Development 126, 4933-4942 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV2476

Compartments and organising boundaries in the *Drosophila* eye: the role of the homeodomain Iroquois proteins

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Accepted 9 September; published on WWW 21 October 1999

SUMMARY

The *Drosophila* eye is patterned by a dorsal-ventral organising centre mechanistically similar to those in the fly wing and the vertebrate limb bud. Here we show how this organising centre in the eye is initiated – the first event in retinal patterning. Early in development the eye primordium is divided into dorsal and ventral compartments. The dorsally expressed homeodomain *Iroquois* genes are true selector genes for the dorsal compartment; their expression is regulated by Hedgehog and Wingless. The organising centre is then induced at the interface between the *Iroquois*-expressing and non-expressing cells at the eye midline. It was previously thought that the eye develops by a mechanism distinct from that operating in other imaginal discs, but our work establishes the importance of lineage compartments in the eye and thus supports their global role as fundamental units of patterning.

Key words: *Drosophila melanogaster*, Compartment, Eye development, Iroquois proteins, Dorsal-ventral patterning

INTRODUCTION

Compartments are the fundamental units used in building pattern in the epidermis of *Drosophila* (García-Bellido et al., 1973; García-Bellido, 1975). These units are demarcated regions of the adult insect epidermis that contain all the descendants of the founder cells set aside at an earlier stage. Their existence has been demonstrated by cell-lineage studies; descendants of marked cells from one compartment never cross the boundary to enter an adjoining compartment (García-Bellido et al., 1973; García-Bellido, 1975). In the imaginal discs, the primordia of the adult epidermis, the first event of patterning is their binary subdivision into anterior and posterior compartments by the expression of the selector gene *engrailed* in posterior cells (Morata and Lawrence, 1975). Wing and haltere discs become further subdivided into dorsal and ventral compartments by the action of the selector gene *apterous* expressed by dorsal cells (Díaz-Benjumea and Cohen, 1993; Blair et al., 1994).

The eye-antennal disc complex is subdivided into anterior and posterior compartments, although this subdivision occurs later than in the other discs (Morata and Lawrence, 1978). The eye part is primarily derived from the non-segmented embryonic acron and is 'anterior'. It has been under debate whether there are compartmental subdivisions within this part of the disc. The main criterion to judge whether there are compartments in a disc is the existence of cell-lineage restrictions. Cell-lineage studies revealed that even first instar-induced clones do not cross the dorsal-ventral (DV) midline in the eye (Becker, 1957; Baker, 1978; Campos-Ortega and Waitz, 1978). This clonal restriction boundary seemed homologous to the DV compartmental subdivision of the wing: *Ophthalmoptera* mutations cause replacement of eye tissue by wing, with an anterior wing margin forming along the eye DV midline (Postlethwait, 1974). However, the developmental significance of this cell-lineage restriction remained unproven. Genetic mosaics demonstrate the non-clonal origin of the ommatidia. Similarly, the mirror image symmetry above and below the equator is not established by a strict clonal mechanism (Ready et al., 1976; Baker, 1978; Campos-Ortega and Waitz, 1978). Therefore, development of the eye was assumed to proceed through non-compartmental pattern mechanisms (Ready et al., 1976). Alternative mechanisms for pattern formation in the eye that involve the formation of gradients of secreted signals like Wingless (Wg) have been favoured (reviewed by Reifegerste and Moses, 1999). However, the discovery of gene expression patterns restricted to either the dorsal or the ventral halves of the eye disc (Brodsky and Steller, 1996; McNeill et al., 1997; Cho and Choi, 1998; Domínguez and de Celis, 1998; Papayannopoulos et al., 1998) together with the straightness of the boundaries of large lineage clones where they reach the eye midline (Domínguez and de Celis, 1998) are compatible with the restriction boundary first described by Becker (1957) being a true compartment boundary.

A key function of compartment boundaries is to act as

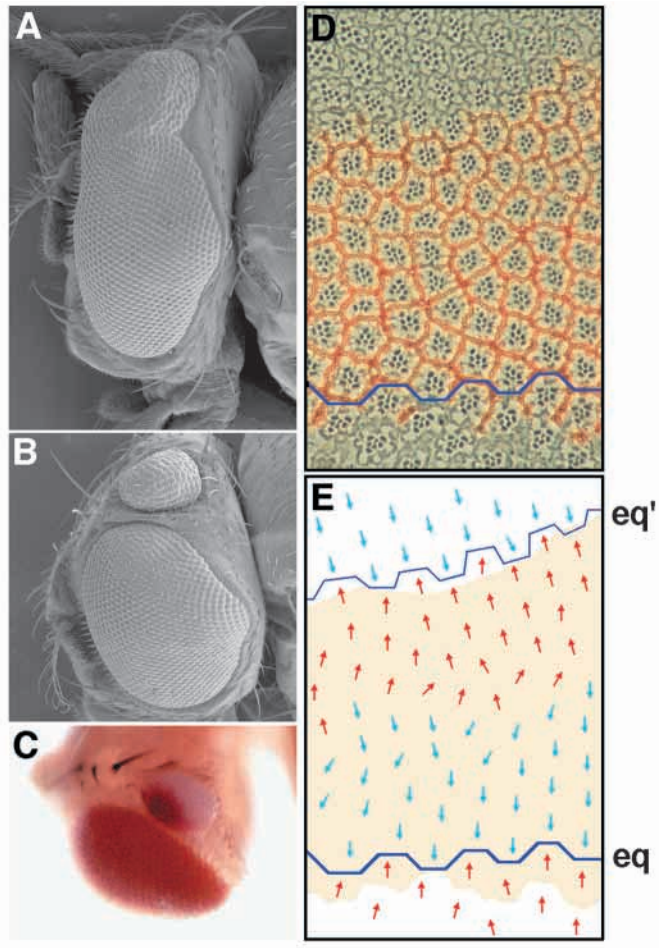

Fig. 1. Consequences of removing *IRO-C* activity in eye development. In all panels, anterior is to the left and dorsal is up. (A-C) Adult eyes harbouring dorsal *IRO-C* clones. Mutant tissue is genetically labelled by the *white* mutation, appearing as a pigmentless tissue against the red-pigmented wild-type tissue. (D) Histological section through an eye carrying a dorsal (upper *white* tissue) and a ventral (lower *white* tissue) clone. (E) Schematic representation of the ommatidial polarity of the eye in D. Dorsal (blue) and ventral (red) ommatidial chirality is represented by arrows. The equator (eq) is outlined by a thick blue line in D and E. The equator runs close but does not coincide with the straight border of the endogenous *IRO-C* expression (not shown). The border of the dorsal *IRO-C* clone defines an ectopic equator (eq', in E). The ventrally located clone does not show phenotypic effect.

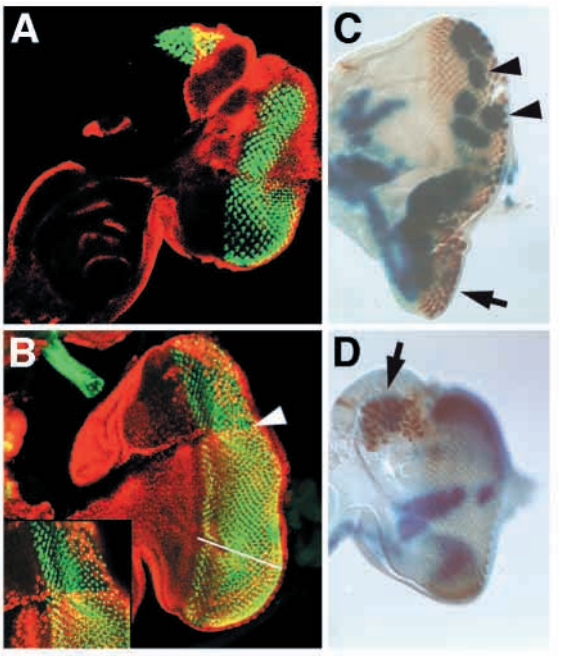

Fig. 2. Analysis of ectopic *IRO-C* borders. Clones of *IRO-C* mutant cells are marked by the absence of anti-Myc (red in A) or anti-β-galactosidase staining (red in B). Discs are double stained with anti-Elav (A) and anti-Spalt (B). (A) The ectopic eye begins development near the margin and progresses inwards, as judged by the maturation of the ommatidial rows and expression of the furrow marker *dpp* (not shown). (B) The orientation of the rows of *spalt*-expressing cells (photoreceptor R3 and R4) marks the position of the endogenous equator (white line) and of the ectopic equator (arrowhead) along the border of the *IRO-C* clone. Inset is a close-up of the disc. (C,D) Ectopic eyes (stained with anti-Elav, brown), associated with *ara*-expressing clones (marked by X-gal, blue), are indicated by arrows. Ectopic *ara* expression within the eye field (arrowheads in C) does not prevent ommatidia development.

pattern-organising centres that govern growth and pattern at both sides of the boundary (reviewed by Lawrence and Struhl, 1996). Likewise, the DV boundary of the eye has organising properties that control growth and DV ommatidial polarity (Cho and Choi, 1998; Domínguez and de Celis, 1998; Papayannopoulos et al., 1998). The eye organiser, like the DV compartment boundary in the wing (Irvine and Wieschaus, 1994), requires the confrontation of *fringe* (*fng*)-expressing and non-expressing cells. *fng* is expressed in the second instar by ventral eye cells and the generation of ectopic *fng* expression borders in mosaic eyes induces ectopic DV organisers at the new borders. Moreover, elimination of the *fng* border by early generalised expression of a *fng* transgene abolishes eye formation. Vertebrate Fng homologues are similarly essential to mediate signalling between dorsal and ventral cells in the limb bud (reviewed by Irvine and Vogt, 1997).

In the wing disc, *fng* is expressed only by dorsal cells and its expression is controlled by the dorsal selector gene *apterous* (reviewed by Irvine and Vogt, 1997). Here we analyse the functions of the dorsally expressed homeoproteins encoded by the *Iroquois* gene complex (*IRO-C*), Araucan (Ara), Caupolican (Caup) and Mirror (Mirr) (Gómez-Skarmeta et al., 1996; McNeill et al., 1997) and suggest that the *IRO-C* genes may act as dorsal selectors in the eye disc. Previous studies were suggestive: *mirr* is expressed in the dorsal half of the eye and *mirr*$^{-}$ clones induce ectopic equators at the boundary of *mirr*-expressing and non-expressing cells (McNeill et al., 1997). But these *mirr* expression borders neither show long range polarising activity nor influence growth, as might be expected for mutations in a true compartment selector gene. Moreover, the putative function of the two Mirr-related proteins, Ara and Caup, which also exclusively accumulate in the dorsal part of the eye disc (Domínguez and de Celis, 1998), must also be considered. Misexpression of *caup* or *mirr* represses expression of *fng* and block eye growth (Cho and Choi, 1998; Domínguez and de Celis, 1998), which suggests

that both Caup and Mirr act to establish the *fng*-dependent organiser.

Here we report that localised expression of the three *IRO-C* genes specifies the identity of dorsal cells in the eye. Using genetic mosaics, we show that juxtaposition of *IRO-C*-expressing and non-expressing cells forms a straight border that promotes growth and serves as a pattern-organising centre in the eye disc. We also present evidence of regulatory relationships between *IRO-C* genes, Wg and Hh that may account for previously reported data. Thus, our results argue in favour of a lineage-compartment model for the regulation of eye growth and patterning and support the importance of compartments as the fundamental units for patterning.

MATERIALS AND METHODS

Fly stocks

Df(3L)iro^{DFM3}, a deficiency null for *ara*, *caup* and *mirr*, and *UAS-caup* are described by Diez del Corral et al. (1999). *mirr^{e48}*, *ptc^{IIw}*, *dsh^{VA153}*, *sgg^{M1-1}*, *smo^{3}*, *hh-lacZ* (*hh^{P30}*), *dpp-lacZ* (*dpp^{P10638}*, an enhancer trap line that reproduces the early expression of *dpp*), *wg-lacZ*, *mirr-lacZ* (*mirr^{P69Df7}*), *fng-lacZ*, *eyeless-GAL4* (*ey-GAL4*), *Actin>y^{+}>GAL4*, *UAS-ara*, *UAS-hh*, *UAS-Notch^{ecd}* (encodes a dominant negative form of Notch), *UAS-fng* and *UAS-lacZ* are described in FlyBase (http://gin.ebi.ac.ak:7081).

Mosaic analysis

Heat shock induction of *FLP* expression (Xu and Rubin, 1993) was performed either at 24-48 hours after egg laying (AEL) or 48-72 hours AEL for 1 hour at 37°C. For crosses involving either *CD2, πMyc* or *NMyc*, third instar larvae were subjected to a second heat shock (1 hour at 37°C) to induce *CD2* or *Myc* expression. Staining with anti-Myc, anti-CD2 or anti-β-galactosidase revealed mutant clones. Genotypes of the larvae analysed were:

y w hsFLP122; mwh Df(3L)iro^{DFM3} (or *mirr^{e48}*) *FRT80B/hsNMyc* (or *tubulin-lacZ* or *P[w^{+}])FRT80B*

y w hsFLP122; dpp-lacZ/+; mwh Df(3L)iro^{DFM3} FRT80B/hsNMyc FRT80B

y w hsFLP122; wg-lacZ/+; mwh Df(3L)iro^{DFM3} FRT80B/hsNMyc FRT80B

y w hsFLP122; ptc^{IIw} sha FRT42D/hsCD2 FRT42D; mirr-lacZ/+

y w dsh^{VA153} FRT18A/w hsπMyc FRT18A; hsFLP1/+

y sgg^{M1-1} FRT18A/w hsπMyc FRT18A; hsFLP1/+

y w hsFLP122; smo^{3} FRT40A/arm-lacZ FRT40A

Misexpression experiments

Clones of cells overexpressing *ara* or *caup* were obtained by heat-treating *y hsFLP122; UAS-ara* (or *UAS-caup*)*/Actin>y^{+}>GAL4, UAS-lacZ* larvae (at 24-48 hours AEL) 7 minutes at 37°C.

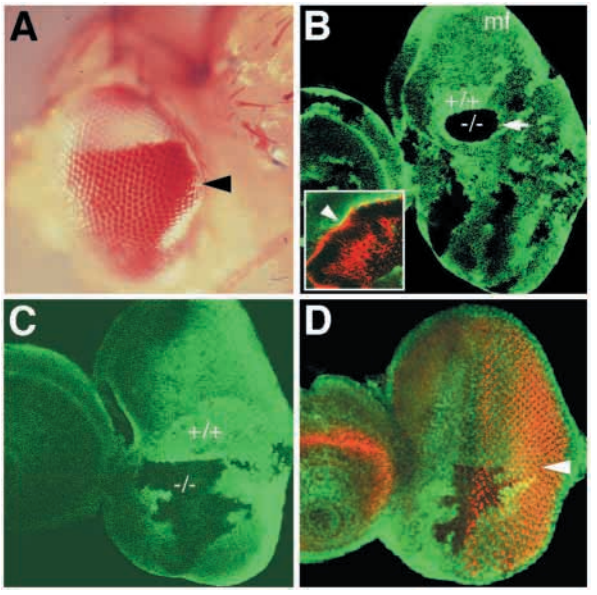

Fig. 3. The interface between *IRO-C*-expressing cells (dorsal identity) and non-expressing cells (ventral identity) is a smooth boundary. (A) Adult eye carrying a dorsal *IRO-C* clone marked with *white*. Note the sharp boundary of the clone. The approximate position of the DV midline is indicated by an arrowhead. (B-D) *IRO-C* clones marked by the absence of anti-Myc (B,C) or anti-β-galactosidase (D) staining (green). Clones were induced at first instar and analysed in late third instar larvae. (B) The outline of an *IRO-C* mutant patch (–/–) is smooth when located in the dorsal part of the eye disc; in contrast, the edge of the twin clone (+/+), recognised by the elevated levels of Myc expression, is wiggly. All dorsally located *IRO-C* clones (n>100) behave similarly. (C,D) The outlines of *IRO-C* clones located in the ventral part of the eye are smooth where they abut the DV midline (arrowhead in D) but wiggly elsewhere. Note in C the dorsal origin of the clone as judged by the position of its twin clone. *IRO-C* clones in the dorsal anterior part are transiently surrounded by an epithelial fold (inset in B), manifested by phalloidin staining (red). The position of the DV midline is revealed by anti-Spalt staining (red in D).

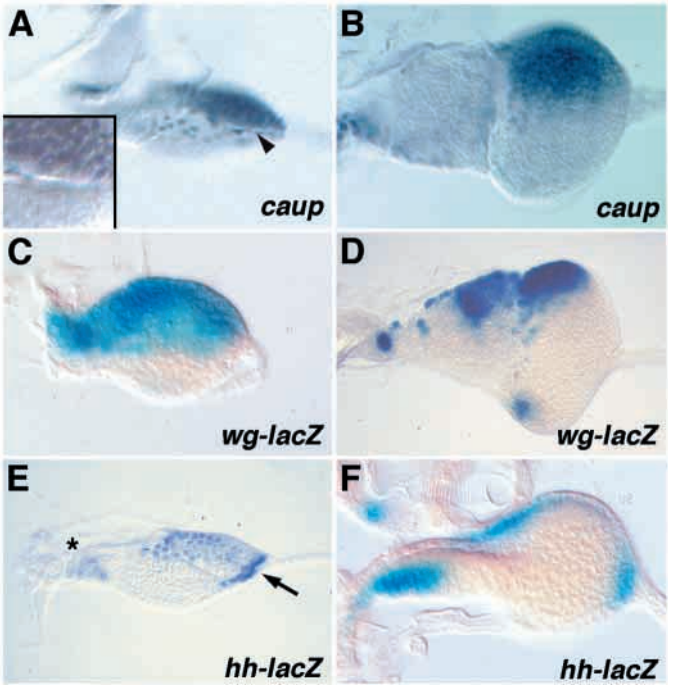

Fig. 4. Early expression domains of *ara/caup*, *wg* and *hh* in eye discs. Wild-type (A,B), *wg-lacZ* (C,D) and *hh-lacZ* (E,F) discs stained with anti-Caup (A,B) and X-gal (C-F) at late-first/early-second (A,C,E) and late second (B,D,F) larval instar. Expression of the three genes is restricted to the dorsal half in the early eye discs (A,C,E). *hh-lacZ* is also expressed at the posteriormost margin (arrow in E) and in the posterior compartment of the antenna (asterisk). The limit of *IRO-C* expression demarcates the DV midline and is associated with a groove (A, arrowhead, and inset).

UAS-caup, UAS-hh, UAS-Notch^{ecd} or *UAS-fng* were crossed to *ey-GAL4; hh-lacZ* or *ey-GAL4; fng-lacZ* at 25°C.

Histochemistry

Imaginal discs were dissected and stained as described by Domínguez and Hafen (1997). Primary antibodies were: rabbit anti-β-galactosidase (Cappel), mouse anti-Myc (BabCo); rat anti-Elav (Developmental Studies Hybridoma Bank), rat anti-Caup (this antiserum cross-reacts with Ara but not with Mirr; Diez del Corral et al., 1999), rabbit anti-Spalt (a gift from R. Barrios), rabbit anti-Atonal (a gift from Y. N. Jan), mouse anti-Wg (a gift from S. M. Cohen) and mouse anti-CD2 (Serotech). Secondary antibodies were from Jackson and Amersham. FITC-phalloidin staining was performed as described by Diez del Corral et al. (1999). *fng* digoxigenin-labelled RNA probe was prepared from a 1.9 kb *fng* cDNA clone (Irvine and Wieschaus, 1994). Images were collected using Zeiss LS310 and BioRad MRC 1024 confocal microscopes.

Scanning electron microscopy and histology

Scanning electron microscopy and histological sectioning of adult eyes were performed as described by Ma and Moses (1995).

RESULTS

IRO-C expression borders exert long range organising activity in growth and patterning of the eye disc

In the *Drosophila* compound eye, the hexagonal array of dorsal and ventral ommatidia is extremely accurate; only rarely the path of the equator deviates from the midline and never by more than one ommatidium (reviewed by Wolff and Ready, 1993). The path of the equator takes alternating dorsal and ventral steps (thick blue line, Fig. 1D), while the path of the DV midline is straight (reviewed by Reifegerste and Moses, 1999). The accuracy of the location of the equator may depend on the shape and position of the DV midline, which itself is defined at an earlier stage. The midline can be visualised by the expression of the transcription factor Mirr in the dorsal cells (McNeill et al., 1997). The two other Mirr-related proteins, Ara and Caup, are also expressed in a dorsal-specific domain (Domínguez and de Celis, 1998). We have examined the functions of the three *IRO-C* proteins in establishing the DV midline by generating clones of cells deficient for the whole *IRO-C* (*Df(3L)iro^{DFM3}* clones, henceforth referred to as *IRO-C* clones) and compared them with similarly induced *mirr^{e48}* clones.

Dorsal *lRO-C* clones are frequently associated with extensive outgrowths of the eye (73 out of 90 clones examined; Fig. 1A), including both mutant and adjacent wild-type cells. A subset of these *IRO-C* clones develops a clearly independent eye (23 out of 73 clones with eye phenotype examined, Fig. 1B,C), consisting of both *IRO-C*$^{-}$ (*white*$^{-}$) and *IRO-C*$^{+}$ (*white*$^{+}$) cells (Fig. 1C). Sections through such mosaic eyes show that the border of *IRO-C* expression always defines an ectopic equator (eq'): wild-type ommatidia, located as far as 7 ommatidial rows away from the clonal border, are repolarised towards the new border of *IRO-C* expression (Fig. 1D,E). The ectopic equators and the independent eye fields are also revealed in eye discs stained with ommatidial cell markers (Fig. 2A,B). All the above described phenotypes of *IRO-C* clones are only observed when the clone abuts the disc margin; in addition, *IRO-C* clones in the dorsal head capsule cause autonomous transformations to ventral cuticle structures (F. C. and S. C., unpublished). *mirr^{e48}* clones only affect ommatidial polarity (McNeill, et al., 1997; our unpublished observations), suggesting that Ara and/or Caup may partially compensate for the absence of Mirr in such clones.

Ectopic borders of *IRO-C* expression can also be generated by targeted expression of *mirr* (McNeill et al., 1997), *ara* or *caup* (Fig. 2C,D; not shown). In the ventral region of the eye disc, *ara*$^{+}$ or *caup*$^{+}$ ectopic borders, like *mirr* borders, reorganise DV polarity (McNeill et al., 1997; not shown) and promote formation of ectopic eye fields, albeit at a low frequency (4 out of 23 clones located in the ventral anterior disc region, Fig. 2C). Dorsally situated clones overxpressing *ara* or *caup* also induce ectopic eyes at the same low frequency (Fig. 2D), suggesting that confrontation of cells with different amounts of IRO-C proteins may be sufficient to generate an organising border.

In summary, the boundaries of *IRO-C* expression exert long range organising activity and can promote the formation of an independent eye, consisting of both *IRO-C*$^{+}$ and *IRO-C*$^{-}$ cells. These boundaries are relatively straight (Figs 1C, 3A), suggesting that *IRO-C* mutant cells do not intermix with neighbouring *IRO-C*-expressing cells.

IRO-C clones in the dorsal part of the eye disc have smooth borders

IRO-C mutant cells differentiate ommatidia normally (Fig. 2A,B) but they form compact patches with smooth borders, as if mutant cells minimise their contact with surrounding cells (Fig. 3A,B). In contrast, their twin (+/+) clones have wiggly borders. The smooth boundaries are probably caused by the confrontation of *IRO-C*-expressing and non-expressing cells. The ventral part of the eye disc lacks *IRO-C* expression and hence ventral *IRO-C* clones have wiggly contours (Fig. 3B,D). Moreover, we have found *IRO-C* clones of dorsal origin (according to the position of their twin clones) located in the ventral part of the disc. Such clones form straight boundaries with dorsal cells but wiggly boundaries with ventral cells (Fig. 3C). The failure of two populations to mix has been ascribed to an autonomous function of a selector gene in specifying a characteristic 'affinity' to the compartment cells where it is expressed (García-Bellido, 1975) which causes them to maximise contact with cells of the same compartment, while minimising contact with cells of the other compartment. The properties of the *IRO-C* clones suggest that the homeodomain IRO-C proteins confer a dorsal-specific cell 'affinity'.

Wg and Hh-mediated inductions maintain the spatial distribution of *IRO-C* expression

The formation of the DV midline has been postulated to appear de novo in an initially homogeneous eye field via a mechanism that involves gradients of secreted signalling molecules, like Wg, expressed at the disc margin (reviewed by Reifegerste and Moses, 1999). Accordingly, the position and shape of the eye midline are defined at the point of lowest concentration of a dorsal (Wg) and a ventral (unidentified) signal and prior to the subdivision of the disc into dorsal and ventral expression domains. One way these signals might enable the DV midline to become the organiser is by inducing

the expression of *IRO-C* genes in the dorsal cells. According to our results, affinity differences between dorsal (*IRO-C*$^{+}$) cells and ventral (*IRO-C*$^{-}$) cells may be the main mechanism responsible for maintaining the straight DV midline. To investigate how the two models are reconcilable, we examined the expression of *IRO-C* and *wg* at first/early second instar stages and studied, by clonal analysis, their putative regulatory relationships.

At late first/early second instar, *IRO-C* expression is already restricted to the dorsal half of the disc (Fig. 4A). A groove marking the limit of *IRO-C* expression (Fig. 4A, inset), resembles that, described previously, along the presumptive DV boundary in the early third instar eye discs (Ready et al., 1976). Differences in affinity between dorsal and ventral cells probably induce this groove because dorsal *IRO-C* clones are also transiently surrounded by a fold (Fig. 3B, inset).

At early second instar *wg* is expressed in the presumptive dorsal region of the eye disc, (Fig. 4C; F. J. Díaz-Benjumea, personal communication). Later this expression evolves to dorsal and ventral anterior marginal domains (Fig. 4D; Ma and Moses, 1995). We assayed expression of *IRO-C* in cells lacking *dishevelled* activity (*dsh*^{VA153}) and therefore unable to transduce Wg signalling (reviewed by Cadigan and Nusse, 1997). Early and late induced *dsh*^{VA153} clones autonomously lack *ara/caup* expression (Fig. 5A-D), indicating that Wg is required continuously for *IRO-C* expression. This expression is normally downregulated in cells posterior to the morphogenetic furrow but it is maintained in dorsal-posterior clones of *shaggy* (*sgg*$^{M1-1}$) cells (Fig. 5E-G, compare with 5H), where the Wg pathway is constitutively active (Cadigan and Nusse, 1997). However, activation of *IRO-C* in ventral *sgg*$^{M1-1}$ clones is seen only occasionally in a subset of the mutant cells (not shown). Hence, we conclude that Wg signalling is necessary but not sufficient to activate *IRO-C* expression.

Another factor required for *IRO-C* expression is Hh. Similar to *wg*, *hh* is expressed in a dorsally restricted domain at late first/early second larval instar (Fig. 4E). Regulation of *IRO-C* by the Hh pathway was assayed in clones of cells deficient for the Hh receptor complex formed by Smoothened (Smo) and Patched (Ptc) (reviewed by Ruiz i Altaba, 1997). In *ptc*^{IIw} cells, a situation equivalent to constitutive activation of the Hh pathway in the receiving cells, *mirr-lacZ* (Fig. 5I-K) and *ara/caup* expression (not shown) are ectopically activated within the mutant cells and in some wild-type adjacent cells (Fig. 5I, inset). Late induced *ptc*^{IIw} clones (at 72-96 hours AEL) do not derepress *mirr-lacZ* (not shown). In *smo*^{3} clones, where Hh reception is blocked (Chen and Struhl, 1996), *ara/caup* expression is absent in the centre of the clone and strongly decreased in its periphery (Fig. 5L). This result, and the non-autonomous effect of *ptc*^{IIw} clones, suggest that a secreted signal, induced by Hh, rescues the loss of *hh* in the *smo*^{3} mutant cells. This factor could be Wg, as *wg* is derepressed in *ptc*^{IIw} clones in the anterior region of the eye disc (Domínguez and Hafen, 1997).

Early generalised expression of *hh* (in *ey-GAL4*; *UAS-hh*) dorsalises the eye (Fig. 6C,E,F), severely reducing its size (Fig. 6B). Similar effects have been reported for early misexpression of *wg* (Heberlein et al., 1998). Together, these observations and the previous data support a model in which both Wg and Hh signalling organise DV patterning by directing *IRO-C* expression. However, Wg and Hh do not meet the complete requirement for the postulated gradient model by Reifegerste and Moses (1999). First, their expression is already asymmetric in the early disc. Second, ubiquitous and high expression of Wg or Hh should prevent the formation of the straight DV boundary, while this is not the case (Fig. 6E,F; Heberlein et al., 1998).

IRO-C organising borders define the position of morphogenetic furrow initiation

Retinal differentiation is associated with the passage of the morphogenetic furrow which normally begins at the intersection of the DV midline with the posterior margin. The site of furrow initiation is widely assumed to be specified at the lowest point of concentration of Wg activity (Reifegerste and Moses, 1999). Our results show that *IRO-C* expression borders can non-autonomously recruit mutant and wild-type cells to form an eye provided they are located close to the disc margin. Thus, *IRO-C* may induce retinal differentiation through the local repression of *wg* at the disc margin, causing a sink of the Wg gradient. We therefore examined the expression of *wg* in relation to *IRO-C* borders.

At late second/early third instar, *wg* is expressed around the anterior dorsal and ventral disc margins (Ma and Moses, 1995; Treisman and Rubin, 1995). *wg* expression is not impeded within marginal *IRO-C* mutant clones (Fig. 7A). Thus, we conclude that an *IRO-C* expression border is sufficient to promote furrow initiation, even in the presence of *wg*.

In the wild-type eye, this process requires the positive action of Decapentaplegic (Dpp; Chanut and Heberlein, 1997; Pignoni and Zipursky, 1997) and Hh (Domínguez and Hafen, 1997; Borod and Heberlein, 1998). *dpp* is expressed around the posterior and posterior-lateral disc margin (Masucci et al., 1990; Blackman et al., 1991), symmetrically across the *IRO-C* expression border (Fig. 7B). Similarly, *dpp-lacZ* is activated straddling the border of an *IRO-C* clone abutting the disc margin (Fig. 7C). *hh* is expressed along the dorsolateral and posterior margin of the early third instar eye disc (Domínguez and Hafen, 1997; Borod and Heberlein, 1998). Just before morphogenetic furrow initiation, *hh* expression increases at the posteriormost region (Domínguez and Hafen, 1997 and Fig. 8D), which is the site where the *IRO-C* border intersects with the disc margin (M.D., unpublished). We investigated this modulation of *hh* expression in eye discs where the *IRO-C* border has been eliminated (by generalised expression of *ara* using the *ey-GAL4* driver). *hh-lacZ* expression initiates normally (Fig. 8A), but its levels fail to increase at the posteriormost domain (Fig. 8B). At mid/late third instar, *hh-lacZ* expression is completely eliminated from the posterior disc margin (Fig. 8C), a loss not due to generalised cell death, since *wg* expression around the posterior margin (Heslip et al., 1997) is not impeded in the mutant late third instar discs (not shown). Nor is the failure to maintain *hh* expression a consequence of the absence of ommatidial differentiation because *hh-lacZ* posterior expression is not eliminated in *atonal* mutant eye discs, where eye neurogenesis fails to initiate (Borod and Heberlein, 1998). Thus, an *IRO-C* expression border is needed to maintain and upregulate *hh* expression at the posteriormost margin, which is necessary for furrow initiation.

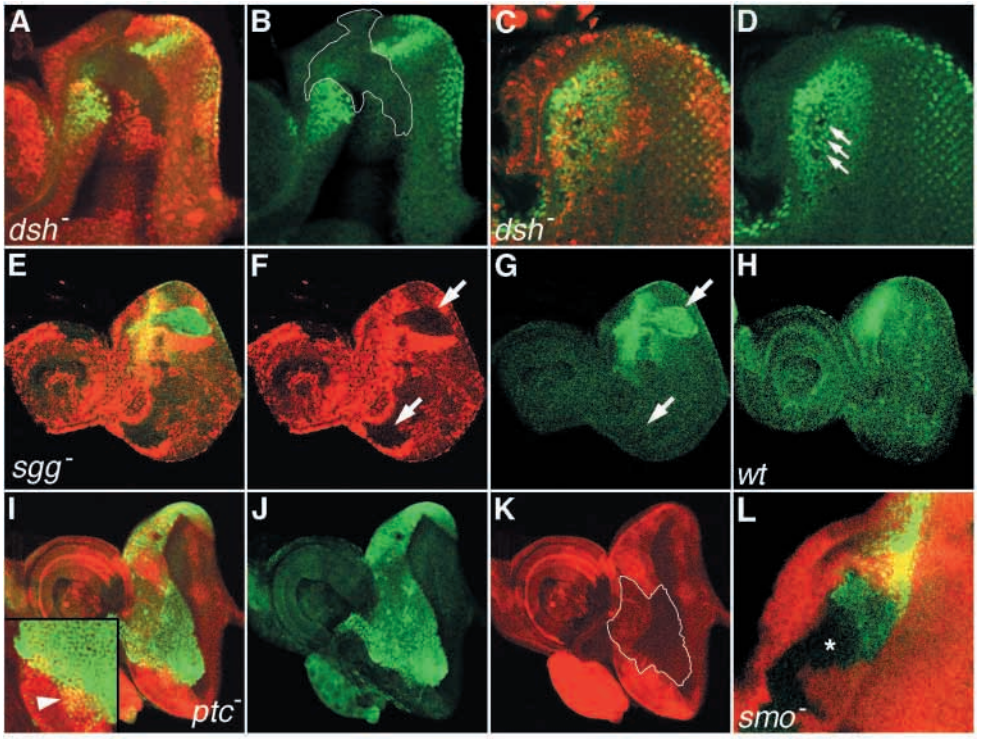

Fig. 5. *IRO-C* gene expression depends on Wg and Hh signalling pathways. (A-D) Caup/Ara expression (green) disappears in *dsh*^{VA153} clones induced at 24-48 hours (A,B) or 48-72 hours (C,D) AEL marked by the absence of anti-Myc staining (red). Arrows in D indicate some small clones. (E-G) Caup/Ara (green) are activated only within dorsal *sgg*$^{M1-1}$ clones. Clones were induced at 24-48 hours AEL and marked as above. (H) Caup/Ara expression in late third instar wild-type disc is restricted to the anterior dorsal region and some posterior differentiating ommatidia. (I-K) *mirr-lacZ* (green) expression is ectopically activated in ventral *ptc*^{llw} clones marked by the absence of anti-CD2 (red) staining. Note the non-autonomous *mirr-lacZ* induction (inset in I, arrowhead). (L) Caup/Ara (green) expression within an anterior-dorsal *smo*^{3} clone marked by the absence of anti-β-galactosidase staining (red). The levels of Caup/Ara expression within the clone are decreased in the periphery and absent in the centre of the clone (asterisk). White outline, in B and K delimit the clones.

The organising activity of *IRO-C* borders is mediated by the Fng/Notch pathway

Our analyses demonstrate that an *IRO-C* border is essential and instructive for growth, DV polarity and initiation of eye morphogenesis at both sides of the border. Nevertheless, the *IRO-C* is only expressed at the dorsal half of the eye disc (McNeill et al., 1997; Domínguez and de Celis, 1998) and encodes transcription factors (Gómez-Skarmeta et al, 1996; McNeill et al., 1997). Consequently, their non-autonomous effects should be mediated through a signalling pathway with long-range activity. It has been proposed that *fng* acts downstream of the *IRO-C* in the formation of the DV organiser (see Introduction). Consistently, dorsal *IRO-C* mutant cells exhibit autonomous derepression of *fng* expression (Fig. 7E).

Thus, eye patterning requires a dorsal expression of *IRO-C* that establishes a *fng* expression border. This leads to the localised activation of *Notch* along the DV midline (Cho and Choi, 1998; Domínguez and de Celis, 1998; Papayannopoulos et al., 1998). Accordingly, the elimination of the *fng* expression border (in *ey-GAL4; UAS-fng*) or the block of *Notch* activation (in *ey-GAL4; UAS-Notch*^{ecd}) produces a loss-of-eye phenotype equivalent to that caused by misexpression of *caup* (Domínguez and de Celis, 1998). This effect on eye development is likely caused by the failure to maintain *hh* (Fig. 8E; not shown). In *ey-GAL4; UAS-Notch*^{ecd} the restricted expression of *IRO-C* is unaffected (Domínguez and de Celis, 1998), supporting the proposal that the Fng/Notch pathway acts downstream of the *IRO-C* border.

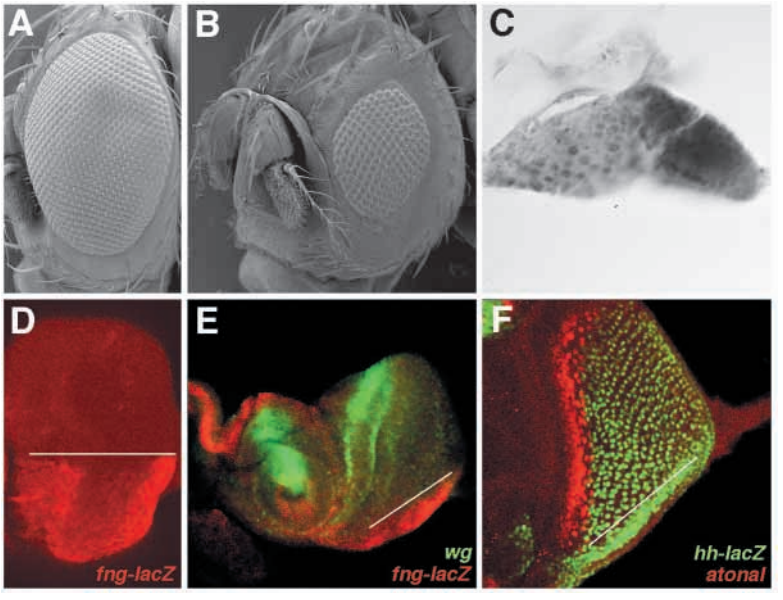

Fig. 6. Early and generalised expression of *hh* dorsalises the eye. Scanning microscope images of wild-type (A) and *ey-GAL4; UAS-hh* (B) adult eyes. (C,E,F) *ey-GAL4; UAS-hh* eye discs are stained with anti-Caup (C), anti-Wg and anti-β-galactosidase (*fng-lacZ*, E) and anti-Atonal and anti-β-galactosidase (*hh-lacZ*, F). The white lines mark the position of the DV midline. Early generalised expression of *hh* expands *caup*/*ara* expression ventrally. *fng* expression domain is restricted (compare E to wild type D). (F) Most ommatidia have dorsal chirality, monitored by the orientation of the rows of *hh-lacZ* expression (green) in R2/R5 cells. The furrow (marked by *atonal* staining) has progressed normally. All discs shown are at mid to late third instar.

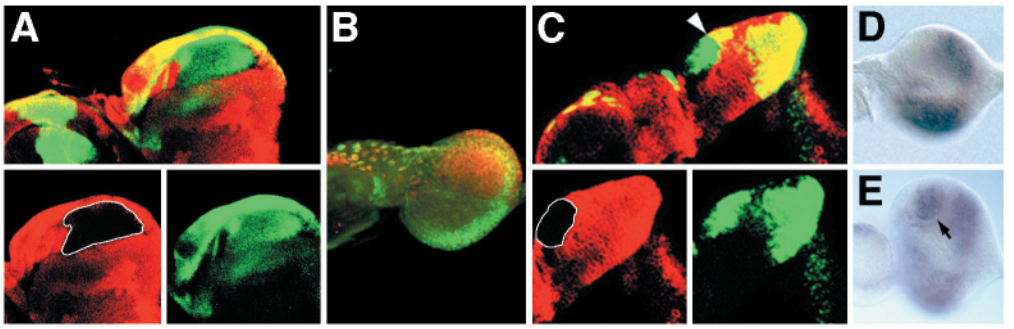

Fig. 7. Effect of *IRO-C* clones on *dpp*, *wg* and *fng* expression. Clones are marked by the absence of anti-Myc staining (red in A,C) and by the epithelial fold that surrounds it (arrow, E). (A) *wg-lacZ* expression (green) is detected within the *IRO-C* clone. (B) Early third instar *dpp-lacZ* disc stained with anti-Caup (red) and anti-β-galactosidase (green). (C) *dpp-lacZ* expression (green) is activated straddling the *IRO-C* clone border (arrowhead). (D) Wild-type *fng* expression monitored by in situ hybridisation at mid-third instar stage. (E) Eye disc of a similar stage carrying an anterior-dorsal *IRO-C* clone, showing ectopic *fng* expression. White outlines, delimit the clones.

DISCUSSION

We have shown that eye patterning relies on true DV compartments and that the *IRO-C* acts as the dorsal selector gene. IRO-C proteins play two major functions: (1) the establishment of distinct cell affinities in dorsal versus ventral cells and (2) the generation of the *fng* expression border. This dual function ensures the position and shape of the DV organiser.

IRO-C acts as the eye dorsal selector gene

Our study indicates that in the eye disc the *IRO-C* fulfils the requirements for a compartment selector gene (García-Bellido, 1975). A prediction of the compartment selector hypothesis is

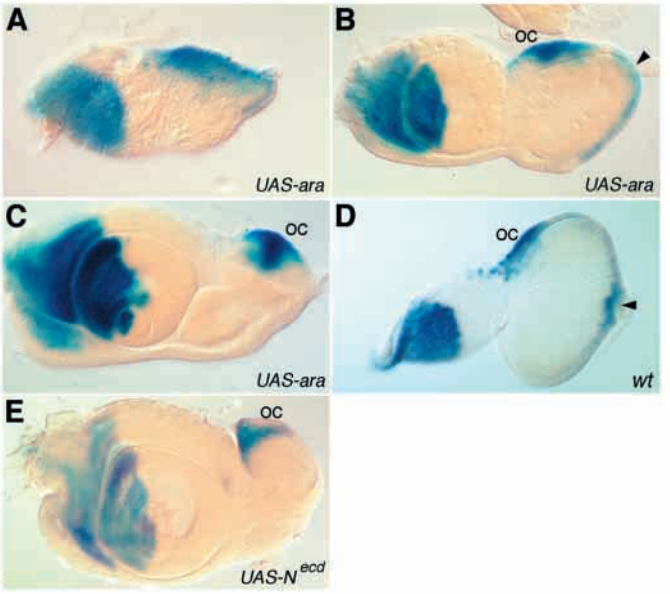

Fig. 8. *hh* expression during the onset of furrow initiation depends on induction by the DV boundary. *hh-lacZ* expression in combination with different UAS lines and *ey-GAL4* (A-C and E) and in wild type (D). Discs shown are at early (A), mid (B and D) and late third (C and E) instar. A and B show that *hh-lacZ* expression initiates normally in the mutant discs but fails to increase at the posteriormost region (arrowhead in B) of the eye disc at mid third instar (compare B to wild type in D). Posterior *hh* expression disappears at late-third instar and only remains around the ocellar (oc) region (C,E).

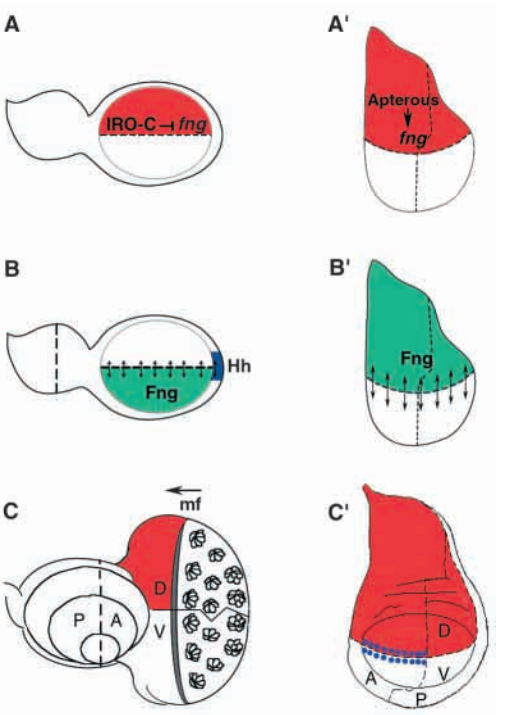

Fig. 9. A model for eye development and its comparison with wing development. (A-C) Three sequential stages of eye development and (A′-C′) similar stages in the wing. (A,A′) At late first/early second instar, the expression of the dorsal selector genes *IRO-C* and *apterous* (red) bisects the eye (A) and wing (A′) discs into dorsal and ventral compartments. The different affinity between *IRO-C*-expressing and non-expressing cells (A) and *apterous*-expressing and non-expressing cells (A′) establishes straight DV compartment boundaries (dashed lines). Restricted expression of the selector genes also establishes the *fng* expression domain (green). At both sides of the *fng* expression border Notch is activated (double arrows). Notch activation along the DV midline is required for eye disc growth and for upregulation of *hh* expression (dark blue) at the posterior disc margin (the 'firing centre'), a prerequisite for morphogenetic furrow (mf) initiation. As cellular differentiation progresses in the eye disc (C), *IRO-C* expression is downregulated posterior to the furrow. In contrast, *apterous* expression (C′) is heritably maintained throughout development. Posterior repression of *IRO-C* expression may be necessary for normal polarisation of the ommatidia since misexpression of *ara* or *caup* (driven by photoreceptor cell-specific GAL4 drivers) disrupts ommatidial polarity (M. D., unpublished results). (C,C′) The anterior (A) and posterior (P) compartments of the eye-antennal disc and wing disc are also indicated.

that the activity of a selector gene confers to cells a specific affinity, such that cells expressing the selector do not intermingle with non-expressing cells. This leads to a straight boundary between adjacent compartments (reviewed by Blair, 1995). The borders of *IRO-C* clones in the dorsal half of the eye disc are smooth, contrary to the wiggly boundaries of their twin (*IRO-C*$^{+}$) clones, and the endogenous DV limit of *IRO-C* expression is a straight line. Previous cell lineage studies showed that clones run straight along the eye disc midline but have wiggly boundaries when located away from the border (Domínguez and de Celis, 1998). We suggest that this cell lineage restriction is associated with the *IRO-C* expression border.

Compartment selector genes are also expected to control the type of structures the compartment cells will make (García-Bellido, 1975). The dorsal and ventral ommatidia are identical structures, making it difficult to firmly establish whether loss of *IRO-C* function causes a transformation from dorsal to ventral cell identity. However, such transformation is suggested by the activation of the ventral-specific gene *fng* by dorsal *IRO-C* mutant cells. Moreover, generalised expression of *caup* in the eye disc causes duplication of dorsal bristles in the ventral head region (Domínguez and de Celis, 1998) and the loss of *IRO-C* causes transformation of dorsal head capsule into ventral structures (F. C. and S. C., unpublished), supporting the dorsal selector function of *IRO-C* in the eye-antennal disc.

Organising activities of the DV compartment boundary of the eye

It has been shown that an organiser centre, established by localised activation of *Notch* at the DV midline before the onset of retinal differentiation, regulates eye growth and polarity (Cho and Choi, 1998; Domínguez and de Celis, 1998; Papayannopoulos et al., 1998). The restricted dorsal expression of *IRO-C* genes and the small eye phenotype resulting from *IRO-C* early generalised expression suggested that the border of *IRO-C* expressing and non-expressing cells would be critical to establish this organiser (Cho and Choi, 1998; Domínguez and de Celis, 1998). This role is now demonstrated by the finding that any new border of *IRO-C* expressing and non-expressing cells is essential and instructive for eye growth and differentiation. Thus, both removal of *IRO-C* activity in the dorsal half of the eye and clones of cells expressing *IRO-C* genes in any position promote outgrowths and ectopic ommatidia fields that encompass wild-type and mutant territory. Development of these eyes initiates from the intersection of the disc margin and the *IRO-C* expression border and progresses orthogonally to the *IRO-C* border as in the normal eye.

Ommatidial polarity is reorganised around the new dorsal *IRO-C* clonal borders. This effect is similar to that of dorsal *mirr*^{e48} clones (McNeill et al., 1997) and ventral *fng* clones (Cho and Choi, 1998; Domínguez and de Celis, 1998; Papayannopoulos et al., 1998), though the borders of *mirr*^{e48} clones only caused repolarisation of adjacent ommatidia. An organising activity of *IRO-C* has also been shown for the notum-wing hinge border of the wing disc although the patterning of this region around the *IRO-C* expression border does not involve a compartmental lineage-restriction boundary (Diez del Corral et al., 1999).

In the eye, the *IRO-C* organising activity is probably mediated by its ability to restrict the expression of *fng* to the ventral region of the eye disc, thus establishing a boundary of *fng*-expressing and non-expressing cells at the DV midline. This conclusion is derived from the reciprocal patterns of expression of *IRO-C* and *fng* in early eye discs, the loss of ventral *fng* expression after generalised expression of *mirr* (Cho and Choi, 1998) or *caup* (Domínguez and de Celis, 1998) and the derepression of *fng* in dorsal *IRO-C* mutant cells (this work). The weak effects of *mirr* clonal borders are most likely due to the presence in *mirr* clones of Ara and Caup, which would partially substitute for Mirr activity and repress *fng* expression. Note also that dorsal *mirr* clones do not induce development of ectopic eye fields. Thus, expression of *fng* should be totally derepressed only in *IRO-C* clones and, only in this case, the maximal activation of *Notch* would be attained. Moreover these results suggest that the polarising activity of the DV midline depends on a lower level of Notch activation than the growth promoting activity of such a border.

Nevertheless, we note that the effects of *IRO-C* borders on polarity and growth are much stronger that those described for *fng* borders (Cho and Choi, 1998; Domínguez and de Celis, 1998; Papayannopoulos et al., 1998). In addition, high levels of ectopic expression of *IRO-C* genes in clones of cells, using the UAS/GAL4 system, occasionally promotes ectopic eye development in the dorsal part of the eye disc. Thus, factors other than *fng* may help mediate IRO-C organising activity.

The DV organiser participates in the positioning of the 'firing centre' for eye development

The site of initiation of retinal differentiation ('firing centre') has been suggested to depend on the balance of positive and negative signals such as Dpp, Wg and Hh (reviewed by Treisman and Heberlein, 1998). Our study shows that an *IRO-C* border is instructive and decisive for this event. Expression of *dpp* is activated de novo by *IRO-C* borders even though expression of the *dpp* repressor Wg is not eliminated. *hh* expression is upregulated at the posterior margin of the eye disc just prior to induce the initiation of the morphogenetic furrow (Domínguez and Hafen, 1997). Our results indicate that this upregulation is a consequence of Notch activation at the *IRO-C/fng* border (Fig. 9). When activation of Notch is not attained (either by the ubiquitous expression of *ara* or *fng*) or when Notch signalling is blocked (by expression of a dominant negative form, *Notch*^{ecd}), *hh* expression at the posterior region of the disc is neither enhanced nor maintained and the eye fails to form. Thus, the development of non-eye or small-eye in these situations could be attributed to the failure to maintain Hh signalling from the posterior marginal cells. Conversely, development of ectopic eyes associated with *IRO-C* clones would result from the generation of ectopic *fng*-expression borders leading to ectopic activation of Notch and concomitant upregulation of *hh* expression in the marginal cells.

Compartment boundaries and pattern formation in the eye

Our results build a strong case for eye development occurring by the same basic strategy as other discs and argue against the previous models on eye development, claiming that eye patterning is somehow unique and different. Our findings support a model for eye development based on the compartment hypothesis (reviewed by Lawrence and Struhl,

1996) in which formation of an affinity boundary influences pattern formation and growth in a developmental field. Differential expression of a selector gene in a field of cells generates an interface between cells with different identities (Fig. 9). According to our model, the DV midline is a cell lineage restriction border which controls growth and patterning in the entire eye disc and which is established by the early expression of the *IRO-C* genes in the dorsal half of the eye disc. Interactions at the interface between dorsal (*IRO-C*$^{+}$) and ventral (*IRO-C*$^{-}$) cells can indeed generate non-autonomously an independent eye.

IRO-C genes are expressed throughout larval eye development only in dorsal cells and this expression is maintained by both Hh and Wg signalling pathways. Expression of *ara/caup* disappears in *dsh*^{VA153} mutant clones, whereas it is maintained in dorsal-posterior *sgg*$^{M1-1}$ clones, demonstrating a requirement for the Wg signalling pathway in the maintenance of *IRO-C* expression. This is in accordance with the reported regulation of *mirr-lacZ* by Wg (Heberlein et al., 1998). Since *wg* is expressed at both margins of the eye disc from late second larval stages, the presence of a repressor of *mirr-lacZ* expression in the ventral region of the disc was proposed (Heberlein et al., 1998). However, we suggest this repressor would not be necessary since here we show that *wg* is expressed in a restricted dorsal domain at earlier stages. We have also found that *IRO-C* gene expression is scarcely activated in ventral *sgg*$^{M1-1}$ clones but is readily activated in cells where Hh signalling is constitutively activated (assayed in *ptc*^{llw} clones). Thus, we propose that high levels of both Wg and Hh pathways are required for the expression of *IRO-C* in the early eye disc, this being only attained in the dorsal region of the disc. These regulatory interactions between Hh, Wg and *IRO-C* may account for the effects of the misexpression of Wg (Ma and Moses, 1995; Treisman and Rubin, 1995; Heslip et al., 1997) and Hh (Chanut and Heberlein, 1995; Heberlein et al., 1995) in DV ommatidial polarity and morphogenetic furrow initiation.

A mechanistically similar DV organiser has been found to control the development of the vertebrate limb bud (Laufer et al., 1997; Rodríguez-Esteban et al., 1997). In this primordium, a border of *Radical-fringe* expression establishes the apical ectodermal ridge (AER), a specialised group of cells that controls limb bud outgrowth (reviewed by Irvine and Vogt, 1997). *Radical-fringe* expression is regulated by the LIM-homeodomain protein Lhx2, expressed only by the dorsal cells (Rodríguez-Esteban et al., 1998). The analogy of the two systems extends to the requirement of the AER for the positioning and maintenance of posterior *Sonic hedgehog* expression (Zuñiga and Zeller, 1999). Thus, it seems that the principles identified in the insect eye and wing may apply more widely to animal development.

We are most grateful to J. Modolell and P. Lawrence for their constant help and advice; to other colleagues and members of our laboratories for positive criticisms; to R. Barrios, Y. N. Jan and S. M. Cohen for antibodies; and to J. D. Wasserman and the Bloomington Stock Centre for stocks. Predoctoral fellowships from the Ministerio de Educación y Ciencia and Comunidad Autónoma de Madrid to F. C. and R. D del C., respectively, are acknowledged. This work was supported by grants 07B/0033/1997 from Comunidad Autónoma de Madrid to S. C., PB93-0181 (from Dirección General de Investigación Científica y Técnica) and RG 0042/98B (from Human Frontier Science Program) to J. Modolell, an institutional grant from Fundación Ramón Areces to the Centro de Biología Molecular Severo Ochoa and a TMR Marie Curie Research Training Grant to M. D.

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