

REVIEW ARTICLE

The Iroquois family of genes: from body building to neural patterning

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SUMMARY

The Iroquois (Iro) family of genes are found in nematodes, insects and vertebrates. They usually occur in one or two genomic clusters of three genes each and encode transcriptional controllers that possess a characteristic homeodomain. The Iro genes function early in development to specify the identity of diverse territories of the body, such as the dorsal head and dorsal mesothorax of *Drosophila* and the neural plate of *Xenopus*. In some aspects they act

in the same way as classical selector genes, but they display specific properties that place them into a category of their own. Later in development in both *Drosophila* and vertebrates, the Iro genes function again to subdivide those territories into smaller domains.

Key words: Iroquois genes, Patterning, Neural specification, *Drosophila*, Vertebrate

INTRODUCTION

The Iroquois (Iro) genes were discovered in *Drosophila* in the course of a mutagenesis designed to identify genes that affected the patterning of external sensory organs, essentially bristles and other types of sensilla (Dambly-Chaudière and Leyns, 1992; Leyns et al., 1996). The first mutant allele recovered (*iro¹*; Fig. 1) suppressed all the lateral bristles of the dorsal mesothorax (notum) of the fly, leaving only a wide band of both large and small bristles in the central region of the notum. This pattern resembled the hairstyle of the Iroquois American Indians (also known as Mohawk) – hence the name of the locus. It was further shown that the suppression of bristles was due to the failure of the proneural genes of the *achaete-scute* (*ac-sc*) complex (AS-C) to be expressed in the imaginal disc epithelium that gave rise to the lateral notum. As a consequence, the sensory organ precursor cells (SMCs) were not formed. These results suggested that the Iro locus might encode a factor(s) that allowed the expression of AS-C in the presumptive lateral notum.

The molecular characterization of the Iro genes in *Drosophila* (Gómez-Skarmeta et al., 1996; McNeill et al., 1997) allowed the identification of homologs in *C. elegans* and several vertebrates, namely, *Xenopus*, mouse, zebrafish and chick (Bao et al., 1999; Bellefroid et al., 1998; Bosse et al., 2000; Bosse et al., 1997; Christoffels et al., 2000; Cohen et al., 2000; Funayama et al., 1999; Gómez-Skarmeta et al., 1998; Goriely et al., 1999; Peters et al., 2000; Tan et al., 1999). At the level of their products, the strong similarity of the homeodomains and the universal presence of a characteristic motif possibly involved in protein-protein interactions (the Iro box, similar to the central part of the EGF repeats of the Notch

receptor protein) indicated that the Iro products constituted a new family of homeodomain proteins within the TALE class (Bürglin, 1997; Fig. 1). In addition, the genomic organization of the Iro genes is also apparently conserved (Fig. 1). Although only part of their functions in *Drosophila*, *Xenopus*, chick and mouse have been discovered, the emerging view is that the Iro genes, in both the fly and vertebrates, are required at early stages of development to define large territories. Examples are the dorsal regions of the eye, head and mesothorax of *Drosophila*, and the neural ectoderm of *Xenopus* (Fig. 2). Later in development, the Iro genes function again to subdivide these territories into smaller domains. As expected, these early and late functions correlate with the patterns of expression of the genes; they are initially expressed in large domains and are later restricted to subdomains of these large territories. In addition, new domains of expression appear late in development and are required for additional patterning activities. This article outlines present understanding about the function of the Iro genes, and discusses the mechanisms that regulate them during development and some of the properties of Iro-expressing cells. To our knowledge, this is the first specific review of this group of genes (see Mann and Morata, 2000; McNeill, 2000, for related topics).

GENOMIC ORGANIZATION OF THE Iro GENES

Drosophila has three Iro genes. Together they form the Iroquois complex (Iro-C) that spans approx. 130 kb of DNA (Netter et al., 1998) (Fig. 1). The individual genes have been named *araucan* (*ara*), *caupolican* (*caup*) and *mirror* (*mirr*); Gómez-Skarmeta et al., 1996; McNeill et al., 1997). The Ara

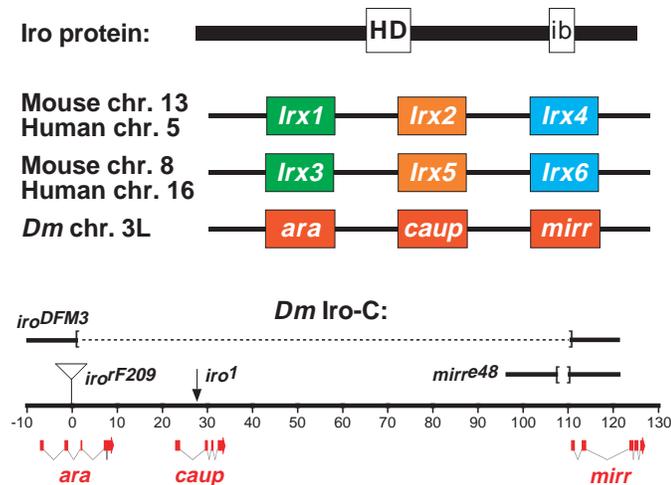
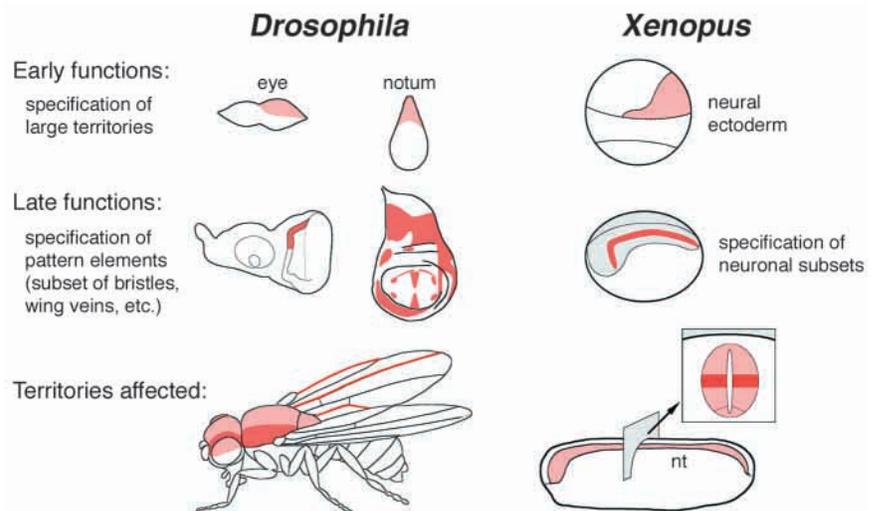


Fig. 1. Iro proteins and genomic organization of the Iro genes. All Iro proteins conform to the same structure with two strongly conserved domains: a homeodomain of the TALE class (HD) and the Iro box (ib), a motif reminiscent of the central part of the EGF repeats of the Notch receptor protein. Generally, Iro genes are chromosomally arranged in groups of three genes. Vertebrates paralogous genes are labelled with similar colours. A detailed physical map of the *D. melanogaster* (*Dm*) Iro-C is shown at the bottom of the figure. Molecular lesions associated with *iro*¹ (chromosomal breakpoint), *iro*^{F209} (P element insertion), and *iro*^{DFM3} and *mirre*⁴⁸ (chromosomal deletions) are indicated. Transcription units of the three *Drosophila* genes are shown in red. Coordinates are in kb. (Data taken from Bosse et al., 2000; Gómez-Skarmeta et al., 1996; McNeill et al., 1997; Netter et al., 1998; Peters et al., 2000.)

and Caup proteins are closely related, whereas Mirr is more divergent. The similarity between Ara and Caup and their essentially identical patterns of expression (Gómez-Skarmeta et al., 1996) suggest that these proteins may functionally replace each other. This is supported by the fact that all the Iro mutations so far analyzed remove the function of both genes

Fig. 2. Comparison of Iro early and late functions in *Drosophila* imaginal discs and *Xenopus* embryos. Top line, early functions. Iro genes are expressed in the dorsal-most regions of the early second instar eye and wing discs, respectively, and in the neural ectoderm of stage 9-10 *Xenopus* embryos (light pink). Absence of these early functions prevents proper development of these regions. The common functional 'leitmotif' is apparently the specification of large territories. Middle line, late functions. Iro genes are expressed in more resolved patterns (dark pink). Their absence removes pattern elements like sensory bristles and wing veins and probably causes transformation among different types of neurons in the vertebrate CNS. Bottom line, territories affected by early and late functions are denoted by light and dark pink, respectively. nt, section of neural tube. Iro genes are also expressed in several regions of the *Drosophila* embryo, other imaginal discs, and many other regions and tissues of the vertebrate embryos (Bellefroid et al., 1998; Bosse et al., 2000; Bosse et al., 1997; Calleja et al., 2000; Christoffels et al., 2000; Cohen et al., 2000; Gómez-Skarmeta et al., 1998; Goriely et al., 1999; McNeill et al., 1997; Tan et al., 1999), which suggests additional functions.



from at least some of their domains of expression, and that overexpression of one gene can rescue defects in imaginal discs associated with the removal of *ara* and *caup* or of *ara*, *caup* and *mirror* (Diez del Corral et al., 1999; F. C., unpublished). This last finding suggests that functional redundancy may even extend to *mirr* to some degree. However, the rescue of the mutant phenotype has not been examined in adults, as overexpression of Iro family members is generally incompatible with viability. Conclusive evidence on their redundancy awaits the availability of point mutations in each gene. The identical pattern of expression of *ara* and *caup* is apparently due to their sharing of common enhancers within the Iro-C (Gómez-Skarmeta et al., 1996). As *mirr* also shows similar expression patterns in some regions of the embryo and imaginal discs, part of these enhancers may also act on *mirr*.

Six Iro genes (*Irx1-Irx6*) have been identified in the mouse (Bosse et al., 2000; Bosse et al., 1997; Bruneau et al., 2001; Christoffels et al., 2000; Cohen et al., 2000; Peters et al., 2000). They are clustered in two groups of three genes each located in chromosomes 8 (*Irx1*, *Irx2* and *Irx4*) and 13 (*Irx3*, *Irx5* and *Irx6*) (Bosse et al., 2000; Peters et al., 2000). The two groups most probably originate from a duplication of an ancestral three-member cluster, as suggested by the similarities among paralogous genes (*Irx1-Irx3*, *Irx2-Irx5* and *Irx4-Irx6*). Similarly to the mouse, six genes can be found in human databases that correspond to *Irx1* to *Irx6*. Analyses of their genome organization indicate that they are also grouped in two clusters of three genes each in chromosomes 5 and 16, in positions equivalent to those of the mouse genes (Bosse et al., 2000; Peters et al., 2000). Orthologs of at least four of these six mouse/human genes have been found in other vertebrates. Thus, the cluster duplication probably occurred in an ancestor of the vertebrate lineage. The genome organization and number of Iro genes in animals such as *Amphioxus* should help determine when these duplication events occurred during the evolution of chordates.

Comparison of vertebrate and *Drosophila* Iro family members indicates that the vertebrate proteins are more similar

to one another than to the *Drosophila* family (Bosse et al., 2000; Peters et al., 2000). Thus, in contrast to the Hox genes (Mann and Morata, 2000), the gene duplications that generated the *Drosophila* Iro-C and the vertebrate Iro clusters may have occurred independently and therefore do not derive from a common ancestral cluster. Moreover, *Caenorhabditis elegans* has only one Iro gene (Bürglin, 1997).

The partially overlapping domains of expression of paralogs suggest that some enhancers may be conserved within the two vertebrate clusters. Moreover, the expression of orthologs in some similar domains suggest that at least part of these enhancers may be conserved in different vertebrate lineages. For example, *Xiro3*, chick *Irx3*, mouse *Irx3* and the zebrafish ortholog *iro3* are expressed in an equivalent region of the dorsoventral axis of the neural tube and in the lateral mesoderm (Bellefroid et al., 1998; Bosse et al., 1997; Briscoe et al., 2000; Tan et al., 1999). Hence, the clustered organization and partially overlapping patterns of expression of vertebrate and also of *Drosophila* genes suggest that genome organization is important for their regulation.

Iro EARLY FUNCTIONS: THE DEFINITION OF LARGE TERRITORIES

The *Drosophila* eye

The Iro-C is essential for the growth and patterning of the pair of eye/antenna imaginal discs of *Drosophila*. These discs give rise to the eyes, most of the head capsule, the antennae and the maxillary palpi of the fly. From at least the late first larval instar, the three Iro-C proteins accumulate in the territory that will give rise to the dorsal half of the eye and the dorsal head capsule. The expression in the dorsal eye territory establishes a smooth interface between Iro-C-expressing and non-expressing cells that has proven to be essential for the development of the whole eye (Cavodeassi et al., 1999; Domínguez and de Celis, 1998; McNeill et al., 1997; Yang et al., 1999; Fig. 3A). This interface corresponds to a dorsoventral (DV) pattern organizer, which in the adult is topologically associated with the equator, a mirror-image axis of symmetry that bisects the eye (reviewed by Reifegerste and Moses, 1999). Ommatidia on each side of the equator have enantiomorphic polarity.

The first evidence pointing to a role of the Iro-C in eye development came from the analysis of *mirr* loss-of-function cell clones (McNeill et al., 1997), which, when located in the dorsal region of the eye, induced ectopic equators at the border between *mirr*-expressing and non-expressing cells. Afterwards, several groups showed that the spatially restricted expression of the Iro-C was required for proper eye development, as generalized misexpression of either *ara*, *caup* or *mirr* throughout the eye disc led to loss-of-eye phenotypes (Cavodeassi et al., 1999; Cho and Choi, 1998; Domínguez and de Celis, 1998). These data were confirmed by the finding that dorsal clones of cells that completely lacked Iro-C function induced an ectopic DV organizer at the new Iro-C⁺/Iro-C⁻ interface and gave rise to ectopic eyes with their equators running along the ectopic organizer (Cavodeassi et al., 1999; Fig. 3).

The molecular bases for the eye disc DV organizer have to some extent been clarified and are very similar to those that

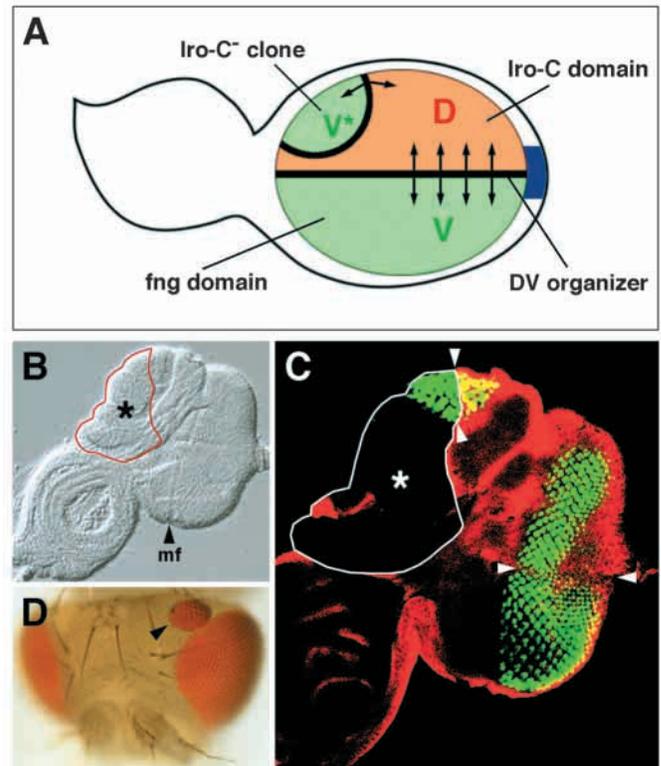


Fig. 3. Iro-C promotes and organizes *Drosophila* eye growth. (A) The three Iro-C genes are expressed in the dorsal (D) half of the eye disc (Iro-C domain). This expression is necessary to establish the eye DV organizer (thick horizontal black line). Iro-C represses within its domain the expression of *fng* and the apposition of ventral (V) *fng*-expressing and D non-expressing cells allows the activation of the N receptor at this interface. N activity is essential for eye growth and patterning, but the nature of the signals (arrows) downstream of this activity are unknown. Iro-C⁻ clones within the Iro-C domain allow expression of *fng* and establish ectopic organizers that promote development of ectopic eyes. (B,C) Conventional optic and confocal view of an eye-antenna disc bearing an Iro-C⁻ clone (asterisk and outlined in red or white) on which the drawing in A has been modelled. (C) In the confocal view, the developing extant and ectopic eyes are visualized by Elav staining (green or yellow) that reveals developing photoreceptors. White arrowheads show positions of eye DV axes of symmetry. mf, morphogenetic furrow. (D) Ectopic eye (arrowhead), which results from an Iro-C⁻ clone like the ones shown in B,C. (Data taken from Cavodeassi et al., 1999.)

support the DV organizer of the wing disc (reviewed by Irvine and Vogt, 1997). In both eye and wing discs, this organizer is prefigured by the activation of the transmembrane Notch receptor in a thin stripe of cells. This activation is effected by Fringe (Fng), which modulates the response of Notch to its ligands Serrate and Delta (reviewed by Irvine and Vogt, 1997; see also Brückner et al., 2000; Moloney et al., 2000). In the eye disc, *fng* is expressed in the ventral region (Fig. 3A) and leads to the activation of Notch exclusively along the interface between *fng*-expressing and non-expressing cells (Cho and Choi, 1998; Domínguez and de Celis, 1998; Papayannopoulos et al., 1998). Iro-C confines the expression of *fng* to the ventral domain by negatively regulating the transcription of this gene in the dorsal half (Cavodeassi et al., 1999; Cho and Choi, 1998; Domínguez and de Celis, 1998; Yang et al., 1999).

In addition to helping establish the dorsoventral organizer, the *Iro-C* is essential for the specification of the dorsal territory of the head as opposed to the ventral one. *Iro-C*⁻ clones in the prospective dorsal head give rise to typically ventral structures, such as suborbital bristles, ptilinum and rostral membrane, in place of dorsal structures like head capsule, dorsal bristles or ocelli (Cavodeassi et al., 2000; Pichaud and Casares, 2000).

The *Drosophila* dorsal mesothorax

Similar to the eye/antenna disc, the *Iro-C* is expressed, at least from the early second instar, in the entire dorsalmost part of the wing disc, that which will give rise to the dorsal mesothorax (essentially the notum, Fig. 4A). Again, similar to the eye/antenna disc, this early expression in the wing disc has a dual role: helping to confer identity and setting up an organizing center that patterns the tissue at both sides of the boundary of *Iro-C* expression (Diez del Corral et al., 1999). In this disc, this boundary corresponds to the border between the developing notum and the wing hinge (Fig. 4A).

The early expression of *Iro-C* is necessary for specifying the notum fate, as its removal causes prospective notum cells to autonomously adopt a wing hinge fate (Diez del Corral et al., 1999), as revealed by the generation of diverse typical hinge structures, i.e. ectopic tegulae and sclerites. Paleontological data on the structure of primitive fossil pterygote hinges suggests that the hinge structures have originated from the tergal lobes (lateral projections of the tergum; Kukalova-Peck, 1978); that is, phylogenetically, they are probably body wall structures. Thus, the *Iro-C* may help establish a subdivision between two tergal territories and may do so by antagonizing genes that specify hinge fate.

The pattern organizing properties of *Iro-C* expression boundaries are manifested by the effects of *Iro-C*⁻ clones located within the prospective notum in the surrounding wild-type tissue (Diez del Corral et al., 1999; Fig. 4A,C). These clones surround themselves with a smooth fold, which is formed by wild-type and *Iro-C*⁻ cells and is most similar to the fold that separates, in the third instar disc, the notum and hinge territories. Using appropriate disc markers it has been found that the mutant clones cause the surrounding mediolateral notum cells to acquire properties that are typical of cells located near the notum/hinge border (Fig. 4C). The signal(s) that arise from this border and organize pattern in neighbouring tissue are unknown, although the Notch/Fringe signaling system is most probably not involved (F. C., unpublished).

The vertebrate neural plate

In *Xenopus*, three *Iro* genes have so far been identified (Bellefroid et al., 1998; Gómez-Skarmeta et al., 1998). Two of them, *Xiro1* and *Xiro2*, are expressed at the beginning of gastrulation in the dorsal ectoderm. By injection of mRNAs that encode wild-type or modified forms of the *Xiro* proteins it has been found that, in the wild type, these proteins are essential for development of the neural plate (Bellefroid et al., 1998; Gómez-Skarmeta et al., 1998; Gómez-Skarmeta et al., 2001). Thus, their overexpression enlarges the neural plate and reduces the neural crests (Fig. 5). Conversely, interference with *Xiro* function, by means of dominant negative derivatives, transforms neural territory towards epidermis. Moreover, the *Xiro* proteins may act redundantly, as suggested by their highly related homeodomains, their lookalike patterns of

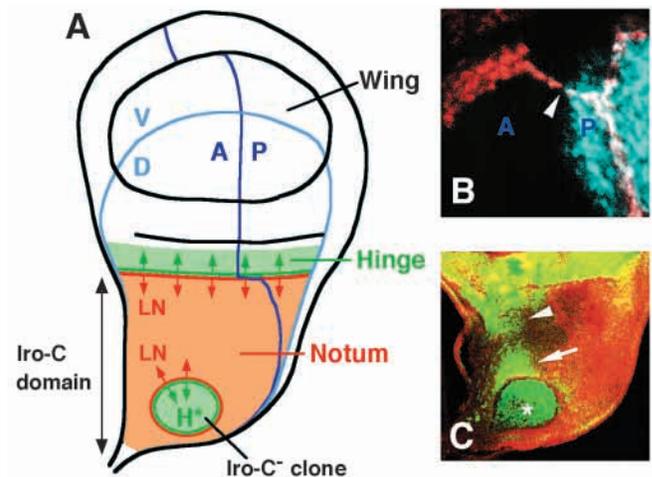


Fig. 4. *Iro-C* helps to specify the *Drosophila* notum and establishes a pattern organizer at the notum/wing hinge border. (A) Cartoon of a mature wing disc with indication of the fates of the relevant regions. Dark and light blue lines indicate compartment borders (D, dorsal; V, ventral; A, anterior; P, posterior compartments). *Iro-C* domain of expression is coextensive with the presumptive notum in young wing discs. This expression is necessary for the specification of the notum, as *Iro-C*⁻ clones transform notum into wing hinge (Diez del Corral et al., 1999; indicated by a green H). The border of notum *Iro-C*-expressing and hinge *Iro-C*-non-expressing cells is a pattern organizing center (arrows), as revealed by the effects of *Iro-C*⁻ clones on the surrounding wild-type tissue. This acquires properties of lateral notum (LN; see also below). (B) Clones of cells overexpressing Ara in the wing pouch stained with anti Ara antibody (red in the A compartment and white in the P compartment). The cells show strong affinity for each other, as indicated by the string-like shape of the clones that can establish contact even across the A/P compartment boundary (arrowhead). Blue and white, *engrailed* expression (data courtesy of R. Diez del Corral). (C) *Iro-C*⁻ clone (*iro*^{DFM3}) on which the cartoon in A has been modeled. Green: *l(2)09261* marker specific for the wing, hinge and anterior lateral notum (arrowhead). This marker is not or is very weakly expressed in the rest of the presumptive notum. The presence of the *Iro-C*⁻ clone (asterisk) causes the surrounding wild-type tissue to express the marker (arrow), indicating a fate transformation towards lateral notum. (Data taken from Diez del Corral et al., 1999.)

accumulation and the similar effects of their overexpression on neural development.

Some inroads have been made towards understanding the mechanism by which *Xiro* acts on neural plate specification (Gómez-Skarmeta et al., 2001). In *Xenopus*, neural fate acquisition requires removal of BMP-4 function from the dorsal ectoderm. *Bmp-4* is initially expressed throughout the embryo but, soon after gastrulation begins, its expression disappears from the dorsal mesoderm and ectoderm. This downregulation depends on Wnt signaling, which should activate one or more repressors to accomplish this downregulation (reviewed by Harland, 2000). One of the repressors seems to be *Xiro1*, because expression of *Xiro1* is activated by Wnt signaling and overexpression of *Xiro1* represses *Bmp-4*. Moreover, the presence of a dominant negative form of *Xiro1* promotes ectopic *Bmp-4* expression. However, BMP-4 signaling also represses *Xiro1*. It seems likely that *Xiro1* directly represses *Bmp-4*, as it binds to the *Bmp-4* promoter. Moreover, *Xiro1* (and most likely *Xiro2* and

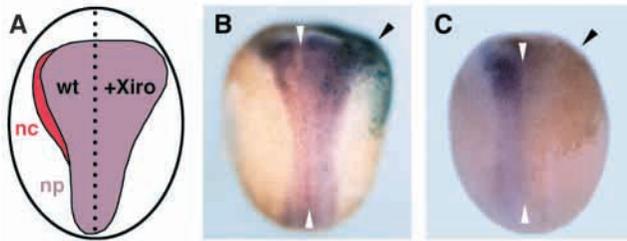


Fig. 5. Early *Xiro* function is required for neural plate formation. (A) Drawing of a dorsal view of a *Xenopus* embryo at the neurula stage. *Xiro* overexpression on the right side (injected with *Xiro1* mRNA) expands the neural plate (np). This expansion is associated with a reduction of the adjacent neural crest territory (nc). (B) *Xenopus* embryo injected with *Xiro1* and *lacZ* mRNAs. Compare the size of the neural plate, as determined by expression of the *Sox2* marker, in the uninjected left side with the injected right side (black arrowhead; green, X-gal-staining to reveal injected side). (C) Interference with early *Xiro* function using a dominant negative construct suppresses neural differentiation on the injected side (black arrowhead; brown, Myc staining, which reveals localization of dominant negative protein). (Data taken from Gómez-Skarmeta et al., 1998; Gómez-Skarmeta et al., 2001; Bellefroid et al., 1998.) White arrowheads in B,C indicate the position of the midline of the neural plate.

3) appears to act mechanistically as a repressor (Gómez-Skarmeta et al., 2001). Thus, the Xiro proteins, by repressing *Bmp-4*, help define the territory of the neural ectoderm. It is of interest that in the absence of both BMP-4 signaling and Xiro1 function, neural fate is not attained (Gómez-Skarmeta et al., 2001). This indicates that Xiro1 not only represses *Bmp-4*, but downregulates other factors that repress neural fate. These factors remain to be determined. The reported ability of ectopic Xiro to expand the domains of expression of proneural genes (Gómez-Skarmeta et al., 1998) may be an indirect consequence of its enlargement of the neural plate. The finding that Xiro1 acts as a repressor also suggests an indirect effect on proneural genes.

The chick ortholog of *Xiro2* (*Irx2*) is initially expressed at the prospective neural plate in a pattern largely complementary to that of *Bmp-4* (Goriely et al., 1999). Thus, it is possible that, similarly to Xiro1, *Irx2* and BMP-4 interact antagonistically. However, in the chick and mouse, inhibition of BMP-4 activity is not a clear requisite for neural plate formation. Hence, it will be of interest to determine whether *Irx2*, or other chick *Irx* genes, are required for proper neural plate specification, similar to their *Xenopus* counterparts.

LATE Iro FUNCTIONS: SUBDIVISION OF TERRITORIES

The dorsolateral subdivision of the *Drosophila* notum

Although the Iro-C is initially required for the specification of the whole notum, at later stages its expression is restricted to the lateral domain (Figs 2 and 6A). Accordingly, late Iro-C clones do not affect the medial (dorsalmost) region, but prevent the proper specification of the lateral notum, as demonstrated by the formation of vesicles that detach from the adult notum cuticle (Diez del Corral et al., 1999). The removal of Iro-C

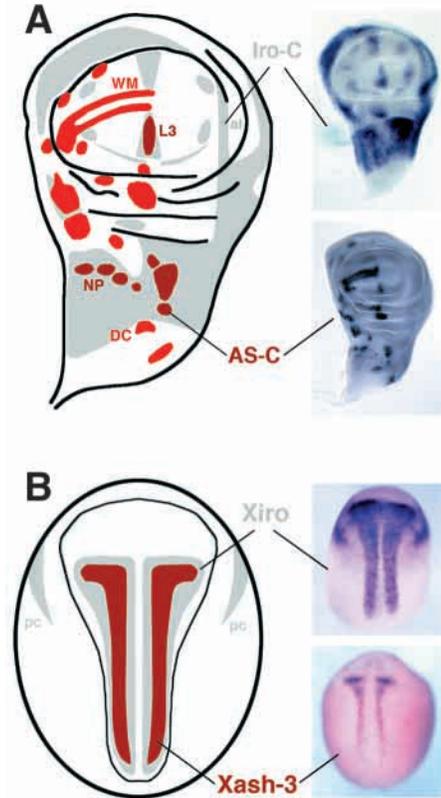


Fig. 6. Proneural genes are expressed within the larger domains of Iro expression in both *Drosophila* imaginal disc and *Xenopus* embryos. (A) Expression of *caup* and *sc* in late third instar wing discs and a drawing showing the overlap between both patterns of expression. *ara* and *ac* expressions are indistinguishable from those of *caup* and *sc*, respectively. Proneural clusters whose presence is known to depend on Iro-C are shown in brown. Other proneural clusters are denoted in red. al, allula; DC, dorsocentral cluster; L3, L3 proneural cluster; NP, notopleural clusters; WM, wing margin. (B) Overlapping patterns of expression of *Xiro1* and *Xash-3* in the neural plate of the *Xenopus* embryos at the neurula stage. *Xiro2* is co-expressed with *Xiro1*. *Xiro3* is expressed in a similar pattern although in slightly narrower bands. In addition, both *Xiro1* and *Xiro2* are expressed in the prospective placode region (pc), whereas *Xiro3* is expressed in lateral mesoderm. Other *Xenopus* proneural genes such as *Xnrg-1* are expressed in domains that partially overlap with those of *Xiro* genes. (Data taken from Gómez-Skarmeta et al., 1996; Gómez-Skarmeta et al., 1998; Bellefroid et al., 1998.)

expression from the medial region seems to depend on the *pannier* (*pnr*) gene. *pnr*, which encodes a GATA transcriptional regulator, is expressed in this medial region and helps specify its identity (Calleja et al., 2000). Directly or indirectly, Pnr downregulates Iro-C, as *pnr*⁻ clones within the *pnr*-expressing domain generally upregulate Iro-C, and ectopic expression of *pnr* within the Iro-C domain suppresses its expression. Although overexpression of *pnr* in the lateral notum interferes with its proper development (Calleja et al., 2000; García-García et al., 1999), it is unclear whether removal of Iro-C is also a requisite for correct specification of the medial notum.

Subdivisions into dorsal and dorsolateral domains demarcated by the largely exclusive expression of *pnr* and Iro-C also occur in the head, tergites and late embryos. However,

their functional significance has been examined only in the tergites, where Iro-C is required for proper pigmentation and normal morphology of bristles in their lateral region (Calleja et al., 2000).

The patterning of notum bristles and wing veins

The late function of Iro-C in the lateral notum is also necessary for the development of its sensory organs. In the presumptive notum of *iro¹* flies, the expression of *ara* and *caup* is strongly decreased (Gómez-Skarmeta et al., 1996). This is most probably responsible for the lack of *ac-sc* expression in the lateral notum and, as a result, the absence of sensory organs (Leyns et al., 1996). (The patterns of expression of *sc* and *caup* are shown in Fig. 6.) The depletion of *Mirr* alone (i.e., homozygous *mirr^{B1-12}* allele) also removes two out of seven lateral large bristles and it genetically interacts with *iro¹* and other Iro recessive alleles (Kehl et al., 1998). Thus, the three Iro-C proteins appear to promote the activity of *ac-sc* and suggest, given the functional redundancy of the Iro-C proteins, that *ac-sc* expression requires a certain level of overall Iro-C function. As the Ara protein binds in vitro to an evolutionarily conserved, functionally indispensable sequence of an *ac-sc* cis-regulatory element, it has been proposed that the Iro-C proteins directly activate these proneural genes (Gómez-Skarmeta et al., 1996). However, overexpression of a chimeric protein containing the Ara homeodomain fused to the Engrailed repressor domain, which should function as a repressor, expanded the proneural cluster governed by that regulatory element. This expansion was similar to that obtained by overexpressing wild-type Ara (J. L. G. S. and E. de la Calle-Mustienes, unpublished), suggesting that this, and other Iro proteins, act as transcriptional repressors. Hence, their positive effect on *ac-sc* should be indirect and probably related to the proper specification of the notum territory.

Besides allowing development of the notum lateral bristles, the Iro-C genes also help confer identity to these sensory organs (Grillenzoni et al., 1998). The sensory neurons innervating the medially located bristles send an axonal branch that crosses the CNS midline (medial identity), while the ones that innervate the lateral bristles do not send such a branch (lateral identity). The lateral identity depends on the presence of Iro-C products. It is not known whether this requirement occurs during neuronal differentiation or is a consequence of the earlier Iro-C function in lateral notum specification.

At third instar larvae, new Iro domains of expression appear in the prospective wing pouch. Iro-C is expressed in the presumptive veins L1, L3 and L5, it is required to activate the provein gene *rhomboid/veinlet* (*rho/ve*) and it is necessary for the differentiation of these veins (Gómez-Skarmeta et al., 1996). Similar to the activation of the proneural genes, it is possible that these requirements underlie a role of the Iro-C in the proper specification of the vein territory. The allula, in whose anlage the Iro-C is also expressed (Fig. 6), requires its function for its development (Gómez-Skarmeta et al., 1996; Kehl et al., 1998).

The subdivision of the vertebrate neural tube

During neurulation, the pattern of expression of *Xiro1* and *Xiro2* becomes restricted to two stripes within the neural plate that extend posteriorly from the midbrain-hindbrain boundary. *Xiro3* is also expressed within the *Xiro1/2* domain, but in

narrower stripes (Fig. 6B). These patterns of expression suggest that the Xiro proteins function in the subdivision of the neural territory. This has been demonstrated in the chick embryo, where *Irx3*, the ortholog of *Xiro3*, together with other homeodomain factors, effect the dorsoventral patterning of the neural tube (Briscoe et al., 2000). *Pax6*, *Pax7*, *Dbx1*, *Dbx2*, *Irx3*, *Nkx6.1* and *Nkx2.2* are expressed in partially overlapping domains along the dorsoventral axis of the neural tube in response to an activating/repressing gradient of Shh molecules emanating from the notochord and floor plate. The combinations of these transcription factors define five regions, each of which will give rise to one of five types of neurons (V0, V1, V2, MN and V3). For example, the region that generates V2 neurons expresses both *Irx3* and *Nkx6.1*, while that which forms MN neurons expresses *Nkx6.1* alone. *Irx3* overexpression in the MN domain transforms MN into V2 neurons. This and other ectopic expression experiments support the model that combinations of homeoproteins define the identity of the different neurons (Briscoe et al., 2000). Mutual repression between some of the homeodomain-encoding genes allows formation of sharp boundaries among their expression domains. It is not known whether *Irx3* helps generate such sharp borders, as its *Drosophila* counterparts do in the notum/hinge and dorsal/ventral eye boundaries.

Heart patterning

The Iro genes also participate in the subdivision of the vertebrate heart into smaller territories (Bao et al., 1999). The developing heart derives from a single tubular structure that gives rise to two types of chambers: the ventricles and the atria. In chick, *Irx4* is expressed mainly in the developing ventricle. Ectopic expression of *Irx4* in the atria activates ventricle-specific genes. Conversely, expression of a dominant negative form of *Irx4* in the ventricle downregulates ventricle-specific genes and activates atria-specific genes. However, these alterations of the sites of expression of chamber-specific genes are not associated with morphological atria/ventricle transformations. Thus, rather than defining heart chamber identity, *Irx4* probably imparts specific regional characteristics such as contractile and electrophysiological properties. Indeed, mice with targeted disruption of *Irx4* exhibit normal heart morphology, but aberrant ventricular gene expression and maturity onset cardiomyopathy (Bruneau et al., 2001). In contrast to *Xenopus* Iro proteins, *Irx4* appears to act as a transcriptional activator (Bao et al., 1999).

CONTROL OF Iro GENE EXPRESSION

Some progress has been made in unraveling the control of Iro genes expression at early and late stages of development. The emerging view is that although some signalling pathways are recurrently used in their control, the details of their regulation vary widely among different tissues. Sequence and functional comparison of the Iro enhancers will be needed to decide whether the parallels in regulation have a common ancestry or have originated independently. This seems particularly intriguing, given the most likely possibility that the tandem duplications that have given rise to the *Drosophila* and vertebrate Iro clusters are independent events.

In *Drosophila*, the Iro genes are activated early in the dorsal

domain of the eye disc by Wg and Hh signaling (Cavodeassi et al., 1999). The JAK/STAT pathway has also been found to positively regulate *mirr* (Zeidler et al., 1999); it probably also regulates *ara/caup* expression. In the prospective notum, early activation of Iro-C appears to be promoted by the EGFR signaling pathway (Wang et al., 2000). The Vein (Vn) EGFR-activating ligand is mostly restricted to the prospective notum and may be responsible for the Iro-C activation in this region. Thus, several signalling pathways participate in the early activation of Iro-C in the imaginal discs. In the case of the early expression of vertebrate Iro genes, the Wnt and BMP-4 signaling pathways have so far been implicated (see above).

The regulation of late Iro gene expression in *Drosophila* has been examined in detail only in the region of the third instar wing disc that will give rise to the wing vein L3, where *ara* and *caup*, but not *mirr*, are expressed (Gómez-Skarmeta and Modolell, 1996; Fig. 6A). This control has provided an excellent example of how inputs from different signaling systems and partially overlapping distributions of factors (a prepattern) are integrated to create smaller and more precise spatial domains. Indeed, Hh signaling is responsible for *ara/caup* activation and this is mediated by the Gli protein Cubitus interruptus (Ci). High levels of Dpp signaling are also required. Accordingly, *ara-caup* expression occurs within the band where the Ci protein acts as an activator and where Dpp signaling is maximal. However, the *ara-caup* domain does not encompass the whole Ci band because it is delimited posteriorly by the repressor Engrailed, which accumulates in a narrow wedge that overlaps with the Ci band, and it is split into two subdomains by Wg, which accumulates at the prospective wing margin and blocks expression of *ara-caup*. In the wing pouch, outside the domain of Ci accumulation, *ara-caup* are repressed by the zinc-finger factors Spalt and Spalt-related (Sal/Salr; de Celis and Barrio, 2000). The posterior edge of the Sal/Salr domain apparently delimits the anterior edge of the *ara-caup* expression in the L5 prospective vein territory.

Little is known about the control of late expression of vertebrate Iro genes. In *Xenopus*, the refinement of the early *Xiro* domain comprising most of the neuroectoderm (Fig. 2) to two narrow bands located at each side of the midline (Fig. 5) probably depends on Hh signaling mediated by Gli proteins, while posteriorizing signals such as retinoic acid and FGF seem to limit them to the region posterior to the midbrain/hindbrain boundary (Gómez-Skarmeta et al., 1998; Bellefroid et al., 1998). Chick Iro genes seem also to depend on Hh signaling (Briscoe et al., 2000).

CELL-CELL AFFINITY AND Iro EXPRESSION

In the *Drosophila* wing imaginal disc, the best characterized pattern organizing centers are associated with compartment borders. Compartments are defined by cell lineage restriction boundaries and are associated with the expression of a selector gene, *engrailed* (*en*) in the posterior (P) and *apterous* (*ap*) in the dorsal (D) compartment, that provides identity and a specific affinity to the cells of the compartment (reviewed by Blair, 1995; Mann and Morata, 2000). The distinct affinities of cells of opposing compartment (i.e., A versus P or D versus V, Fig. 4A) are thought to help maintain the integrity of compartment boundaries. The confrontation of cells that

express a selector gene with cells not expressing it at the other side of the compartment boundary induces specific signals across this boundary that organize pattern in both compartments.

We have discussed how the apposition of Iro-C-expressing and non-expressing cells generate the DV organizer of the eye disc and the notum/hinge organizer of the wing disc. Are these organizers also associated with compartment borders? Although strictly the answer should be no, the situation in the eye disc warrants some qualifications. In this disc, there is an early cell lineage restriction that, in the adult fly, bisects the eye and head in dorsal and ventral regions. However, this restriction is not absolute, as would be expected from a classical compartment boundary (Baker, 1978; Campos-Ortega and Waitz, 1978). Still, the expression of the Iro-C in the dorsal half of the early eye disc, which is necessary for conferring its dorsal identity and for generating the eye DV organizer, suggests that Iro-C is the selector responsible for that cell lineage restriction (Cavodeassi et al., 1999; Domínguez and de Celis, 1998). This interpretation is supported by the finding that, in addition, the Iro-C apparently confers a specific affinity to the cells in which it is expressed. Indeed, Iro-C⁻ clones surrounded by Iro-C-expressing cells have smooth contours, in contrast to the wiggly contours of clones whose cells have the same state of expression of Iro-C as the surrounding tissue (Cavodeassi et al., 1999; Yang et al., 1999). Moreover, Iro-C⁻ clones of dorsal origin can trespass the DV boundary and end up located within the ventral compartment (Cavodeassi et al., 1999). That the Iro-C proteins endow cells with a specific affinity is dramatically manifested by clones of cells that overexpress Ara (Fig. 4B); these seek to contact each other even when they belong to different compartments.

However, in contrast to classical selector genes, whose expression is maintained throughout development, the expression of the Iro-C fades away from the dorsal compartment during the third instar, coinciding with the onset of ommatidial differentiation (Cavodeassi et al., 1999). This suggests that Iro-C homeoproteins must be removed for cell differentiation to take place. Conceivably, the differences of cell affinity across a sharp border of Iro-C expression might interfere with the proper assembly of the crystalline arrangement of ommatidia. Moreover, because individual ommatidia can recruit cells of dorsal and ventral origin at the equator (Lawrence and Green, 1979; Ready et al., 1976), compartment-specific differential affinities might also interfere with ommatidial assembly. The disappearance of Iro-C products may lead to the breakdown of the cell-lineage restriction border, which might help, early in eye development, to maintain the integrity of the DV organizer.

In contrast to the eye disc, in the wing disc, the smooth and relatively straight border of Iro-C-expressing and non-expressing cells associated with the notum/hinge organizer is not a cell lineage restriction border (Diez del Corral et al., 1999). Most likely, the differences in affinity between Iro-C-expressing and non-expressing cells, manifested by the smooth borders of Iro-C⁻ clones in the presumptive notum (Fig. 4), are important to maintain the separation of two cell populations. However, the fact that descendants from cells in either population can cross the border suggests that the state of expressing or non-expressing Iro-C is not inheritably maintained. Rather it must be imposed by other agents.

Epidermal growth factor signaling probably delimits the early Iro-C-expressing domain, but it is not known whether it also helps maintain the straight border at later stages.

Another difference between Iro-C and typical selector genes is that the ectopic expression of the Iro-C genes in the ventral compartment of the eye disc does not induce transformations reciprocal to their loss-of-function phenotypes (Cavodeassi et al., 2000). The Iro homeodomains are related to those of the Pbx/Meis proteins (Gómez-Skarmeta et al., 1996; McNeill et al., 1997), which act as cofactors of many Hox proteins (Mann and Affolter, 1998), and have the Iro box that probably mediates protein-protein interactions. Thus, they could act as transcription factors in multimeric complexes. Accordingly, their absence would impair the function of these complexes, thereby causing dorsal-to-ventral transformations, but their ectopic expression in ventral cells would not reproduce their normal function if other members of the complexes were unavailable in these cells. Hence, the reciprocal transformations should not be accomplished.

In vertebrates, it is not known whether the Iro genes define cell affinity properties and organizing centers. However, most (if not all) of the Iro genes already identified show sharp boundaries of expression in the midbrain/hindbrain junction (Bellefroid et al., 1998; Bosse et al., 2000; Bosse et al., 1997; Cohen et al., 2000; Gómez-Skarmeta et al., 1998; Goriely et al., 1999; Peters et al., 2000; Tan et al., 1999). This territory acts as an organizer for both the midbrain and hindbrain. Thus, similar to the *Drosophila* notum/hinge and dorsal/ventral eye organizing borders, the vertebrate Iro genes perhaps participate in the specification of this and other organizing boundaries (reviewed by Simeone, 2000). Their function in rhombomeres, which are known to behave as cell-affinity compartments (reviewed by Lumsden and Krumlauf, 1996), may also be of special interest. It is possible that, again as in *Drosophila*, the vertebrate Iro proteins help to create differences of cell affinities between the Iro-expressing and non-expressing rhombomeres.

PERSPECTIVES

The complexity of the patterns of expression of the Iro genes in both *Drosophila* and vertebrates suggest many not yet characterized functions in the development of other territories. Specially tantalizing may be their functions in vertebrate limbs, mesodermal placodes and in rhombomeres, as well as in the *Drosophila* embryo, where Iro members show restricted patterns of expression in several regions and tissues (McNeill et al., 1997; Calleja et al., 2000).

Although several genes have been suggested as candidates for Iro regulation (*Drosophila* *fng*, *ac-sc*, *rho/ve* and *Xenopus* *Bmp-4*, *Xash-3*), it is not known whether the control is direct. The characterization of the Iro DNA-binding preferences and the identification and functional analyses of the binding sites in the *cis*-regulatory elements of the putative Iro regulated genes may help clarify this point. Evidently, many additional target genes remain to be identified.

Both *Drosophila* and *Xenopus* data suggest partial redundancy of the Iro genes (Bellefroid et al., 1998; Gómez-Skarmeta et al., 1996; Gómez-Skarmeta et al., 1998). However, in the mouse developing heart, each *Irx* gene analyzed is

expressed in a distinct pattern that comprises different subterritories (Christoffels et al., 2000; Cohen et al., 2000; Bao et al., 1999; Bruneau et al., 2001). Considering that chick *Irx4* specifies ventricular properties (Bao et al., 1999; Bruneau et al., 2001), we speculate that each mouse *Irx* gene has a specific function and defines properties of different territories. If this were the case, *Irx* genes would be unable to substitute for one another, indicating non-redundant functions. The fact that the similarity among different *Irx* proteins is limited to small regions of their sequences (the homeodomain and the Iro box, Fig. 1) also suggests non-redundant functions. The remaining, non-conserved regions of these proteins may be required for specific modulation of their activity by means of different protein-protein interactions. We have discussed that the presence in Iro proteins of motifs apparently involved in this type of interactions, together with the resemblance of their homeodomains to those of the Pbx/Meis family, suggest that the Iro proteins act as multiprotein complexes. It is therefore of interest to clarify whether these proteins act in fact as complexes and, if so, to identify their partners. Association with different partners could explain why some Iro proteins act as repressors (*Xiro1*) and others as activators (*Irx4*). Finally, a detailed analysis of how these genes are regulated will integrate Iro genes in developmental genetic cascades and help us to understand the importance of their genomic organization.

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