

Control of *Drosophila* eye specification by Wingless signalling

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SUMMARY

Organ formation requires early specification of the groups of cells that will give rise to specific structures. The Wingless protein plays an important part in this regional specification of imaginal structures in *Drosophila*, including defining the region of the eye-antennal disc that will become retina. We show that Wingless signalling establishes the border between the retina and adjacent head structures by inhibiting the expression of the eye specification genes *eyes absent*, *sine oculis* and *dachshund*. Ectopic Wingless signalling leads to the repression of these

genes and the loss of eyes, whereas loss of Wingless signalling has the opposite effects. Wingless expression in the anterior of wild-type discs is complementary to that of these eye specification genes. Contrary to previous reports, we find that under conditions of excess Wingless signalling, eye tissue is transformed not only into head cuticle but also into a variety of inappropriate structures.

Key words: *Drosophila*, Wingless, Axin, Eyes absent, Sine oculis, Dachshund, Eye, Transdetermination, Transdifferentiation

INTRODUCTION

Organ development comprises a cascade of fate decisions, typically starting with a broad regional specification, followed by increasingly elaborate differentiation. The *Drosophila* imaginal disc provides a useful system in which to address these processes; we focus on the regional specification of cells that will form the compound eye. One of the best characterised mechanisms for defining regional identities involves compartment formation by selector genes – transcription factors that are necessary and sufficient to direct a particular compartmental fate. For example, in the *Drosophila* wing disc, anteroposterior (AP) and dorsoventral (DV) compartments are specified by the differential expression of the selector genes *engrailed* and *apterous* in the posterior and dorsal compartments, respectively (Diaz-Benjumea and Cohen, 1993; Garcia-Bellido et al., 1973; Morata and Lawrence, 1975). The borders between compartments then acquire ‘organiser’ characteristics, and regulate cell proliferation and patterning (Sanicola et al., 1995; Tabata et al., 1995; Zecca et al., 1995). In addition to the compartmentalisation of imaginal discs, there is a process of regional subdivision, for example between wing-blade and notum, and Wingless and epidermal growth factor receptor signalling contribute to this subdivision (Baonza et al., 2000; Ng et al., 1996; Wang et al., 2000; Zecca and Struhl, 2002a; Zecca and Struhl, 2002b).

The process of compartmentalisation of the eye-antennal imaginal disc differs significantly from the mechanism used by other imaginal discs. The eye-antennal discs are primordia of much of the adult head capsule, as well as of the eyes and antennae. Clonal analysis indicates that AP subdivision only occurs in the antennal part of the disc (Morata and Lawrence,

1979). In the eye anlage, patterning is associated with a wave of neural differentiation that sweeps across the disc, from posterior to anterior. This wave of development is preceded by an indentation called the morphogenetic furrow (Ready et al., 1976). In many ways, the furrow represents a dynamic AP border and, like the AP border of the wing disc, it has long-range patterning abilities (Baonza and Freeman, 2001; Heberlein and Moses, 1995; Treisman and Heberlein, 1998). Prior to retinal development, which is initiated by the furrow, the eye-antennal disc is regionally subdivided into head, eye and antennal domains.

Several genes and signalling pathways are known to contribute to the subdivision of the eye/antennal disc. The Notch and EGFR pathways are required for the eye/antennal division (Kumar and Moses, 2001). The retinal cells are specified during embryonic and larval stages by the action of the Pax6 transcription factors Eyeless (Quiring et al., 1994) and Twin of eyeless (Czerny et al., 1999), in conjunction with the downstream transcription factors Eyes absent (Bonini et al., 1993), Sine oculis (Cheyette et al., 1994; Serikaku and Ootsuma, 1994) and Dachshund (Mardon et al., 1994). This hierarchy of transcription factors, which act in a complex series of feedback loops, comprises a ‘cassette’ of eye specification genes (Curtiss and Mlodzik, 2000; Halder et al., 1998; Hazelett et al., 1998). Finally, in a role that resembles its function in regional specification in the wing disc, Wingless participates in distinguishing cells that will form eye and adjacent head cuticle (Royet and Finkelstein, 1997), although the mechanism of this subdivision has been unclear.

Like several other pathways, Wingless signalling has multiple functions in eye development. In the third instar disc, Wingless expression is restricted to the lateral margin, anterior

to the progressing furrow, where it prevents ectopic furrow initiation (Ma and Moses, 1995; Treisman and Rubin, 1995). Earlier, the localised repression of Wingless by Dpp is responsible for triggering the initiation of the furrow at the posterior margin of the disc (Chanut and Heberlein, 1997; Domínguez and Hafen, 1997; Pignoni and Zipursky, 1997). In contrast to these processes, little is known about the function of Wingless in restricting the extent of the eye field (i.e. defining the border between eye and head cuticle). The observation that ectopic Wingless signalling prevents eye development (Lee and Treisman, 2001; Royet and Finkelstein, 1997), implies that Wingless might regulate the eye specification genes, but current evidence suggests otherwise: Wingless appears to be genetically downstream of them (Hazelett et al., 1998).

In this work, we have analysed the relationship between the Wingless signalling pathway and the eye specification genes. Our data imply that Wingless signalling initiates the border between eye and head, and thereby controls the specification of the retinal territory, by negatively regulating the expression of eye specification genes. Moreover, we show that Wingless activity can promote developmental plasticity, leading to transdetermination of eye cells.

MATERIALS AND METHODS

Genetic strains

We have used the stocks *axn^h* (H. Musisi and M. Bienz, personal communication); *fz^{H51}* and *fz-2^{C1}* (Chen and Struhl, 1999); and *sine oculis-lacZ* and *wingless-lacZ*, both of which are described in FlyBase (<http://fly.ebi.ac.uk:7081/>). The UAS lines used were *UAS-armK45* (Brunner et al., 1997) and *UAS-eyal* (Pignoni et al., 1997).

Generation of mosaics

Mitotic clones were generated by Flp-mediated mitotic recombination (Xu and Rubin, 1993). Recombination was induced different times during the development (60, 84 and 108 hours after egg laying) and by a 1 hour 30 minutes heat shock at 37°C. Mutant clones in a *Minute* background for *axn^h* were marked by the absence of β -galactosidase staining, using *y w hsp70-flp; FRT⁸² arm-lacZ M(3)/TM6B* stock (Domínguez et al., 1998). These flies were crossed to *y w; FRT⁸² axn^h/TM6B*. Mutant clones for *fz* and *fz2* were marked by the absence of GFP crossing males *hsp70-flp; tub-GFP FRT^{2A}* by females *yw fz.fz2 FRT^{2A}/TM6B*.

Clones of cells expressing GAL4 were induced 24–48 or 48–72 hours after egg laying by 12–15 minute heat shocks at 37°C in flies of the following genotypes:

(1) *y w hsFLP1.22; Act5C<FRT yellow⁺ FRT> GAL4 UAS-GFP/UAS-arm** and

(2) *y w hsFLP1.22; Act5C<FRT yellow⁺ FRT> GAL4 UAS-GFP/+; UAS-arm*/so-lacZ*.

The flip-out of the *<FRT yellow⁺ FRT>* cassette results in the expression of the transcriptional activator GAL4 gene under the control of the *Act5C* promoter (Ito et al., 1997). Clones were detected by expression of GFP, and were analysed in third instar larvae.

axin⁻ clones and *axin⁻* clones expressing *UAS-eyal* were generated using the GAL4/GAL80 system (Lee and Luo, 1999). *UAS-eya FRT⁸² axn^h/TM6B* and *FRT⁸² axn^h/TM6B* females were crossed to *yw Hs flp tub GAL4 UAS-GFP; tub GAL80 FRT⁸²/+* males.

Scanning electron microscopy

Scanning EM was performed as previously described (Domínguez et al., 1998).

Immunohistochemistry

Eye imaginal discs from third instar larvae were stained as described (Gaul et al., 1992). The following antibodies were used: rabbit and mouse anti- β -galactosidase (Cappel); mouse and rat anti-Elav (used at 1:50 and 1:100, respectively) (O'Neill et al., 1994); and mouse anti-Dll (Díaz-Benjumea and Cohen, 1995) and anti-So (Cheyette et al., 1994). Anti-Elav, anti-Eya, anti-Wg and anti-Dac were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. Alexa 488- and 594- (Molecular Probes) and Cy5- (Jackson ImmunoResearch) conjugated secondary antibodies were used at dilutions of 1:200.

FACS analysis

FACS was performed as described previously (Neufeld et al., 1998).

RESULTS

Ectopic activation of the Wingless pathway transforms eye cells to other fates

In order to analyse the effect of ectopic activation of the Wingless pathway during the development of the eye-antennal imaginal disc, we induced clones either mutant for the negative regulator of Wingless signalling, Axin, or expressing an activated form of Armadillo (*Arm**) (Brunner et al., 1997). Axin is a scaffold protein necessary for the phosphorylation of Armadillo/ β -catenin by the glycogen synthase kinase 3b homologue, Shaggy (Hamada et al., 1999; Ikeda et al., 1998; Willert et al., 1999; Yanagawa et al., 2000). Phosphorylated Armadillo is ubiquitinated and degraded by the proteasome (Jiang and Struhl, 1998). Thus, the loss of Axin causes the ectopic activation of the Wingless pathway.

Consistent with earlier results in which Wingless signalling was ectopically activated (Lee and Treisman, 2001; Royet and Finkelstein, 1997), we find that *axin⁻* or *arm** cells show considerable overgrowth and cannot differentiate as ommatidia. But in contrast to previous reports of ectopic Wingless signalling early in eye development, we find that the mutant tissue induces a variety of inappropriate developmental fates (Fig. 1A–C). Previously, the mutant tissue has been described as always differentiating as dorsal head (Royet and Finkelstein, 1997). In addition to the frons cuticle with a characteristic ridged appearance that corresponds to dorsal head, we frequently find other structures including naked cuticle and tube-like overgrowths with macrochaetae (1C), none of which correspond to recognisable head structures. Some of the tube-like overgrowths resemble legs or antennae, although we have not seen specific elements (e.g. bracts) to confirm this. Therefore, ectopic Wingless signalling can respecify eye cells to adopt a variety of fates.

Given the resemblance of some of the structures caused by ectopic Wingless signalling to legs and antennae, we have examined whether *axin⁻* or *arm** clones express *Distal-less*. This gene is required to specify the distal domains of the leg, antenna and wing discs and is never expressed in the eye disc during the third larval instar (Kumar and Moses, 2001). Thus, its ectopic expression would indicate a change of fate from eye to leg, antenna or wing. Approximately 5% of clones ($n > 100$) did indeed express *Distal-less* (Fig. 1D); notably, the ectopic expression of *Distal-less* was always associated with tube-like overgrowth in the disc (Fig. 1E–G). This result confirms our conclusion that eye cells receiving ectopic Wingless signal are

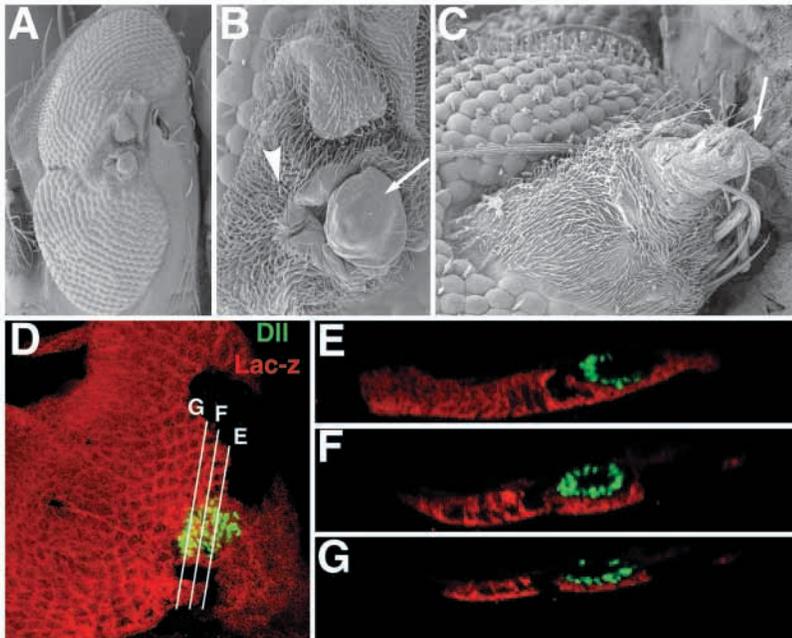


Fig. 1. Activation of the Wingless signalling pathway blocks eye differentiation. (A-C) Scanning EM images of adult eyes with *axin*⁻ clones. *axin* mutant cells induce the differentiation of frons cuticle corresponding to the dorsal head (arrowhead in B), but also naked cuticle (arrow in B). (C) Large *axin* mutant clone causing a tube-like overgrowth with multiple macrochaetae (arrow). (D-G) In third instar eye discs, *axin* mutant clones (which lack red anti-*lacZ* staining) occasionally express Distal-less (green); anterior towards the left. The expression of Distal-less is associated with tube-like overgrowth. These overgrowths appear in a different focal plane that can be seen in transverse sections of the disc (E-G; the positions of the sections are indicated by the white lines in D).

not only transformed to dorsal head structures. Instead, Wingless signalling respecifies eye cells into a variety of fates, which include head cuticle, but also include more dramatic transformations to cells with properties of legs and/or antennae. This phenomenon resembles transdetermination, which has been shown to be promoted by Wingless in other *Drosophila* tissues (Johnston and Schubiger, 1996; Maves and Schubiger, 1998).

Ectopic Wingless signalling disrupts proliferation in the eye disc

One of the phenotypes caused by the ectopic activation of Wingless in the eye discs is tissue overgrowth. In the eye discs, as in other tissues, differentiation is accompanied by the cessation of cell proliferation; all the cells are arrested in G1 in the morphogenetic furrow. After the furrow, those cells not incorporated into the precluster undergo one more division, known as the second mitotic wave (Ready et al., 1976). We compared the cell cycle state of wild-type and *axin*⁻ cells from eye/antennal discs containing large numbers of clones with ectopic Wingless activation; in these discs, the mutant cells expressed GFP [using the Gal4/Gal80 system (Lee and Luo, 1999)] so could be separated from wild-type cells by FACS sorting. Forty-two percent of wild-type cells were in G1, whereas 24% and 34% were in S and G2, respectively (Fig. 2A, black trace). Note that most of the wild-type cells in S or G2 are actually in the antennal region of the disc or anterior to the morphogenetic furrow: immunostaining shows few in the posterior eye region, these being limited to the second mitotic wave (Baker and Yu, 2001). In the same eye/antennal discs, *axin*⁻ cells (Fig. 2A, red trace) showed a significant increase in the proportion of cells in S and G2 (30% and 38%, respectively), at the expense of cells in G1 (32%). In conjunction with the observation that overgrowth is seen in *axin*⁻ and *arm*^{*} clones anterior to the furrow, but is much greater on average in clones posterior to the furrow, this indicates that cells receiving excess Wingless signalling

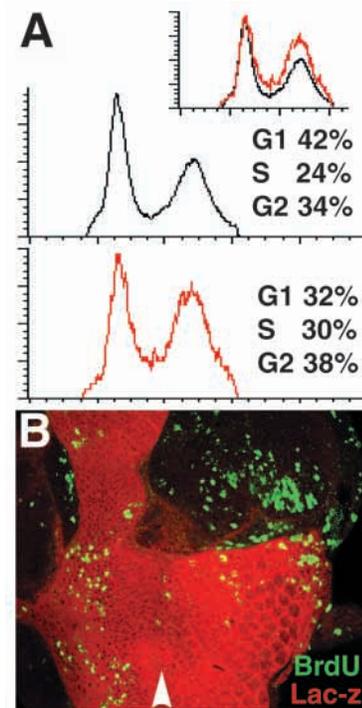


Fig. 2. Excess proliferation in *axin*⁻ mutant clones. (A) *axin*⁻ mitotic clones were induced 60 hours after egg laying (AEL) using the GAL4/GAL80 system and eye/antennal discs were analysed by FACS at 108 hours AEL. Using this marker system, the mutant cells are positively marked by GFP. The black trace represents GFP-negative (wild-type) cells and red line represents GFP-positive (*axin*⁻) cells. The proportion of cells in G2 and S is increased in *axin*⁻ cells, consistent with the hyper-proliferation phenotype observed in these clones. (B) An *axin*⁻ *Minute* clone (marked by absence of red) stained with anti-BrdU (green). The focal plane shown is at the level of the clonal overgrowth so the S-phase associated with the second mitotic wave (just posterior to the morphogenetic furrow, arrowhead) is not seen.

overproliferate and are not arrested by the passage of the furrow. Consistent with this conclusion, we observe substantial excess BrdU incorporation posterior to the furrow in *axin*⁻ clones (Fig. 2B).

Ectopic Wingless signalling represses eye selector genes

The loss of eye identity caused by the ectopic activation of Wingless, suggests a possible function for Wingless in the regulation of the eye selector genes. The top of the genetic hierarchy involved in eye specification appears to be the Pax6 homologue, Eyeless (Halder et al., 1995; Quiring et al., 1994). In the third instar eye disc the expression of Eyeless is restricted to the region anterior to the furrow and, despite the Wingless-induced inhibition of eye development, the expression of Eyeless in this region is not affected by *axin*⁻ clones (Lee and Treisman, 2001). This lack of an effect anterior to the furrow, despite the overgrowth and abnormal Distal-less expression in the same region, implies that misregulation of Eyeless is not the primary cause of the transformations caused by ectopic Wingless activity.

Downstream of Eyeless (although feedback relationships makes the epistatic relationship complex) are other transcription factors required for eye specification, including Eyes absent, Sine oculis and Dachshund (Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994; Serikaku and Otousa, 1994). A phenotype similar to *axin*⁻ clones of excess proliferation and consequent overgrowth is caused by loss of Eyes absent and Sine oculis (Pignoni et al., 1997). Moreover, as in *axin*⁻ clones (Lee and Treisman, 2001), clones mutant for *sine oculis* ectopically express Eyeless in the region posterior to the furrow [see fig. 3G by Pignoni et al. (Pignoni et al., 1997)]. The similar mutant phenotypes shown by the loss of function of these genes and the ectopic activation of Wingless signalling make them good candidates to be regulated by the Wingless pathway.

We therefore analysed the expression pattern in third instar eye discs of Eyes absent, Sine oculis and Dachshund in *axin*⁻ and/or *arm*^{*} mutant clones. At this stage, Dachshund is expressed at high levels on either side of the morphogenetic furrow, whereas Eyes absent and Sine oculis are expressed in all the cells of the eye primordium (see Figs 5, 6) (Bonini et al., 1993; Cheyette et al., 1994; Curtiss and Mlodzik, 2000; Mardon et al., 1994). In order to produce large patches of mutant tissue, we have used the *Minute* technique (Morata and Ripoll, 1975). We find that in *axin*⁻ *M*⁺ clones the expression of Eyes absent in front of the furrow is always autonomously eliminated (Fig. 3). This effect is not only seen in large clones that touch the eye margin but also in small internal clones (Fig. 3A,I-K). Identical results were obtained with Sine oculis and Dachshund: their expression was autonomously lost from anterior *axin*⁻ *M*⁺ clones (not shown). Consistent with these results, in *arm*^{*}-expressing clones Eyes absent, Dachshund and *sine oculis* (detected with a *lacZ* reporter construct) were

similarly autonomously eliminated (Fig. 4A-C). We therefore conclude that Wingless signalling represses the expression of the eye selector genes *eyes absent*, *dachshund* and *sine oculis* anterior to the morphogenetic furrow. Posterior to the furrow, however, some clones express high levels of Eyes absent (e.g. Fig. 3A,B,F-H; Fig. 4C), and Dachshund (e.g. Fig. 4B). This effect is always associated with overgrowth, and this expression is restricted to only some cells in these clones.

In order to analyse the temporal requirement of the Wingless pathway in the regulation of Eyes absent, we have induced *axin*⁻ *M*⁺ clones at different stages of eye development. We

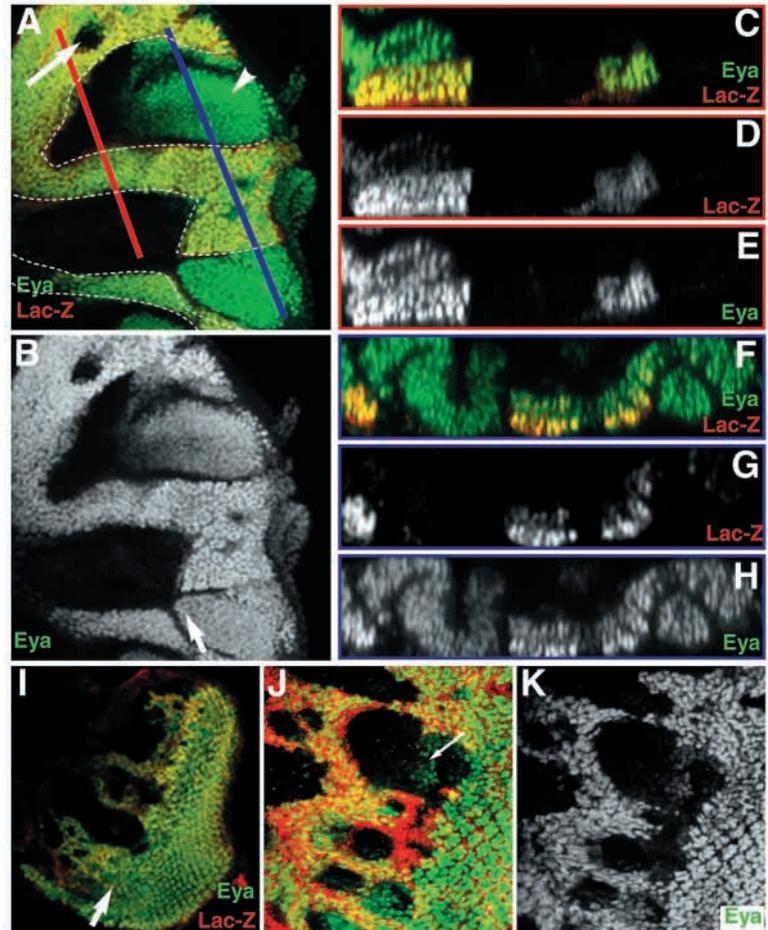


Fig. 3. Wg signalling regulates the expression of Eyes absent. *axin*⁻ *M*⁺ clones were induced 60±12 (A-H) and 108±12 hours AEL (I-K). In all panels, Eyes absent protein is shown in green and mutant clones are indicated by absence of red anti-*lacZ* staining. (A,B) In *axin* mutant clones (surrounded by broken lines) the expression of Eyes absent is abolished in the region anterior to the morphogenetic furrow (approximate furrow positions are marked with arrows in B and I); note, however, that Eyes absent is present in cells in the posterior region of the eye discs (arrowhead). Eyes absent expression is also eliminated in small internal clones anterior to the furrow (arrow in A). (C-E) A transverse section in the anterior region of the disc shown in A (red line) confirms that Eyes absent expression is autonomously eliminated from the clone. (F-G) In a more posterior transverse section from the same disc (blue line), we observe that Eyes absent is expressed in mutant cells. The overgrowth of the mutant cell leads to the folded morphology of the tissue. (I-K) Small clones induced late in eye development also prevent the expression of Eyes absent. However, in some mutant cells close to the morphogenetic furrow (position indicated with arrows in I). Eyes absent is still present at low levels (arrow in J). Panels J and K show a different disc from I, at higher magnification.

find that ectopic expression of Wingless is sufficient to repress Eyes absent throughout the whole of eye disc development. However, in some clones anterior to the furrow that were induced very late [from mid-third instar onwards (108 hours AEL); Fig. 3I-K], we saw a small number of cells expressing low levels of Eyes absent (Fig. 3J, arrow). We do not understand the basis of this expression – in all other contexts the loss of Eyes absent was complete – but the effect was weak and the number of cells small. The fact that it is only seen in the latest induced clones suggests that it may represent slight perdurance of Axin within some cells in the clone.

We also examined *axin*⁻ clones in a non-*Minute* background, using the Gal4/Gal80 expression system (Lee and Luo, 1999) to mark the mutant clones positively (red in Fig. 5). This approach complemented the above analysis in three ways. First, it allowed us to confirm that the excess growth in *axin*⁻ clones was independent of the *Minute* background (Fig. 5A, blue section). Second, it allowed us to see the mutant cells more clearly as they were marked in red (Fig. 5A,B,D); this allowed a clearer visualisation of the overgrowth, especially when it was outside the plane of the normal disc epithelium (e.g. arrow in blue, transverse section of Fig. 5A). Third, it confirmed the autonomous loss of Eyes absent (Fig. 5A, red

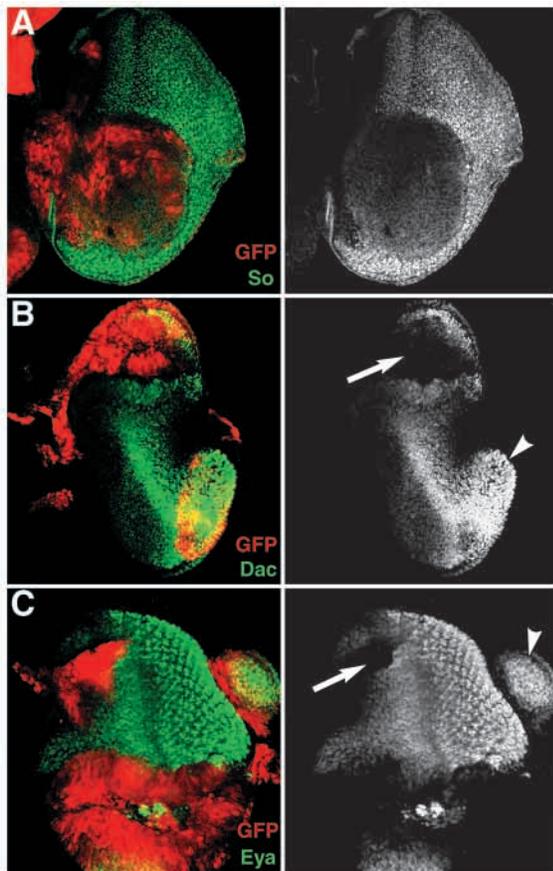


Fig. 4. Clones of *arm*^{*}-expressing cells (red) abolish the expression of *sine oculis-lacZ* (A), Dachshund protein (B) and Eyes absent protein (C) (all shown in green and white in right-hand panel). As observed in *axin*⁻ mutant clones, the expression of these genes is eliminated in anterior clones (arrows in B and C), but is often ectopically expressed (arrowhead in C) or not eliminated (arrowhead in B) in posterior clones.

transverse section), Dachshund (Fig. 5C, arrowhead) and Sine oculis (Fig. 5D and inset in E) in even small clones anterior to the morphogenetic furrow.

Eyes absent and Sine oculis have complementary expression patterns to Wingless in the eye disc

The conclusion that Wingless signalling negatively regulates the expression of Eyes absent, Dachshund and Sine oculis anterior to the furrow leads to the prediction that in normal development, domains of high Wingless activity in the anterior region of the eye disc will be associated with low expression of these genes. Previous work indicates that their expression is broadly non-overlapping, but to analyse this precisely we have double-labelled discs to detect the expression of Wingless and Eyes absent or Sine oculis throughout the third instar larval stage. The expression of these eye specification genes is precisely complementary to that of Wingless in the anterior lateral margins of the eye throughout the third instar (Fig. 6). This is consistent with a role for Wingless signalling in initiating the borders between eye and other head structures. Note that in posterior lateral regions we observe slight overlap between the expression of Wingless and these genes; this is presumably analogous to the expression of eye specification genes we see in some posterior *axin*⁻ clones, and confirms that in posterior regions of the eye disc, Wingless signalling is not incompatible with the expression of these genes.

In the most anterior region of the eye portion of the disc, there is a domain in which Eyes absent, Sine oculis and Wg-lacZ are expressed (e.g. Fig. 6B). Although this could imply other factors being necessary for the repression of Eyes absent and Sine oculis in this region, we favour the idea that Wingless protein reaches these cells from the adjacent lateral expression domains. This is supported by our observation that loss of Wingless signalling in this domain (in *fz*⁻, *fz2*⁻ clones) leads to the ectopic expression of eye specification genes (see below).

Loss of Wingless signalling causes the ectopic expression of Eyes absent and Dachshund

The data presented above analyse the effects of ectopic activation of Wingless signalling. The results suggest that in normal development Wingless signalling is responsible for blocking the expression of eye selector genes like Eyes absent and Dachshund, thereby regulating the extent of the eye field. However, drawing firm conclusions from the consequences of ectopic signalling is unreliable, so we examined the consequences of loss of Wingless signalling, which would be predicted to cause the ectopic expression of the Wingless-repressed eye specification genes. In order to generate a complete loss of Wingless reception, we made clones lacking both Wingless receptors: Frizzled and Frizzled 2 (Bhanot et al., 1999; Chen and Struhl, 1999). We find that in these double mutant clones, Eyes absent and Dachshund are ectopically expressed in the lateral margin anterior to the furrow and the most anterior region of the eye primordium (vertex primordium) (Fig. 7A-C). With low frequency these clones also cause the differentiation of ectopic ommatidia on the dorsal adult head (not shown). This is consistent with a previous observation that loss of *dishevelled* (Heslip et al., 1997), which encodes a component of the Wingless signal transduction pathway (Klingensmith et al., 1994; Theisen et al., 1994), also leads to the formation of ectopic ommatidia. These

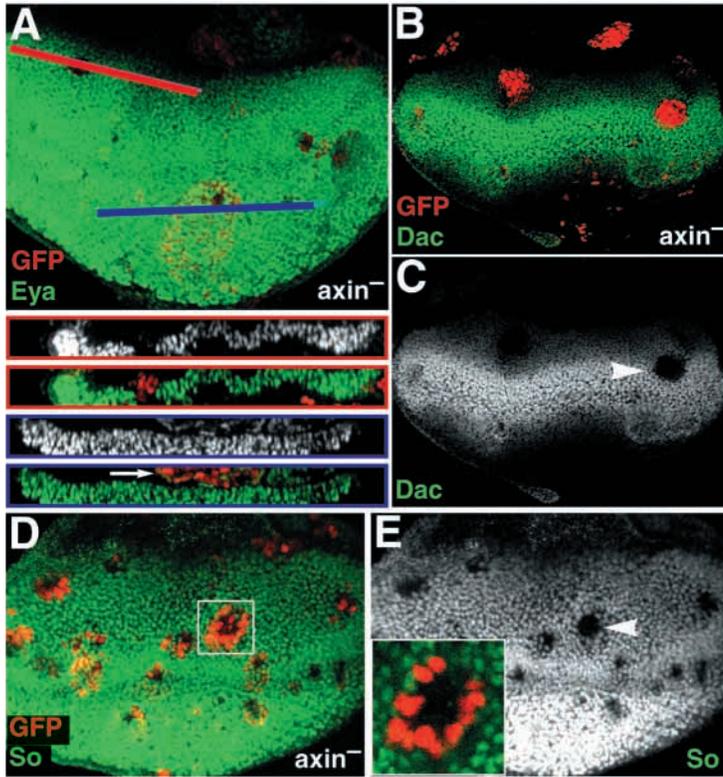


Fig. 5. *axin*⁻ clones induced in a non-*Minute* background also cause overgrowth and loss of eye specification genes. In this case, mutant clones are positively marked in by GFP staining (in red, for consistency with other figures), using the Gal4/Gal80 system. (A) Eyes absent expression (green) is abolished in anterior but not posterior *axin*⁻ clones. Red box: transverse section of a small anterior clone indicated by red line; mutant cells have grown out of the plane of the epithelium and do not express Eyes absent. Blue box: transverse section of a posterior clone indicated by blue line; mutant cells show extensive overgrowth (arrow) and some express Eyes absent. (B,C) Dachshund expression (green) is autonomously abolished in anterior *axin*⁻ clones (e.g. arrowhead in C). (D,E) *Sine oculis* expression is autonomously abolished in anterior *axin*⁻ clones (e.g. arrowhead in E). (D,E) Projections of several confocal sections; to demonstrate the autonomy of this loss, the inset in E shows a single confocal section of the clones indicated by a box in D (the central cells in the clone are not seen as they have grown out of the plane of the section).

double mutant clones also show slight overgrowth. These results confirm the conclusions of the ectopic expression experiments and demonstrate that Wingless signalling inhibits the inappropriate expression of eye specification genes in normal eye development.

The phenotype of ectopic Wingless activation is not rescued by the expression of Eyes absent

The hierarchy of genes required for the eye specification is complex but there is strong evidence to place Eyeless at the top of the cascade. Eyeless activates the expression of *eyes absent* and *sine oculis*, which in turn trigger the initiation of *dachshund* expression; positive feedback between these genes stabilises and maintains their expression (Bonini and Choi, 1995; Chen et al., 1997; Curtiss and Mlodzik, 2000; Halder et

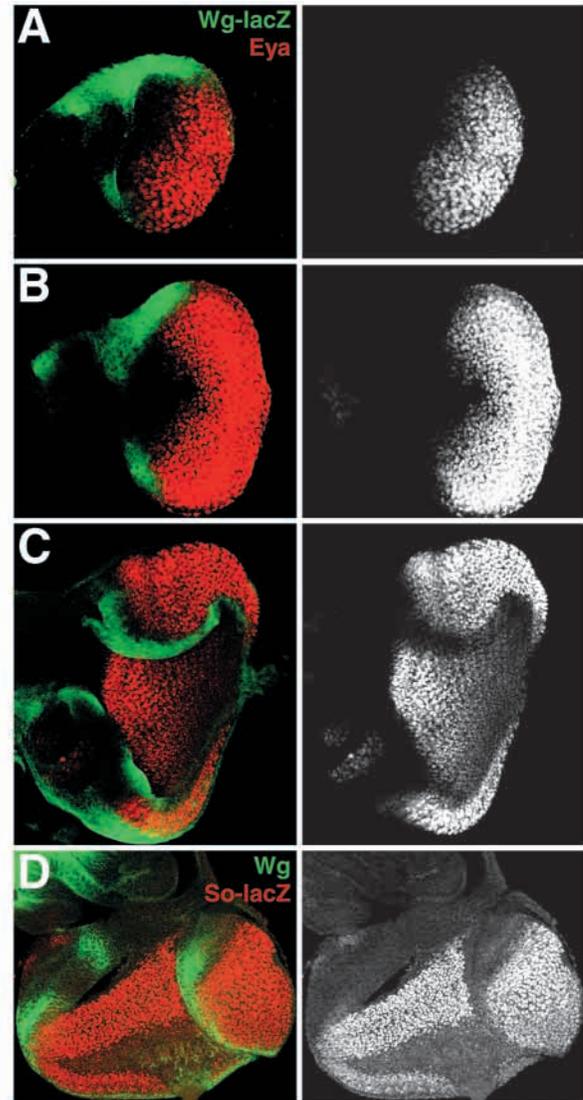


Fig. 6. Eyes absent and *sine oculis* have complementary expression patterns to Wingless. (A-C) Expression of *wg-lacZ* (green) and Eyes absent protein (red and white in right-hand panels) during third instar eye disc development. (A) In the early third instar eye disc, before initiation of the morphogenetic furrow, Wingless is expressed in the anterior lateral margin of the eye disc, whereas Eyes absent is expressed throughout the eye primordia. (B) In the mid-third instar eye disc, when the morphogenetic furrow has started its posterior to anterior progression, the expression pattern of both genes remains similar. (C) A basal view (single confocal section) of a late third instar eye disc, showing that Eyes absent is missing from the anterior lateral margin where Wingless is expressed. Note that in posterior regions some overlap is seen (indicated by yellow staining). (D) Expression of *sine oculis-lacZ* (red, white in right-hand panels) and Wingless protein is precisely complementary in the anterior region, but slightly overlap in the posterior lateral region (single confocal section).

al., 1998; Niimi et al., 1999; Pignoni et al., 1997; Zimmerman et al., 2000). We have shown that Wingless activity represses the expression of *eyes absent*, *sine oculis* and *dachshund* (Figs 3, 4, 5). The repression of *dachshund* is presumably a

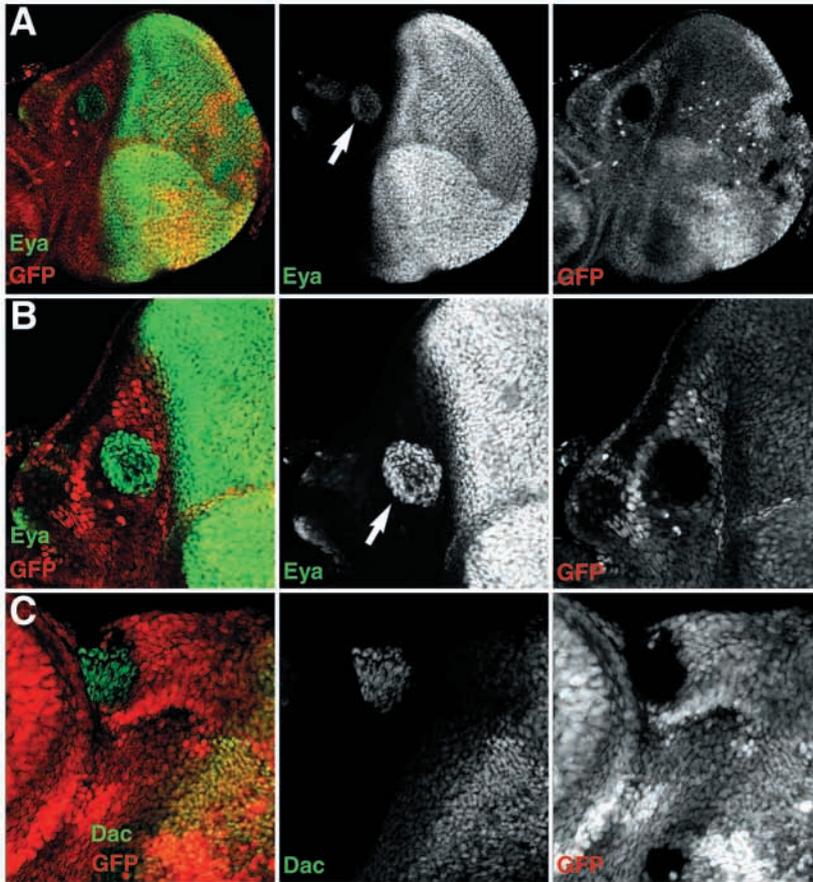


Fig. 7. Wingless signalling is necessary to prevent the expression of Eyes absent and Dachshund in the anterolateral margin of the eye disc. (A,B) Eyes absent (green) is ectopically expressed (arrows) in a *fz1 fz2* mutant clone (lack of red staining); B shows a higher magnification of the relevant region of A. (C) A *fz1 fz2* mutant clone in a similar position to the one shown in A ectopically expresses Dachshund (green).

consequence of the loss of Sine oculis and Eyes absent, but the epistatic relationship between *sine oculis* and *eyes absent* is complicated and it has not been determined whether they act in parallel downstream of *eyeless*, or if *sine oculis* is downstream of *eyes absent* (Curtiss and Mlodzik, 2000; Desplan, 1997; Halder et al., 1998; Pignoni et al., 1997). To address the issue of where Wingless acts in this network, we tested whether the expression of Eyes absent in *axin*⁻ clones [using the Gal4/Gal80 system (Lee and Treisman, 2001; Lee and Luo, 1999)] was sufficient to rescue their phenotype. The phenotype of these clones is very similar to the *axin*⁻ control clones (compare Fig. 8 with Fig. 5): neural differentiation is abolished (not shown) and large tube-like overgrowths are observed. However the expression of Dachshund (Fig. 8A) and Sine oculis (Fig. 8B) is partially rescued in at least some clones anterior to the furrow, implying that Eyes absent can be sufficient to trigger their expression, even when

Wingless signalling is high. This is confirmed by the fact that Sine oculis is often ectopically expressed in these clones in the antennal region of the disc (Fig. 8B, inset). We conclude that the expression of Eyes absent is not sufficient to rescue the whole phenotype caused by ectopic Wingless activity in the eye but can activate the expression of Sine oculis and Dachshund at least to low levels.

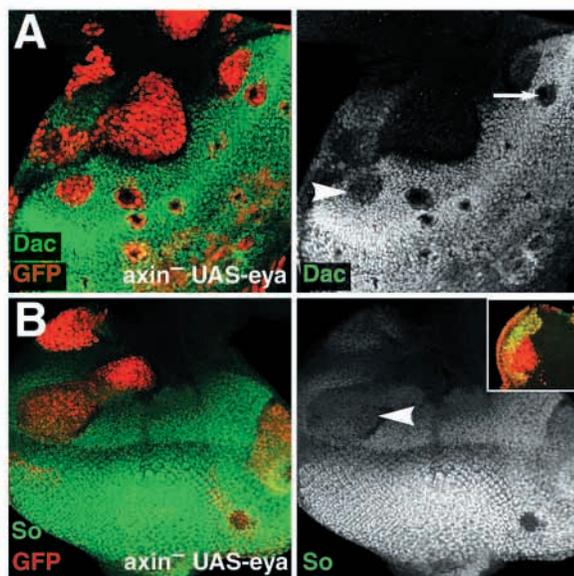


Fig. 8. Ectopic expression of *eyes absent* partially rescues expression of Sine oculis and Dachshund in *axin*⁻ clones. *axin*⁻ clones that simultaneously express *eyes absent* (using the Gal4/Gal80 system) are shown in red. (A) Dachshund expression (green) is partially rescued (arrowhead) in some, but not all (arrow), of these clones (compare with Fig. 5B,C). (B) Sine oculis expression is also partially rescued in these clones (arrowhead); compare with Fig. 5D,E. The inset shows a clone in the antennal region of the disc with ectopic expression of Sine oculis (in green).

DISCUSSION

Our results, consistent with previous observations (Lee and Treisman, 2001; Royet and Finkelstein, 1997), indicate that ectopic activation of Wingless signalling is sufficient to change the fate of eye cells, suggesting a function of Wingless in the regulation of the eye specification genes and thereby in the control of the size of the eye field. The identity of eye cells depends on the function of the transcription factors Eyeless, Eyes absent, Sine oculis and Dachshund. Eyeless occupies the highest position in the hierarchy of competing 'master genes', whereas Eyes absent and Sine oculis act as two mediators of Eyeless (Curtiss and Mlodzik, 2000; Halder et al., 1998; Halder et al., 1995; Pignoni et al., 1997). Finally, several lines of evidence suggest that Dachshund lies downstream of the other early eye genes. The expression of Eyeless is not dependent on Wingless signalling (Lee and Treisman, 2001). Instead, our results indicate that Wingless regulates the final size of the eye field of cells by controlling the expression of *eyes absent*, *sine oculis* and *dachshund*. The expression pattern

of these genes in the anterior eye margin is complementary to the expression of Wingless throughout the third instar, indicating that in anterior regions, high activity of Wingless signalling corresponds to absence of these gene products. Moreover, ectopic activation of Wingless signalling represses their expression anterior to the furrow (where they act to specify the eye field) throughout eye development. Finally, the loss of Wingless signalling causes ectopic expression of Eyes absent and Dachshund.

Further support for our model is derived from previous analysis of eye specification genes. For example, ubiquitous expression of Eyeless in the wing imaginal disc causes activation of Eyes absent and Sine oculis, but only in cells close to the AP border that do not express Wingless (Halder et al., 1998). Furthermore, loss of Eyes absent and Sine oculis cause very similar overgrowths to those we have observed in *axin⁻* or *arm^{*}* clones. This overgrowth presumably represents a combination of hyper-proliferation in the anterior regions, and the loss of eye identity in mutant cells so that they no longer respond to the passage of the morphogenetic furrow by arresting in G1; instead they continue to proliferate, as evidenced by the increase of cells we observe in G2.

We therefore propose that the initial expression of Eyes absent, Sine oculis and Dachshund is negatively regulated by Wingless signalling in the eye disc, and that this regulation initiates the border between the eye field and adjacent head cuticle. We have attempted to define whether Wingless represses the eye specification genes independently or whether *eyes absent* is the primary target but our data confirms earlier reports of the complexity of the regulatory relationships between *eyes absent*, *sine oculis* and *dachshund*. Our observation that Eyes absent is able partially to restore the expression of the other two genes but cannot rescue the overgrowth and differentiation phenotype of *axin⁻* clones has two possible explanations. Either Wingless represses eye development through at least one additional gene, or high level Wingless signalling blocks eye development later in the developmental program [e.g. it is known to inhibit morphogenetic furrow initiation (Ma and Moses, 1995; Treisman and Rubin, 1995)], even after its earlier effects are rescued by *eyes absent* expression.

Despite the clear evidence for Wingless repressing the eye specification genes anterior to the morphogenetic furrow, we find some mutant cells that express Eyes absent, Sine oculis and Dachshund posterior to the furrow. The fact that these mutant cells do not differentiate as eye indicates that this late expression is not enough to induce eye differentiation. We do not fully understand this phenomenon, but we speculate that eye specification requires these genes to be expressed only in front of the furrow, whereas behind the furrow they may have a separate function in differentiation and be regulated differently. Consistent with this hypothesis, the late expression of Eyes absent behind the furrow is required for differentiation of the photoreceptors (Pignoni et al., 1997), and the paramount eye selector gene Eyeless is expressed only anterior to the furrow at this stage (Quiring et al., 1994). Furthermore, this interpretation is consistent with our observation that in posterior regions of the eye disc, there is a slight overlap between the expression of Wingless and the eye specification genes.

During eye disc development, Wingless signalling represses

Dpp activity and vice versa (Domínguez and Hafen, 1997; Hazelett et al., 1998; Ma and Moses, 1995; Treisman and Rubin, 1995; Wiersdorff et al., 1996). In addition to the mutual repression of these two pathways during morphogenetic furrow initiation, it has been proposed that in the early eye disc, Dpp prevents head fate by repressing Wingless (Royet and Finkelstein, 1997). Can the data we present here be explained by this mutually repressive relationship between Wingless and Dpp? Although the loss of Dpp during the early stages of eye disc development resembles the activation of Wingless signalling in some regards, the *axin⁻* and *arm^{*}* clones have other phenotypes that do not correspond to loss of Dpp signalling. Thus, after the furrow is already initiated, Mad clones downregulate Eyes absent and Dachshund only when they are at the margin of the disc (Curtiss and Mlodzik, 2000). This contrasts with our observation that Wingless represses the expression of *eyes absent*, *sine oculis* and *dachshund* in all anterior cells throughout eye disc development, regardless of whether they are marginal or interior. Furthermore, the overgrowth phenotypes caused by the ectopic activation of Wingless signalling are not found in *mad⁻* clones (Curtiss and Mlodzik, 2000; Hazelett et al., 1998). Therefore, although some of the effects of ectopic Wingless activity may be a consequence of the downregulation of Dpp signalling, others must be caused by Dpp-independent mechanisms.

Note that, while highlighting the role of Wingless signalling as an important physiological regulator of the size of the eye, our data do not address how direct the effect of Wingless is on the expression on *eyes absent*, *sine oculis* and *dachshund*. It is possible that this represents a direct transcriptional repressor function for Wingless signalling, but it is equally possible that Wingless induces the expression of a repressor of these eye specification genes.

Very recently, Lee and Treisman reported the phenotype of *axin⁻* clones in the eye imaginal disc. They too reported the overgrowth phenotype that we have described, but otherwise they examined different aspects of the phenotypes of these clones. Based on that analysis they proposed a different model: that Wingless signalling normally promotes the proliferation of cells anterior to the morphogenetic furrow, and that the ectopic activation of this pathway behind the furrow is sufficient to maintain cells in an anterior state in which they proliferate, fail to differentiate and continue to express anterior markers (Lee and Treisman, 2001). Some of our results, such as the high levels of Eyes absent and Dachshund found in posterior *axin⁻* clones, are consistent with this model but several others are not. Thus, the loss of eye identity in *axin⁻* clones is not consistent with those cells being held in an anterior eye state; nor is the loss of expression of eye specification genes that are normally expressed in the anterior of the eye field; nor, finally, is the ectopic expression of Eyes absent caused by loss of Wingless signalling. For these reasons, we believe that our interpretation of Wingless activity being a regulator of the eye specification genes more completely fits the existing experimental evidence.

Wingless and transdetermination

Wingless signalling is required to distinguish wing pouch cells from notum cells (Ng et al., 1996). We and others (Heslip et al., 1997; Royet and Finkelstein, 1997) have described a similar function of Wingless during eye disc development in defining the border between retina and adjacent head.

However, we have found that in addition to dorsal head cuticle, the *axin* mutant cells can transform the eye cells into other tissues. For example, we have shown that the *axin*⁻ clones sometimes express *Distal-less*, a gene not expressed in the third instar eye but specific to the leg, wing and antennal discs. This fits with previous reports that ectopic expression of Wingless during the development of other imaginal discs can induce transdetermination – the change of cell identity from one fate to another (Johnston and Schubiger, 1996; Maves and Schubiger, 1998). The plasticity of mammalian cells during development is a hotly debated issue that has important implications for the potential utility of stem cells. It may be that, as in other fields, *Drosophila* genetics can shed some light on the mechanisms of developmental plasticity and how they are regulated.

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