or Raf (**C**,**C′**) (green) activate IroRE^1-*lacZ* (red) at the prospective lateral notum (arrows) but not at the wing hinge, wing pouch or proximal notum (arrowheads). *lacZ* expression (red) strongly decreased (arrows) in *tkv*^{a12} (**B**,**B′**) or *UAS-Raf*^{DN} (**D**,**D′**) clones.

coexistence at the prospective lateral notum of Iro-C expression and Dpp pathway activity, notwithstanding the negative regulation of Iro-C by such pathway (Fig. 9).

Identification of notum-specific regulatory elements

The transcriptional regulation of the Iro-C genes is modular. Thus, the non-coding Iro-C genomic DNA contains a series of five separate enhancers that control the expression of a reporter gene in sub-domains within the realm of Iro-C expression in the prospective notum region of the wing disc. None of the identified fragments reproduces on its own the entire pattern of expression of Iro-C in the prospective notum. However, IroRE^1 and IroRE^2 promote expression in complementary domains that entirely cover the territory of the presumptive lateral notum. Furthermore, IroRE^2-mediated transcription recapitulates expression of Iro-C at the whole prospective notum at the second larval instar. We hypothesize that the combined activity of both REs would be responsible for a great part of the regulation of Iro-C expression in the notum territory. Moreover, although IroRE^3, IroRE^4 and IroRE^5 mediate *lacZ* expression in patterns only partly related to that of the Iro-C genes, these REs probably contribute to the complex regulation of the Iro-C. In addition, we cannot exclude the possibility of other RE(s) located outside the tested region that would help to establish the final pattern of Iro-C expression. Indeed, *Iro*^{DFM3}, a deficiency obtained by imprecise excision of the *iro*^{rF209} P-*lacZ* element that extends up to the *mirr* promoter (Gomez-Skarmeta et al., 1996), maintains some *lacZ* expression in part of the central notum (not shown).

The identified REs might act simultaneously on *ara* and *caup* expression to give rise to their almost coincident patterns of expression. As previously proposed (R. Diez del Corral, PhD thesis, Universidad Autónoma de Madrid, Spain, 1998), such coincidence cannot be attributed to cross-regulation between *ara* and *caup* since in *iro*^{rF209} mutant discs (*iro*^{rF209} is an *ara* null allele, Fig. 1B) expression of *caup* is unmodified. Regulation of *ara/caup* would be, accordingly, similar to that of the *achaete-scute* genes of the AS-C, which show identical patterns of expression due to the use of shared enhancers (reviewed in Modolell and Campuzano, 1998). Expression of the vertebrate *Iroquois* (Irx) genes appears to be similarly regulated. Thus, the analysis of the regulatory potential of highly and ultra conserved non-coding regions present in the intergenic regions of the Irx clusters suggests these genes to be regulated by partially redundant enhancers shared by the components of each cluster (de la Calle-Mustienes et al., 2005).

Expression of *mirr* in the notum region of the wing disc largely coincides with that of *ara/caup* and most likely is under the control of the same REs. Thus, activity of the IroRE^2 may account for the unmodified expression of *mirr* in *iro*^{1} imaginal discs (associated with an inversion breakpoint located within the *caup* transcription unit; Fig. 1B) (R. Diez del Corral, PhD thesis, Universidad Autónoma de Madrid, Spain, 1998). In addition, differences in the expression of *ara/caup* and *mirr* might be due to the presence of repressor RE(s) or insulator sequences (Gerasimova and Corces, 1996) that would prevent the action of the RE(s) controlling *ara/caup* on the *mirr* promoter. This is consistent with the previous observation of ectopic expression of *mirr* in *Mob1* mutants, a regulatory mutation mapped within the Iro-C (Kehl et al., 1998).

The Iro-C regulatory elements and the integration of developmental signals

Our identification of REs present in the Iro-C has allowed us to the unveil some of the molecular mechanisms of its transcriptional regulation at the level of DNA-protein interaction and to analyse the interplay of positive and negative inputs from convergent signalling pathways.

EGFR activation in the proximal region of the wing disc leads to expression of Iro-C (Wang et al., 2000; Zecca and Struhl, 2002a; Zecca and Struhl, 2002b). Here we demonstrate that both IroRE^1 and IroRE^2 mediate positive regulation by the EGFR pathway (Fig. 9A,B). We show that Pnt mediates activation of IroRE^2-*lacZ* by the EGFR pathway. Furthermore, as previously shown for the Iro-C genes (Zecca and Struhl, 2002a; Zecca and Struhl, 2002b), EGFR-dependent activation is cell context dependent. This suggests the existence, in the cells receiving EGFR signalling, of presently unknown factors that would contribute to *ara/caup* activation and/or the presence of counteracting repressing mechanisms, which should prevent their activation. Clearly, the Dpp pathway is so far the best candidate, since it has been shown that it can repress Iro-C (Cavodeassi et al., 2002) and the IroRE^2-*lacZ* transgene (this work).

The molecular mechanism of Dpp-dependent regulation of Iro-C expression appears to be more complex. The Dpp pathway can repress or activate Iro-C through different REs and different effector proteins. IroRE^2 appears to mediate Dpp-dependent repression at the medial notum (most probably through direct binding of the heterodimer Pnr/Ush and Mad) and at the hinge and lateral notum (independently of Pnr, Ush and the GATA factor Grn in these domains). Dpp-dependent repression of Iro-C may be mediated, in addition, through a different RE, namely, through a *brk* silencer

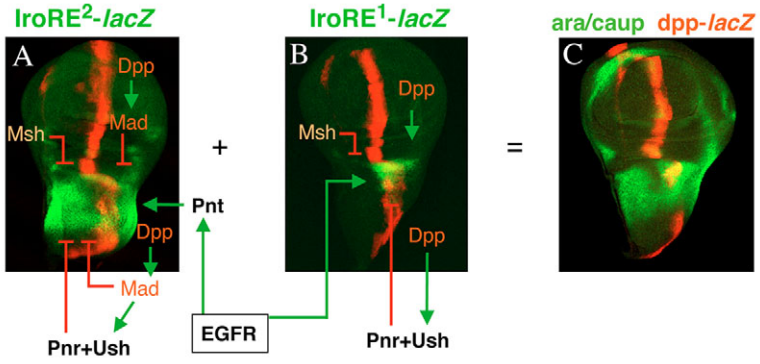

Fig. 9. Proposed regulatory mechanism that controls the expression of *ara/caup* in the prospective notum. Regulation of the activity of Iro-RE^2 (**A**) and Iro-RE^1 (**B**) by the EGFR and Dpp pathways is shown (green arrows, positive regulatory interactions; red T-shaped bars, negative regulatory interactions). Downstream effectors of the pathways are indicated. (**C**) Addition of the actions of both REs largely explains the expression of *ara/caup* in the prospective notum region of the third instar wing disc. Dpp expression was monitored with *dpp-Gal4, UAS-GFP* (A,B) and *dpp-lacZ* (C).

element (brkSE), shown to mediate Dpp-dependent repression of *brk* by binding of a Medea/Mad/Schnurri repressor complex (Pyrowolakis et al., 2004), which is present at the Iro-C within IroRE^5 (A.L. and S.C., unpublished observations).

Despite the Dpp-mediated repression through IroRE^2, a high level of Iro-C proteins accumulates in the lateral region of the notum, near the strong source of Dpp at the AP border (Fig. 9C). Furthermore, in this region of the wing disc Iro-C expression is refractory to Dpp-dependent repression (Cavodeassi et al., 2002). It is noteworthy that, IroRE^1 mediates *lacZ* expression exclusively in that region of the wing disc and it appears to provide a regulatory mechanism for the co-existence of Iro-C expression and Dpp pathway activity, since the Dpp pathway does not repress but, on the contrary, activates IroRE^1-mediated *lacZ* expression (Fig. 9B). Activation is restricted to the lateral notum, most likely because of the presence, in the hinge and medial notum territories, of repressors [Muscle segment homeobox, Msh; also known as Drop (Villa-Cuesta and Modolell, 2005) and Pnr/Ush, respectively] that would counteract activation. Putative binding sites for both Msh [consensus sequence G/C TTAATTG (Kuzuoka et al., 1994)] and GATA proteins are indeed present in IroRE^1 (not shown). Thus, IroRE^1 and IroRE^2 represent two different REs in the same gene that respond in opposite ways to the same positional information, i.e. Dpp signalling. A similar complex level of regulation has been found in the case of segmentation genes such as *even skipped* (*eve*) where the same transcription factor (Hunchback) is able to activate or repress transcription mediated through different *eve* position-specific enhancers (Small et al., 1992; Small et al., 1996).

In addition a Dpp-independent mechanism based in the mutual repression between Iro-C and the homeoprotein Msh helps to maintain the distal border of Iro-C expression (Villa-Cuesta and Modolell, 2005). This repression could be mediated by direct binding of Msh to one putative Msh binding site present in the Iro-RE$^{2\text{-}B}$ sequence.

Further analysis of IroRE^3, IroRE^5, other putative RE(s) located outside of the tested region and putative repressor RE(s) that could not have been identified in our study, would help to establish the final pattern of Iro-C expression, which is essential for the specification of the notum and its subsequent patterning.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/7/1337/DC1

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