The *four-jointed* gene is required in the *Drosophila* eye for ommatidial polarity specification

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Background: The *Drosophila* eye is composed of about 800 ommatidia, each of which becomes dorsoventrally polarised in a process requiring signalling through the Notch, JAK/STAT and Wingless pathways. These three pathways are thought to act by setting up a gradient of a signalling molecule (or molecules) often referred to as the 'second signal'. Thus far, no candidate for a second signal has been identified.

Results: The *four-jointed* locus encodes a type II transmembrane protein that is expressed in a dorsoventral gradient in the developing eye disc. We have analysed the function and regulation of *four-jointed* during eye patterning. Loss-of-function clones or ectopic expression of *four-jointed* resulted in strong non-autonomous defects in ommatidial polarity on the dorsoventral axis. Ectopic expression experiments indicated that localised *four-jointed* expression was required at the time during development when ommatidial polarity was being determined. In contrast, complete removal of *four-jointed* function resulted in only a mild ommatidial polarity defect. Finally, we found that *four-jointed* expression was regulated by the Notch, JAK/STAT and Wingless pathways, consistent with it mediating their effects on ommatidial polarity.

Conclusions: The clonal phenotypes, time of requirement and regulation of *four-jointed* are consistent with it acting in ommatidial polarity determination as a second signal downstream of Notch, JAK/STAT and Wingless. Interestingly, it appears to act redundantly with unknown factors in this process, providing an explanation for the previous failure to identify a second signal.

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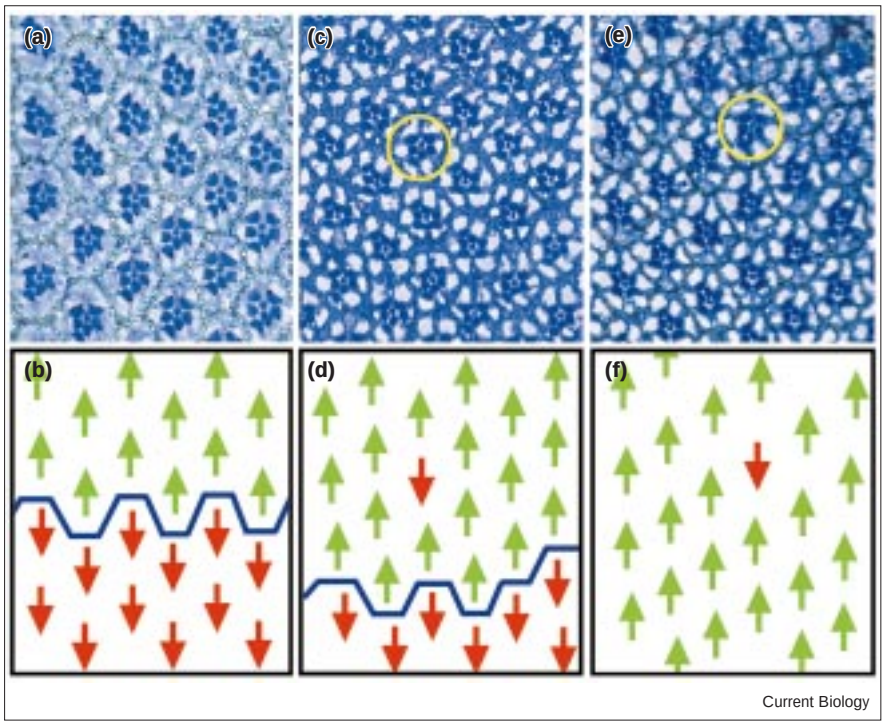
Background

Historically, the insect cuticle has proved an amenable system for the study of patterning processes. A much-studied problem is how cells and groups of cells become polarised in the plane of the cuticle relative to the axes of the organism as a whole. A number of elegant studies (for example [1]) established that many of the experimental results were most easily explained by postulating the existence of gradients of diffusible substances that provide polarity information to individual cells.

In recent years, studies in *Drosophila* have permitted the identification of signalling pathways and molecules involved in polarity determination, particularly in the compound eye. In the wild-type eye, ommatidia occur in two mirror-symmetric forms, a dorsal form found invariably dorsal to the midline, and a ventral form found invariably ventral to it (Figure 1a,b). The dorsoventral midline thus forms an axis of mirror-image symmetry, which is known as the equator, whereas the dorsal and ventral extremes of the eye are referred to as the poles. The ommatidial subunits differentiate during the third larval instar stage from the epithelium of the eye imaginal disc in a wave that moves from posterior to anterior and is marked by the passage of an indentation in the disc epithelium known as the morphogenetic furrow. Dorsoventral polarity is established posterior to the furrow and is first manifest when the nascent ommatidial clusters rotate 90° away from the dorsoventral midline (reviewed in [2]).

The critical step in polarity determination appears to be the point at which the ommatidia decide whether to adopt the dorsal or the ventral mirror-symmetric form, which in turn determines in which direction they rotate. Current models suggest that this decision is determined by a gradient of signalling activity between the equator and the poles (reviewed in [3,4]). Three different pathways appear to be involved in establishing this gradient. During first and second instar larval development, Notch (N) becomes activated at the midline in a narrow band of cells and appears to define the position of the future equator [5–7]. Secondly, an important role has been found for Wingless (Wg): a dorsoventral gradient of its activity in the eye regulates ommatidial polarity such that the point of lowest Wg activity lies at the equator [8,9]. Finally, we have recently shown that a dorsoventral gradient of JAK/STAT activity is present in the eye disc,

Figure 1



The fj homozygous phenotype in the eye. Anterior is to the left and dorsal is uppermost. (a,c,e) Sections through adult eyes at the R7 level. (b,d,f) Schematic representations; green arrows indicate ommatidia with dorsal polarity; red arrows indicate ommatidia with ventral polarity; the blue line indicates the path of the equator, here and in subsequent figures. (a,b) Section through equator of a wild-type eye, revealing the invariant arrangement of dorsal ommatidia dorsal to the equator and ventral ommatidia ventral to the equator. (c–f) Eyes from flies transheterozygous for fj^{d1} and Df(2R)Pcl11B (a deficiency that uncovers the *fj* locus). Occasional examples of ommatidia with inverted polarity relative to their neighbours (circled) are seen at variable distances from the equator.

apparently activated by a localised source of the JAK ligand Unpaired (Upd) at the midline, and that alterations in the direction of this gradient produce corresponding alterations in ommatidial polarity [10].

Currently, the functional relationships between these three pathways are not fully understood. It is clear that Wg and Upd act in parallel, however, as Wg expression cannot repress Upd and vice versa [10]. Furthermore, both the Wg and JAK/STAT pathways have been shown to act across the entire eye disc to regulate ommatidial polarity, whereas the evidence so far indicates only a local effect of the N pathway close to the dorsoventral midline. Therefore, a reasonable working model is that all three pathways act in parallel, with Wg and JAK/STAT together regulating long-range signals and N acting close to the midline to ensure that the equator is tightly defined (see [3,4,10]).

In the case of all three pathways, the data suggest that polarity is ultimately controlled by one or more unidentified signalling molecules, referred to variously as 'factor X', 'the second signal' and 'WntX' (we will use the term 'second signal'). The simplest model that fits all the experimental data suggests that such a molecule should be expressed in a dorsoventral gradient in the eye disc with high levels at the midline and low levels at the poles at a time when ommatidia are differentiating and rotating. Furthermore, it should be a secreted factor, able to signal non-autonomously, that is positively regulated by N and JAK/STAT — which are high at the equator — and negatively regulated by Wg — which is high at the poles (Figure 2a). The independent regulation of a single second signal by all three pathways would provide a mechanism by which their combined patterning functions could be integrated.

A potential candidate for encoding such a factor is the *four-jointed* (*fj*) gene. This has previously been characterised as having an important role in proximodistal axis formation in the adult limbs, where clonal analysis has demonstrated that it can act non-autonomously [11]. Subsequently, enhancer-trap P-element insertions have been recovered in the *fj* locus, which have permitted its molecular cloning and determination of its expression pattern. In addition to expression in the leg and wing discs, the *fj* transcript is expressed in a dorsoventral gradient in the eye disc with peak expression at the midline [12,13] (Figure 2b). Hydropathy analysis suggests that the 583 amino acid predicted protein product is a type II transmembrane protein in which the carboxyl terminus is extracellular; the presence of predicted signal peptidase cleavage sites and the results of *in vitro* canine microsome analysis strongly suggest that the carboxyl terminus is likely to be cleaved to release a secreted peptide that could act as a diffusible signalling molecule [12,13].

We now report that both loss-of-function *fj* clones and ectopic expression of *fj* result in ommatidial polarity

defects and that *fj* expression in the eye disc is regulated by the N, Wg and JAK/STAT pathways. Our results are consistent with *fj* fulfilling the criteria to be a second signal required for the determination of dorsoventral ommatidial polarity.

Results

Mutations in *fj* result in ommatidial polarity defects

Given its expression pattern and the molecular character of its putative gene product, *fj* appeared to be a good candidate for a gene involved in dorsoventral polarity signalling during *Drosophila* eye development. All known *fj* alleles are homozygous viable (including putative molecular nulls such as fj^{d1}, see below), exhibiting visible defects in leg and wing patterning. Sections through the eyes of such viable homozygous mutant individuals revealed reproducible defects in the polarity of individual ommatidia: The phenotype observed was characterised by precise dorsoventral inversions of polarity, such that ommatidia in the dorsal hemisphere of the eye were polarised as if they were in the ventral hemisphere and vice versa (Figure 1c–f). The frequency at which this phenotype occurred was only about once in every 300–400 ommatidia, however. To ascertain that this defect was indeed due to *fj*, we scored eyes from a number of transheterozygous combinations of independently isolated *fj* alleles and deficiencies in the *fj* genomic region (see Materials and methods). We also scored over 4,000 ommatidia in eyes of flies from an Oregon R wild-type stock and observed no ommatidial polarity defects. If ommatidia were normally inverted at a rate of 1 in 400, as seen in *fj* homozygotes, then the probability of seeing no inversions in 4,000 ommatidia would be less than 0.005%.

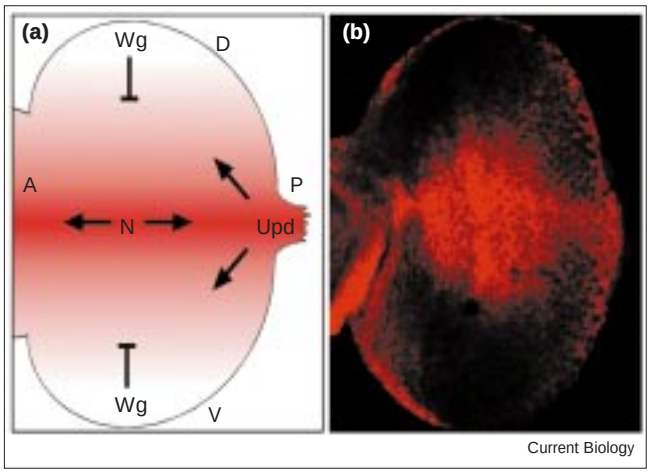
One possibility was that the weak phenotype might be due to the allelic strength of the *fj* mutations being used. We verified the molecular nature of the fj^{d1} mutation used in most of our experiments by PCR-amplifying and sequencing the surrounding genomic region and found that all but the last 57 amino acids are deleted, with no remaining in-frame methionines. We therefore think that it is highly unlikely that any protein product is produced and conclude that this represents a molecular null allele. It was previously reported that individuals homozygous for *fj* occasionally exhibit defects in the shape and size of the eye [13]. We only observed such phenotypes on very rare occasions, and consequently have not attempted to correlate its occurrence with the incidence of dorsoventral ommatidial polarity defects.

Mosaic clones of *fj* result in non-autonomous ommatidial polarity defects

Although we observed only weak ommatidial polarity defects in flies homozygous for *fj* mutations, mosaic clones for the fj^{d1} allele were able to produce strong effects on dorsoventral ommatidial polarity. Typically, clones lying close to the equator in either the dorsal or ventral hemisphere of the eye resulted in dorsoventral inversions of polarity in 1–3 rows of ommatidia straddling the boundary of the clone furthest from the equator (the polar boundary, Figure 3a,b). The effect was non-autonomous at the level of individual ommatidia, as ommatidia beyond the polar boundary of clones were inverted, despite having no homozygous mutant photoreceptors (Figure 3a, inset). Within the remainder of the clone and on the equatorial clonal boundary, ommatidial patterning was normal. These results are consistent with a model in which ommatidia determine their polarity by reference to the gradient of *fj* expression (see model in Discussion).

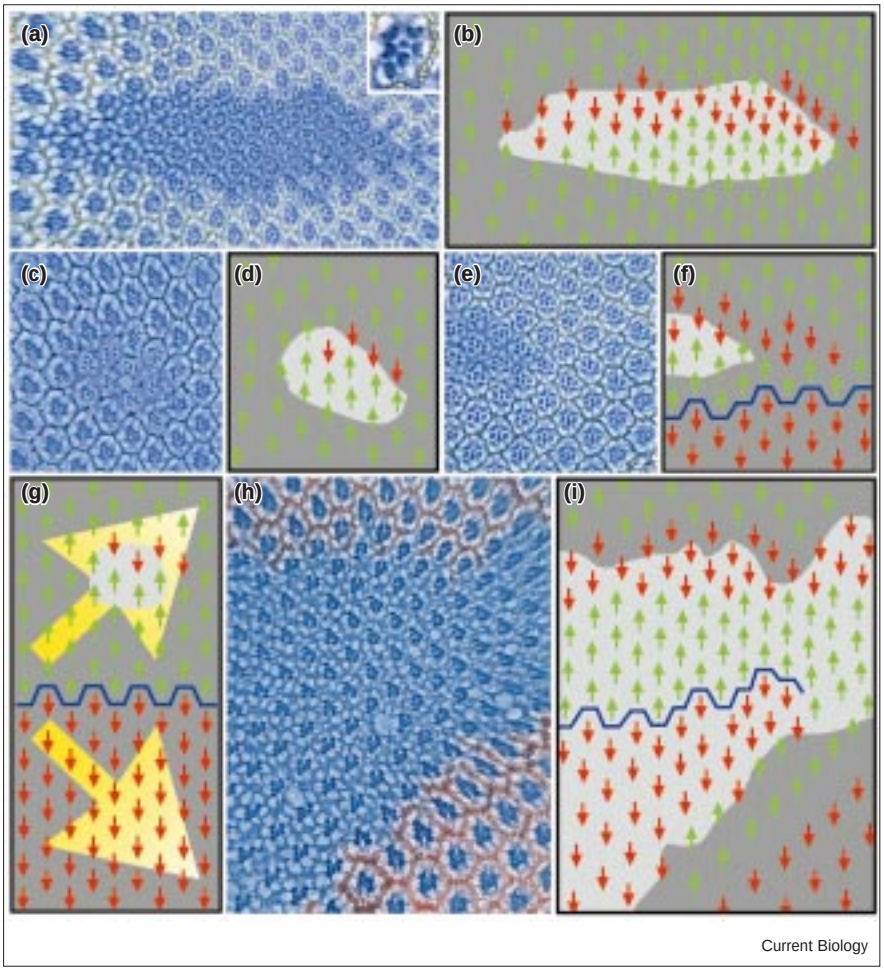
There was little effect of clone size on the strength of the phenotype, but a clear inverse correlation was seen between the distance of the polar boundary of the clone from the equator and the strength of the phenotype (Table 1 and see model). One clone was analysed in which the polar boundary touched the polar eye margin, and in this case no phenotype was observed. These results are consistent with the strongest phenotypes being observed close to the midline, where *fj* expression levels are highest. The strength of the ommatidial polarity defect also varied along the anteroposterior axis within individual clones, being consistently stronger at the posterior edge. Thus, in many clones there were no inverted rows of ommatidia at the anterior edge but 1–2 rows at the posterior edge (Figure 3c,d) and in some cases ommatidia were

Figure 2



The regulation of the 'second signal' and the *fj* expression pattern. Anterior is to the left and dorsal is uppermost. **(a)** Model for second-signal regulation in the eye disc. Wg expressed at the poles of the disc acts as a repressor, whereas activated Notch (N) in a narrow band of cells along the dorsoventral midline and Upd in a small patch at the optic stalk act as activators, producing a gradient of second signal (red) that is high at the midline and low at the poles. **(b)** Third-instar eye disc immunolabelled to show expression of *fj* (red) as revealed by the fj^{P1} enhancer-trap insertion and an anti-β-galactosidase antibody.

Figure 3



The phenotype of homozygous mutant *fj* clones. Anterior is to the left and dorsal is uppermost. (a,c,e,h) Sections containing clones of cells homozygous for fj^{d1} marked by lack of *white* expression. (b,d,f,i) schematic representations of the sections in (a,c,e,h), respectively; lighter grey areas indicate the approximate extent of homozygous mutant tissue. (a,b) A clone close to the equator, in which 1–2 rows of ommatidia on the polar boundary of the clone show inverted polarity. The inset shows an enlargement of an inverted ommatidium from the polar boundary, in which no photoreceptors are homozygous mutant (as indicated by the presence of dark pigment granules). (c,d) A small clone with a weak phenotype extending to the posterior but not the anterior boundary. The small rhabdomeres seen in the mutant photoreceptors are apparently due to another mutation on the chromosome, as they were never seen in other fj^{d1} clones. (e,f) Posterior edge of a clone close to the equator, showing a strong phenotype that extends non-autonomously beyond the posterior boundary. (g) Schematic diagram, summarising data from many clones, showing that direction of non-autonomy on the polar boundary of the clone is strongly skewed to the posterior, consistent with a model in which ommatidial polarity is determined by reference to a vectorial signal (yellow arrows) that is strong at the anterior midline and weaker at the posterior midline. (h,i) A large clone crossing the equator. The endogenous equator is conserved, in addition to inversions observed on both polar boundaries forming ectopic equators. In this example, the path of the endogenous equator is disturbed where it approaches the clonal boundary, and is effectively shifted several rows ventrally.

inverted beyond the posterior edge of the clone (Figure 3e,f). We saw no discernable effect of absolute anteroposterior position of the clone within the eye on the strength of phenotype observed (data not shown).

These observations indicate that although the polarity phenotype observed in *fj* homozygotes and mosaic clones was characterised by precise inversions of ommatidial polarity on the dorsoventral axis, the non-autonomous 'shadow' of polarity inversions observed on the polar boundaries of *fj* clones was clearly skewed on the anteroposterior axis (Figure 3g). Interestingly, clones that crossed the endogenous equator exhibited a 'triple-equator' phenotype (Figure 3h,i), in which the normal equator was preserved and ommatidia were inverted on both polar boundaries. This indicates that, even in the absence of *fj* activity, a peak of some signalling activity remained at the midline (see model).

Localised *fj* expression is required for normal dorsoventral polarity establishment

Both *in situ* hybridisation for *fj* transcripts and the *lacZ* activity patterns revealed by enhancer traps in the *fj* locus indicate that *fj* is normally expressed most strongly in a broad domain around the dorsoventral midline of the eye imaginal disc ([12,13]; Figure 2b). To determine whether this localised expression was functionally significant, we ectopically expressed *fj* during eye development using the GAL4–UAS system [14] and the FLP–FRT system [15]. Ectopic expression of *fj* was driven at the poles of the eye during eye patterning using the *optomotor-blind (omb)*–GAL4 driver. This resulted in dorsoventral inversions of ommatidial polarity at both the dorsal and ventral poles of the eye, often with three or more rows of ommatidia inverted (Figure 4a,b). To express *fj* in smaller groups of cells, an *actin-promoter–FRT–yellow*$^{+}$*–FRT*–GAL4 cassette was used [16]. This also resulted in inverted

Table 1

The relationship of *fj*^{d1} clone size and dorsoventral position to strength of phenotype.

Number of rows inverted on polar boundary	Approximate size of clone (ommatidia)									
	6–15	16–25	26–35	36–45	46–55	56–65	66–75	76–85	86–95	> 96
0										1
1	1		1	1	3	1	1	1	2	
2	3	2	3	4	2	2	1	2		2
3				1		1				

Number of rows inverted on polar boundary	Distance from equator of polar boundary of clone (ommatidia)								
	1–2	3–4	5–6	7–8	9–10	11–12	13–14	15–16	> 16
0									1
1					4	2	3	2	
2	1	2	3	4	10	2	1		
3			1		1				

Approximately 50 homozygous *fj*^{d1} clones of different sizes, shapes and position in the eye were analysed, of which 35 were scorable for size and 37 for distance of polar boundary from equator. Very small clones (< 8 ommatidia) showed no phenotype. There was little correlation between clone size and strength of phenotype (top section). Conversely, there was a clear correlation between the proximity of the polar boundary to the equator and the strength of phenotype (bottom section). When the polar boundary was < 9 rows from the equator, 2–3 rows of ommatidia showed inverted polarity, but when the distance was > 13 rows, only 0–1 rows of ommatidia were inverted.

ommatidial polarity (Figure 4c,d), in this case consistently on the equatorial boundary of the clones of cells expressing *fj*. This is, of course, the opposite to the phenotype observed in *fj* loss-of-function clones, in which inversions of polarity occurred on the polar boundary.

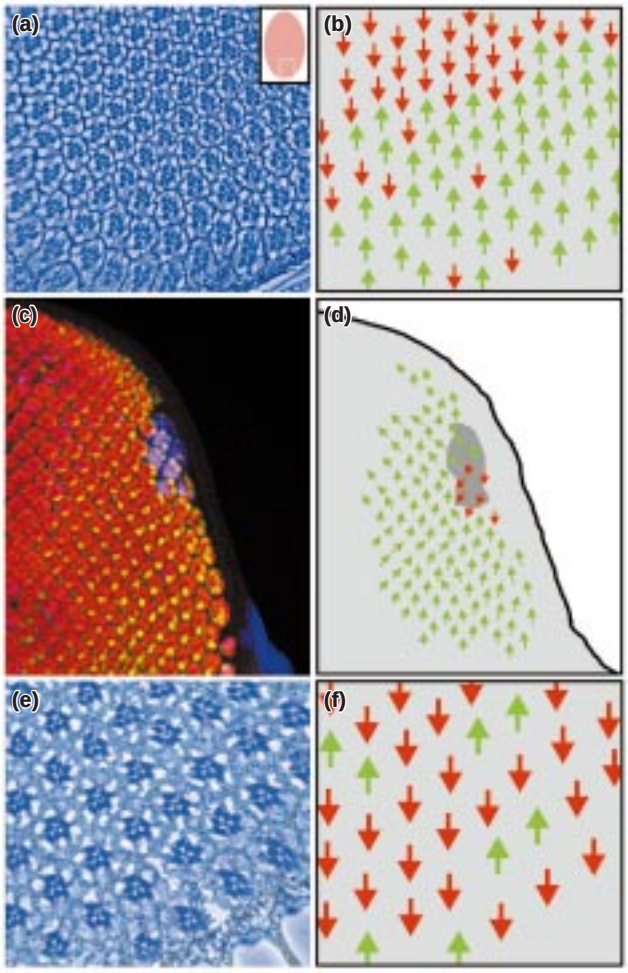
We also used the GAL4–UAS system to attempt to determine the time during eye patterning when *fj* function is required. Expression was driven all along the dorsoventral axis using GAL4 drivers controlled by three different enhancers. No phenotype was observed when the enhancer used (from the *eyeless* gene) drove expression only anterior to the morphogenetic furrow prior to photoreceptor differentiation [17]. When expression was driven using the *sevenless* enhancer [18] and the *glass* multiple reporter [19], however, both of which are expressed posterior to the furrow at the time of photoreceptor differentiation, we observed an ommatidial polarity defect similar to but stronger than the *fj* homozygous mutant phenotype (Figure 4e,f and data not shown).

We conclude that localised *fj* expression is required for normal establishment of ommatidial polarity along the dorsoventral axis and that ectopic sites of *fj* expression result in inversions of ommatidial polarity that phenocopy the endogenous equator (where *fj* expression is normally highest). Additionally, as ectopic expression of *fj* posterior to the furrow results in ommatidial polarity defects, it seems likely that *fj* function is normally required at this stage of development. However, we cannot rule out an earlier role for localised *fj* expression, as it is possible that the *eyeless–GAL4* driver does not drive ectopic *fj* expression at a high enough level to produce a defect.

Expression of *fj* is regulated by the JAK/STAT, Wg and Notch pathways

The expression pattern of *fj*, and the phenotypes that we observed for loss-of-function and gain-of-function of *fj* activity, indicate a role for *fj* function in ommatidial polarity determination along the dorsoventral axis. Recent studies have revealed functions for the N, JAK/STAT and Wg pathways as regulators of ommatidial polarity determination, with the current model suggesting that Notch and Upd are positive regulators of a graded signal that is highest at the equator, whereas Wg is a negative regulator of such a factor (or factors). The *fj* gene is therefore a good candidate for being a downstream target of regulation by one or more of these pathways. Consistent with this, we do indeed observe that *fj* is regulated by the JAK/STAT and Wg pathways. In clones mutant for the *Drosophila* JAK homologue *hop*, which lack JAK function, a reduction in *fj* expression was observed (Figure 5a). Although JAK is a cell-autonomously acting signal-transduction component, the effect on *fj* expression was not cell-autonomous, with greatest downregulation being observed in the centre of the clone. In accordance with downregulation in *hop* clones, clones of cells ectopically expressing the JAK ligand Upd resulted in activation of *fj* expression (Figure 5b). Conversely, ectopic expression of Wg (which is predicted to be a negative regulator) resulted in downregulation of *fj* expression (Figure 5c). It has already been reported that activated N can non-autonomously activate *fj* expression [7], which we also observed (Figure 5d). Taken together, these results indicate that *fj* is regulated by all three of these pathways in a manner consistent with mediating their functions in dorsoventral polarity determination.

Figure 4



The phenotype caused by ectopic expression of *fj*. Anterior is to the left and dorsal is uppermost. (a,e) Sections through eyes in which *fj* was ectopically expressed using the GAL4–UAS system. (c) Immunolabelled third instar eye disc containing a clone of cells ectopically expressing *fj*. (b,d,f) Schematic representations of (a,c,e), respectively; darker grey area in (d) indicates the approximate extent of the *fj*-expressing clone. (a,b) Ventral eye section (white boxed area in inset) of a fly raised at 25°C in which *fj* was ectopically expressed at poles under control of *omb*–GAL4. Ommatidia close to the pole show inverted polarity for several rows. A similar phenotype is seen at the dorsal pole (data not shown). (c,d) Confocal image of dorsal region of a third instar eye disc,containing a clone of cells ectopically expressing *fj* (blue), and double-immunolabelled for the Elav protein to show photoreceptor nuclei (red) and the Prospero protein (green, appears yellow in overlay with red) to reveal ommatidial orientation, using fluorescently labelled secondary antibodies. Ommatidia on the equatorial edge of the clone show inverted polarity; the effect extends non-autonomously outside the clone. Ommatidia on the equatorial edge of the clone show inverted polarity; the effect extends non-autonomously outside the clone. (e,f) Ventral section through the eye of a fly raised at 29°C in which *fj* was expressed under the control of *sevenless*–GAL4. Several ommatidia show inverted polarity (green arrows). The phenotype is strongest at the poles of the eye, where endogenous *fj* expression is lower (data not shown). Occasionally, an additional phenotype is seen, in which an 'achiral' ommatidium is observed, which is symmetric about its anteroposterior axis.

Expression of *fj* is modified by an autoregulatory loop but not by Frizzled activity

One of the noteworthy aspects of *fj* regulation by the Notch and JAK/STAT pathways is that it is non-autonomous, even when it is studied using cell-autonomously acting signalling components such as the intracellular domain of N, N^{intra} [20]. One possible explanation for this non-autonomy would be that *fj* is able to activate its own expression via an autoregulatory loop. To test this hypothesis, we ectopically expressed *fj* in the presence of a *fj* enhancer trap and found that it was indeed able to activate its own expression (Figure 5e). The activation of *fj* expression by ectopic expression of *fj* was non-autonomous, again consistent with the proposed secreted nature of the *fj* gene product.

In addition to the N, JAK/STAT and Wg pathways, the only other gene reported to non-autonomously influence ommatidial polarity is *frizzled* (*fz*) [21]. A possible mechanism for non-autonomy of *fz* function would be via regulation of *fj* expression. We therefore looked at the expression of *fj* in *fz* loss-of-function clones (Figure 5f), and in clones of cells ectopically expressing *fz* (not shown), but in neither case saw any change in *fj* expression.

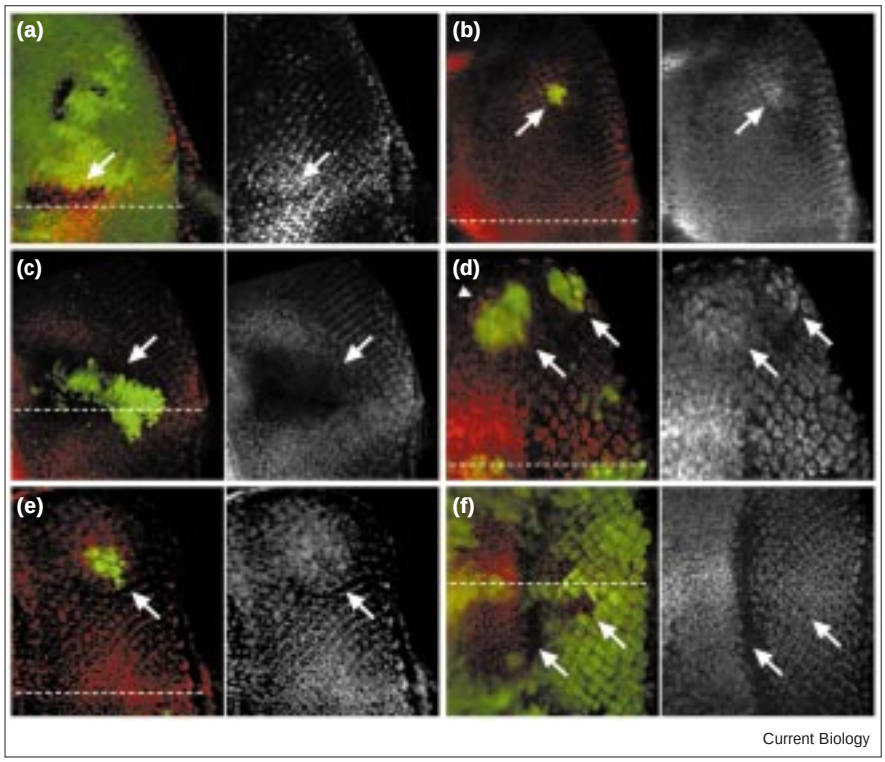
Discussion

Fj is a good candidate for a secreted factor required for dorsoventral ommatidial polarity determination

Our experiments demonstrate that a gradient of *fj* expression from the midline to the poles of the eye disc is necessary for normal dorsoventral polarity determination of ommatidia. Alterations in *fj* expression produced by either loss-of-function mosaic clones or ectopic expression resulted in changes in ommatidial polarity consistent with determination of this polarity by a gradient of *fj*-dependent signalling activity, which is manifested by ommatidia rotating away from a high point of *fj* expression (Figure 6). The non-autonomous nature of the polarity inversions observed on the polar boundaries of *fj* clones indicates that the *fj*-dependent signalling activity is extracellular. Given the molecular nature of the predicted *fj* gene product, we propose that *fj* encodes a secreted or cell-surface factor that is required for dorsoventral ommatidial polarity determination.

An important question is whether *fj* is likely to encode a second signal that acts at the final stages of ommatidial polarity determination, or whether it is an intermediate factor responsible for setting up the gradient of this signal. A number of pieces of evidence support the possibility that *fj* encodes a 'final' signal. Firstly, enhancer-trap activity and transcript *in situ* hybridisation data indicate that it is present in a gradient in the third instar disc at the time that ommatidia are differentiating and rotating to establish their final polarity. Secondly, ectopic expression experiments suggest that only disruption of the *fj* expression gradient posterior to the furrow (where the ommatidia are differentiating), and

Figure 5



The regulation of *fj* expression in the eye disc. All panels are confocal images of third instar eye discs, immunolabelled to show *fj* expression as revealed by the fj^{P1} enhancer trap using an anti-β-galactosidase primary antibody and fluorescently labelled secondary antibody (red, left-hand subpanels; white righthand subpanels), and containing clones marked by green fluorescent protein (GFP) expression. Anterior is to the left and dorsal is uppermost. (b–e) Ectopic expression clones, marked by the presence of GFP. (a,f) Homozygous *hop* and *fz* mutant clones, respectively, marked by the absence of GFP. The dashed lines indicate the approximate position of the equator. (a) A clone of homozygous hop^2 cells lacking JAK function (arrow) downregulates *fj* expression. Note that the effect is stronger in the centre of the clone and, even here, expression is not turned off completely. This is consistent with other factors activating *fj* expression in this region. (b) A clone of cells ectopically expressing Upd (arrow) non-autonomously activates *fj* expression. The non-autonomy appears to be polarised away from the equator, possibly indicating that cells further from the equator are more competent to respond to Upd or that Upd is secreted and/or preferentially moves from the clone in a polar direction. (c) A clone of cells ectopically expressing Wg (arrow) non-autonomously turns down *fj* expression near the equator. The effect is more potent anteriorly. The weak effect more posteriorly may be due to competition with the activator Upd, which is expressed in the posterior region. (d) Clones ectopically expressing activated Notch (arrows) non-autonomously activate *fj* expression away from the equator (arrowhead adjacent to larger clone). Expression of *UAS*–N^{intra} was driven at 18°C. (e) A clone of cells ectopically expressing *fj* (arrow) away from the equator non-autonomously activates *fj* expression. Again, the non-autonomous effect is strongest on the polar side of the clone, as seen with Upd. (f) Clones of homozygous fz^1 cells (arrows) have no effect on *fj* expression.

not anterior to it, is able to alter ommatidial polarity. Thirdly, *fj* expression is regulated by all three of the pathways (N, Wg and JAK/STAT) implicated in the establishment of the signal gradient.

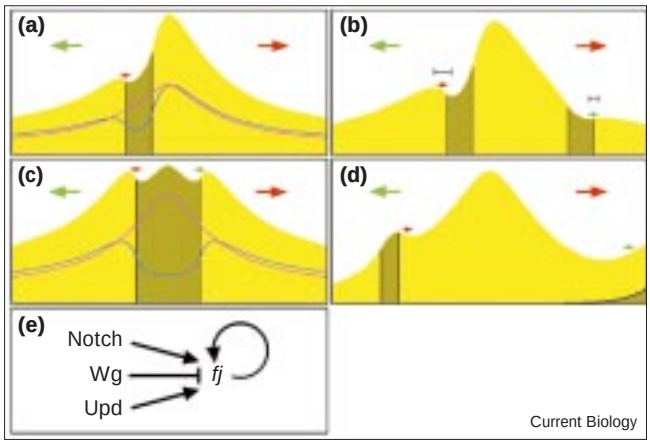
Conversely, it is clear that *fj* does not precisely correspond to the second signal, as previously envisaged. It has largely been assumed that the second signal would correspond to a single factor, and that this factor would act as a ligand for the *Fz* transmembrane receptor. The non-autonomous aspects of *fz* function would then be mediated by regulation of the expression of this factor. The redundancy of *fj* function rules out the possibility that it is a single final factor, and our data indicate that *fz* non-autonomy does not involve regulation of *fj* expression. Indeed, the actual relationship between *fj* and *fz* function remains unclear (see below).

Furthermore, while we favour the possibility that *fj* encodes a secreted factor, it should be noted that in the absence of immunolocalisation studies there is as yet no definitive proof that the carboxyl terminus of the *fj* gene product is cleaved and secreted *in vivo* to give rise to a diffusible ligand. Some other possibilities also fit the results observed. The Fj protein might remain associated with the expressing cell, either because the carboxyl terminus is not cleaved or because the carboxy-terminal peptide does not move once cleaved. In this case Fj could, nevertheless, signal over many cell diameters by a cell–cell relay mechanism. Expression of Fj in a gradient could also produce an activity gradient of another molecule, for instance by trapping or sequestering another secreted ligand. Alternatively, a function as a modifying enzyme that modulates the signalling activity of another pathway is conceivable, similar to the function of the putative glycosyltransferase Fringe in N signalling [22]. Finally, Fj protein might function as a transmembrane receptor that acts non-autonomously by activating a downstream signal.

The relationship of *fj* and *fz* in dorsoventral ommatidial polarity determination

Clonal analysis has indicated that the seven-pass transmembrane protein Fz plays an important role in ommatidial

Figure 6



Models of Fj function in ommatidial polarity specification. (a–d) Graphs of postulated second-signal activity (yellow) across the eye disc from the dorsal pole (left) to the ventral pole (right) with the equator lying at the peak of second-signal activity. Large arrows indicate the normal ommatidial polarity in each half of the eye disc. Ommatidia choose the direction to rotate, and thus their final polarity, according to the direction of slope of the second signal gradient (see [9,10]). Small arrows indicate inversions of ommatidial polarity produced by experimental manipulation of *fj* activity. (a) The final readout of second signal activity is the sum of at least two signals, one of which is *fj*-dependent. In the total absence of *fj*, a gradient is still provided by other signalling systems (pink line), which can determine ommatidial polarity to an imperfect but high degree of accuracy (> 99.5%). A clone of cells lacking *fj* (brown) causes an alteration in the shape of the *fj* activity gradient (blue line), the effect being non-autonomous because Fj is secreted. When the altered *fj*-dependent gradient is summed with that of the other signal, an altered second-signal profile results. Thus, *fj* clones invert the second signal gradient and induce ommatidial polarity reversals, despite the semi-redundant function of *fj*. (b) The strength of the *fj* clone phenotype is weaker in clones further from the equator, consistent with a steeper gradient of *fj*-dependent second-signal activity closer to the equator. The distance over which the gradient will be inverted for clones of equal size at two different positions is indicated (horizontal bars). (c) Clones that cross the dorsoventral midline (brown) still contain the endogenous equator. Preservation of the equator in such clones is due to a peak of second-signal activity that is still present when the *fj*-dependent activity (blue line) is summed with the other signalling system (pink line). (d) Clones of cells that ectopically express *fj* cause inversions of polarity on the equatorial side of the clone, by altering the second-signal profile (left). Expression of *fj* at the poles of the disc inverts ommatidial polarity (right). (e) Summary of the regulatory interactions that modulate *fj* expression.

polarity determination, such that the relative levels of Fz activity in a single pair of photoreceptor cells in each ommatidium determines the polarity adopted by the ommatidium [21]. This led to the proposal that a gradient of a Fz ligand from the midline to the poles of the eye disc was responsible for the determination of ommatidial polarity on the dorsoventral axis. Furthermore, as loss-of-function clones of *fz* also have a weak non-autonomous effect on ommatidial polarity, it has been suggested that Fz might be involved in maintaining a gradient of its own ligand [21].

If this model were correct, the Fz ligand would therefore correspond to the second signal, with Fz acting as its receptor. The gradient of the second signal would produce very small differences in Fz activity between cells in a single ommatidium. Two recent studies have indicated that such small differences could then be amplified into a binary fate choice by a N–Delta-dependent mechanism [23,24].

Although the Fz protein has previously been shown to bind ligands of the Wnt family [25], no Wnt protein has been identified that fulfils the criteria to be a second signal during polarity determination in the eye [9]. Our results raise the possibility that *fj* could encode a ligand that activates Fz during polarity signalling. There is no direct evidence to support this hypothesis, however. There are no reported instances of receptors of the Fz family being activated by ligands other than Wnts, and no obvious similarity between the predicted Fj protein and Wnts. Additionally, we have been unable to detect any genetic interactions between *fj* and *fz* that would support a close interaction of these two genes: for instance, there is no enhancement of a *fz* hypomorphic phenotype in a *fj* mutant background, and similarly no modification of a fz overexpression phenotype by reduction of *fj* gene dosage (D.S., unpublished observations). We also do not see any effect of *fz* clones on *fj* expression, which might be expected if *fz* encoded the *fj* receptor and mediated the autoregulation of *fj* expression. Finally, expression of *fz* under the control of the *sevenless* enhancer produces a randomisation of ommatidial polarity [26], which is distinct from the strict inversions of dorsoventral polarity seen when *fj* is so expressed (although this difference could be due to different potency of these two molecules in this assay).

It is also by no means certain that Fz is the receptor for the final polarity signal in the eye. Clearly, its differential activity in different cells of each ommatidium is critical for polarity determination, but there is currently no evidence that Fz activity varies significantly across the dorsoventral axis of the eye disc. The situation is also made more complex by the observation that *fz* has a weak non-autonomous effect on ommatidial polarity, whereas the known downstream components of its signalling pathway in this context (Dishevelled and RhoA p21 GTPase) have been shown to act autonomously [26]. It is quite conceivable that Fz, Dishevelled and RhoA are autonomously required for the elaboration of a signalling gradient across each ommatidium, as indicated by their function upstream of N and Delta, and that some other unidentified receptor actually receives the critical signal. This would not explain the non-autonomous function of *fz*, although one possibility is that *fz* acts via a different signalling pathway to modulate the expression levels of a different second signal, with which *fj* functions redundantly (see below).

The function of *fj* in ommatidial polarity determination is partly redundant

Given the striking polarity phenotype of *fj* clones, the very weak nature of the homozygous loss-of-function *fj* phenotype is somewhat surprising. We conclude that the role of *fj* in this process must be partially redundant, which also explains why this function has not previously been noted. The simplest hypothesis is that there is another molecule expressed in the same pattern as *fj* that can partly substitute for its function. It is easy to envisage why *fj* clones would still result in a strong phenotype, if the effects of adding together the effects of two signalling gradients are considered (Figure 6a). The existence of this second, parallel-acting factor is further supported by the observation that *fj* clones that cross the equator result in a triple-equator phenotype, suggesting that even in the centre of these clones, there is still a peak of another signalling activity (Figure 6c). Nevertheless, the presence of occasional polarity defects in *fj* homozygous mutant eyes indicates that *fj* is not totally redundant. One possibility is that the redundant factor is encoded by a *fj* homologue, although there is currently no evidence for such a homologue in *Drosophila*.

The recent results indicating that the N, Wg and JAK/STAT pathways are all required for normal dorsoventral polarity determination in the eye suggest that this process is highly regulated. It is interesting that all three of these pathways regulate *fj* expression, but that, nevertheless, *fj* itself is partially redundant with an unidentified signal. The existence of this level of redundancy in a developmental patterning process in *Drosophila* is largely unprecedented. Were all such patterning events so tightly controlled, then most single-loci mutations would reveal, at most, subtle phenotypes, and the genetic analysis of *Drosophila* development would not have been so straightforward. The existence of multiple signals regulating dorsoventral ommatidial polarity determination suggests that there is some survival advantage to the organism in tightly regulating this particular process, such that in a wild-type fly the incidence of errors is essentially zero.

The *fj* clonal phenotype

Two interesting features of the phenotype of *fj* clones deserve comment. Firstly, we note that the phenotype became weaker towards the poles of the eye. This is, in fact, consistent with a situation in which the slope of the gradient of *fj* expression is more shallow towards the poles of the eyes (Figure 6b). As clones right at the polar edge of the eye had no phenotype, this indicates that the unidentified factor with which *fj* functions redundantly is still capable of determining ommatidial polarity in this region.

Secondly, the non-autonomous effect on ommatidial polarity that we observed on the polar side of clones was not oriented strictly dorsoventrally (Figure 3). Instead, the effects of *fj* clones on ommatidial polarity were clearly skewed towards the posterior of the eye — a phenomenon that has not been reported for clones of any of the upstream signalling components. This would appear to result from the gradient of *fj* expression not being strictly dorsoventral. Instead, it is strongly expressed in a broad band anterior to the furrow and then in a wedge-shaped pattern posterior to the furrow that is weaker at the posterior edge of the eye disc. Thus, the gradient of the putative *fj* signalling activity would be expected to decrease towards the posterior polar regions, rather than strictly towards the poles. It is possible that the wedge-shaped pattern of *fj* expression is due to a reduction in the ability of *fj* to autoregulate its own expression towards the posterior of the eye disc following the passage of the furrow. This again provides an explanation for the skewed shadow of *fj* non-autonomy: cells on the polar-posterior edge of the clone would be less able to compensate for the reduced levels of *fj*-dependent signal and so a stronger phenotype would be seen in this region.

Conclusions

Here, we have presented the first characterisation of the function of *fj* in the establishment of polarity in the *Drosophila* eye. We have shown that both localised removal and addition of *fj* expression leads to the non-autonomous repolarisation of ommatidia. Furthermore we demonstrate that *fj* is regulated by the N, JAK/STAT and Wg pathways in a manner consistent with it mediating their functions in ommatidial polarity determination. We have found that localised *fj* expression is required at the time in development when ommatidia first become dorsoventrally polarised. Finally, we show that *fj* can autoregulate, so providing a mechanism for the retention of polarity information imparted at earlier developmental stages. In sum, these data constitute good evidence that *fj* encodes a second signal required for ommatidial polarity establishment.

Materials and methods

Analysis of the fj *homozygous phenotype*

The *fj* homozygous phenotype was examined in the following genotypes: fj^{P1}/fj^{P1}, fj^{d1}/fj^{d1}, fj^{d1}/*Df(2R)PC4*, fj^{d1}/fj^{1}, fj^{d1}/*Df(2R)Pcl11B* (information about these mutations can be found in Flybase, http://fly.ebi.ac.uk:7081/). All eyes were sectioned through the equator for consistency. Ommatidial polarity defects occurred in all these combinations at a rate between 1/100 and 1/800 ommatidia, with no obvious difference due to genotype, sex or temperatures between 20°C and 29°C. In total, 22/6,500 inverted ommatidia were seen in 51 eyes sectioned and no bias in the frequency of inversions relative to the position in the eye was seen: 1 occurred in the 5 ommatidial rows closest to the equator, and the other 11 occurred in rows 6–11. As all sections analysed included the equator, we cannot exclude the possibility that the phenotypic strength is different closer to the poles. In control experiments, 4,100 ommatidia were inspected in the eyes of males and females of the Oregon wild-type stock raised between 18°C and 29°C; no polarity defects were seen in any case.

Genetics

Loss-of-function clones for *fj*, *fz* and *hop* were generated using the FLP–FRT system [27] and marked with *white* (in the adult) or *Ubiquitin*-green fluorescent protein (GFP) (in discs [28]). Ectopic expression

was carried out using the GAL4–UAS system [14] and an *Act–FRT–y⁺–FRT–Gal4* cassette combined with *UAS–GFP* [16], according to methods described previously [10]. GAL4 drivers used were *sevenless*–GAL4 [29], *glass*-multiple-reporter–GAL4 (a gift of Matthew Freeman), *eyeless*–GAL4 [30], *omb*–GAL4 [31]. Other UAS lines used were *UAS*–Wg (a gift of Henry Krause), *UAS*–Upd [10], *UAS*–N^{intra} [32], *UAS*–N^{DN} [33].

Histology and molecular biology

Standard histological methods were used as previously described [10]. *UAS*–Fj was constructed using a full-length *fj* cDNA (gift of Flora Katz). The genomic region flanking fj^{d1} [13] was amplified by PCR using the primers 5′-TCTCTTCGCTCTCCCTCTC-3′ and 5′-GGGCAAGGGCTGATGCTATC-3′. The resulting product was sequenced and aligned to the genomic region (P1 clone DS08374, GenBank accession number AC004295) by standard methods.

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