Decoding vectorial information from a gradient: sequential roles of the receptors Frizzled and Notch in establishing planar polarity in the *Drosophila* eye

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Accepted 17 September; published on WWW 24 November 1999

SUMMARY

The *Drosophila* eye is composed of several hundred ommatidia that can exist in either of two chiral forms, depending on position: ommatidia in the dorsal half of the eye adopt one chiral form, whereas ommatidia in the ventral half adopt the other. Chirality appears to be specified by a polarizing signal with a high activity at the interface between the two halves (the ‘equator’), which declines in opposite directions towards the dorsal and ventral poles. Here, using genetic mosaics, we show that this polarizing signal is decoded by the sequential use of two receptor systems. The first depends on the seven-transmembrane receptor Frizzled (Fz) and distinguishes between the two members of the R3/R4 pair of presumptive photoreceptor cells, predisposing the cell that is located closer to the equator and having higher Fz activity towards the R3 photoreceptor fate and the cell further away towards the R4 fate. This bias is then amplified by subsequent interactions between the two cells mediated by the receptor Notch (N) and its ligand Delta (Dl), ensuring that the equatorial cell becomes the R3 photoreceptor while the polar cell becomes the R4 photoreceptor. As a consequence of this reciprocal cell fate decision, the R4 cell moves asymmetrically relative to the R3 cell, initiating the appropriate chiral pattern of the remaining cells of the ommatidium.

Key words: Frizzled, Notch, Drosophila, Eye, Polarity, Gradients

INTRODUCTION

Gradients play many important roles in biology. Gradients of morphogens can specify distinct scalar outputs, such as the activation of different subsets of genes and the differentiation of distinct cell types to create molecular and cellular patterns. Gradients of nutrients, chemo-attractants or pheromones can provide vectorial information which directs the movement of cells such as bacteria or platelets, or the extension of subcellular processes such as yeast shmoos or neuronal growth cones. Gradients have also been proposed to control the orientation of cells within tissues, so that the specific structures they form all point in the same direction (Lawrence, 1966; Stumpf, 1966). This phenomenon, known as planar cell polarity (Nübler-Jung, 1987), is well illustrated in insects, e.g. the abdominal epidermis of *Drosophila* is formed by a sheet of cells virtually all of which differentiate hairs or bristles that point posteriorly. If gradients are used to establish planar polarity, then cells at any position within a population should have the capacity to detect local, and perhaps small, changes in the concentration of a graded signal, irrespective of the absolute concentration of the signal. Moreover, they should be able to respond by orienting relative to the vector of maximal change in the concentration of this signal, which defines the ‘direction’ of the gradient (Lawrence, 1966; Stumpf, 1966).

In the *Drosophila* eye, planar polarity is not evident as the common orientation of single cells but rather by the asymmetric pattern and orientation of groups of cells – the ommatidia. Ommatidia are clusters of 20 cells precisely arrayed in the crystalline compound eye. The salient asymmetric feature is the arrangement of the eight photoreceptors (Dietrich, 1909). Each ommatidium contains six outer photoreceptors, R1-R6, arrayed in a trapezoidal shape made asymmetric by the different relative positions of the R3 and R4 cells (Fig. 1A). In the middle of the outer rhabdomere grouping lie the inner rhabdomeres, R7 and R8. The R8 cell is also asymmetrically positioned; its cell body is located on one side of the photoreceptor group (between R1 and R2), in strict correlation with the R3/R4 asymmetry (Fig. 1B).

Ommatidia occur as either of two asymmetric forms, one being the mirror reflection of the other. We color code these two different shapes as the red and the blue chiral forms (Fig. 1A). Strikingly, all the ommatidia in the dorsal half of the eye have the same chiral form, whilst all those in the ventral half have the opposite form (Dietrich, 1909). The two types meet at the dorso-ventral midline of the eye: the resulting line of
mirror polarization is called the equator and the dorsal and ventral extremes of the eyes are called the poles (Fig. 1A). In the context of the wild-type eye, ommatidial chirality also correlates with orientation: the apex of the trapezoid formed by the six outer photoreceptors points away from the equator, as indicated by the arrows in Fig. 1A. Hence, in the dorsal half of an eye, ommatidia are all of the same color and point in the same direction, whereas ommatidia in the ventral eye are all of the other color and point in the opposite direction.

Ommatidial chirality appears to be organized by a putative polarizing signal, ‘factor X’ (Wehrli and Tomlinson, 1998), which spreads either inwards from the poles or outwards from the equator. For the sake of simplicity, we refer to factor X activity in the eye as being low at the poles and high at the equator, but recognize that the inverse distribution is equally valid. Up to 15 ommatidia can be aligned in the equatorial-polar axis in each half of the eye, and all decode polarized factor X activity to guarantee that the same chiral forms of ommatidium are produced with very high fidelity.

As indicated in Fig. 1C, factor X is proposed to be distributed as a gradient in each half of the eye under the control of two distinct signaling centers. Fringed (Fng) protein, expressed by ventral, but not dorsal, cells within the eye creates a Fng/non-Fng interface at the equator. This juxtaposition of Fng with non-Fng cells elicits a sharp peak of activity of the receptor Notch in a thin stripe of cells straddling the equator, positively regulating factor X (Papayannopoulos et al., 1998; Cho and Choi, 1998; Dominguez and de Celis, 1998). Conversely, Wg secreted by cells outside of the eye that flank the dorsal and ventral poles appears to diffuse into the retina and negatively regulate factor X activity (Wehrli and Tomlinson, 1998). Hence, these two signaling centers provide an opposing source and sink for factor X activity, which together can account for its graded activity along the equatorial-polar axis. Ommatidia detect the direction of change of this graded activity and use this vectorial information to choose their chirality (Fig. 1C).

The frizzled (fz) gene encodes a serpentine receptor-like protein (Vincent et al., 1989) that is essential for normal planar polarity (Gubb and Garcia-Bellido, 1982) and is a candidate receptor for factor X. Its name refers to the misaligned hairs and bristles observed in mutant flies. Fz has also been implicated as a receptor for Wg, along with a second Fz-like protein Fz2 (Bhanot et al., 1996). However, the available evidence indicates that Wg is not factor X (Wehrli and Tomlinson, 1998) and does not directly control planar cell polarity in the eye or other epidermal derivatives (Struhl et al., 1997). Fz protein is essential for normal eye development and its polarized activity in the R3/R4 photoreceptor pair appears necessary for the acquisition of normal chirality (Zheng et al., 1995).

Receptors of the Lin-12/Notch family and ligands of the Delta/Serrate/Lag-2 (DSL) family (see Greenwald, 1998) have pervasive roles in development, often acting in a process called ‘lateral specification’, in which neighboring cells communicate with each other to choose the subset of cells that will follow one specific fate while the remainder follow an alternative fate. For example, during nematode development, two equivalent cells, Z4.aaa and Z1.ppp, come into contact and compete to become the Anchor Cell (AC) as opposed to the ventral uterine precursor cell (VU). Both cells initially express the DSL ligand Lag-2 as well as the receptor Lin-12, and upon making contact, each cell attempts to suppress the AC fate in the other cell by presenting ligand to activate the other’s receptor (Seydoux and Greenwald, 1989). The receptor and ligand are also coupled by a feed-back loop such that enhanced activity of the receptor leads to upregulation of the receptor and downregulation of the ligand (Wilkinson et al., 1994). As a consequence, as soon as one cell achieves a slight, probably stochastic ascendency in this signaling competition, this small differential is rapidly amplified, causing receptor activity to decline precipitously in one cell (the presumptive AC) while increasing to maximal levels in the other (the presumptive VU).

Ommatidia arise from clusters of 5-7 cells, including the presumptive R3 and R4 cells, which both contact each other and abut the central R8 cell. The chiral pattern of the ommatidia is first apparent as an asymmetric rearrangement of the presumptive R3 and R4 cells in which the presumptive R4 cells separates from the R8 cell whilst the presumptive R3 cell retains contact (Tomlinson, 1985). This difference in cellular behavior reflects an earlier symmetry breaking event in which the two cells of the presumptive R3/R4 pair make alternative cell-fate choices based on their different positions along the equatorial-polar axis. Here, genetic mosaic data are used to determine the roles of Fz and Notch (N) in this process.

Our data provide evidence that symmetry is broken in response to the graded or polarized activity of factor X by the following two-step mechanism. First, a gradient of factor X activity leads to enhanced or polarized activity of the receptor Fz in the presumptive R3/R4 photoreceptor cell that lies closest to the equator where factor X activity is highest. Lateral specification then ensues between the R3/R4 pair using Delta (DI) and N, with the initial difference in Fz signal transduction serving to bias the feedback mechanism and ensure that the cell closest to the equator becomes the R3 cell while the remaining cell becomes the R4. Our findings also indicate that the Notch interaction is an essential amplification step for correctly interpreting an initial, and possibly small, difference in Fz activity between the two members of the R3/R4 pair. We note that this proposed mechanism is also supported by recent findings of Fanto and Mlodzik (1999) and Cooper and Bray (1999). However, our results are distinct from these others in our use of genetic mosaics to assess directly the requirements for Fz and N signaling in each of the two members of the presumptive R3/R4 pair. This information is critical to establish the functional relationships between Fz and DI-N signaling in making the spatially ordered R3/R4 decision upon which chirality depends. It also leads us to suggest different models about the mechanisms that link these two signaling systems.

**MATERIALS AND METHODS**

**Mutations and duplications**

The following amorphic mutations of fz, N, DI and Ser were used: fz<sup>H51</sup>, fz<sup>DK68</sup>, N<sup>XR11</sup>, DI<sup>rev10</sup> and Ser<sup>AX82</sup>; for experiments involving N, we also used the temperature-sensitive mutation N<sup>ts1</sup> and the duplication Dpt (1;2)N<sup>500</sup> (described in Flybase: http://flybase.bio.indiana.edu/).

**Transgenes**

The following previously described transgenes were used: FRT101, FRT80, FRT82 (Chou and Perrimon, 1992; Xu and Rubin, 1993).
w^70, w^90 (Xu and Rubin, 1993).

hs-FLP.1 (Struhl and Basler, 1993),

sev-N^ntra (sev-N^ntra; Fortini et al., 1993).

The following new transgenes were used (details available on request):

1Xsev-fz, mini-w^+; one copy of the sev enhancer (Basler et al., 1989) was placed upstream of the promoter (Bovet et al., 1989) and the coding sequence (Vinson et al., 1989) in the mini-w vector Pw8 (Klemenz et al., 1987).

2Xsev-fz, mini-w^+ is identical to 1Xsev-fz, mini-w^+ except that two copies of the enhancer were placed in tandem upstream of the hsp70 promoter (Basler et al., 1989); the transgene is inserted in the X chromosome.

sev-N^ntrald, mini-w^+; two copies of the sev enhancer were placed in tandem upstream of the hsp70 promoter and the N^ntrald coding sequence in the Pw8 vector; the transgene is inserted in the X chromosome. The N^ntrald coding sequence is a truncated version of the N^ntra coding sequence (Struhl et al., 1993) created by introducing a stop codon immediately downstream from the coding sequence (Struhl et al., 1993) following heat shock-induced technique (Struhl and Basler, 1993) or by Flp-mediated mitotic recombination (Golic, 1991) or the Flp-Mosaic eyes were generated by X-ray induced mitotic recombination, which the coding sequence for the N-terminal Engrailed Repressor to sectioning.

**RESULTS**

**Ommatidial chirality**

Early ommatidia arise as bilaterally symmetric clusters of four presumptive photoreceptor cells, R2-R5, grouped around a fifth, R8. Within the cluster, the R3 and R4 cells begin life as equivalent cells (the presumptive R3/R4 pair) and then make reciprocal cell fate choices, the member of the R3/R4 pair closest to the equator becoming the R3 cell and staying in contact with the R8 cell, while the other member becomes R4 and breaks contact with R8. Throughout this paper, we refer to the cell in the position closer to the equator as the presumptive R3 cell and to the other member of the pair as the presumptive R4 cell. In those experimental cases in which an ommatidium adopts the inappropriate chiral form, the presumptive R3 cell chooses to become an R4 cell and the presumptive R4 cell becomes R3.

**A fate map of the precluster reveals that R3 and R4 arise from cells located at a distance from each other**

The work of Zheng et al. (1995) defined the two cells of the presumptive R3/R4 pair as a focus for determining ommatidial chirality, suggesting that these cells are responsible for decoding the factor X gradient. In the 5-cell precluster, the presumptive R3 and R4 cells are adjacent. However, histological analyses indicate that these cells, as well as the remaining three cells of the cluster, were initially arrowed as a line of cells extending along the equatorial-polar axis (Tomlinson and Ready, 1987; Wolff and Ready, 1993). Moreover, they suggested that the cells that become the presumptive R3 and R4 photoreceptors derive from the cells towards the ends of each line whilst the cells that will form the R8, R2 and R5 cells are located in the middle (Fig. 1C).

To ascertain the spatial relationships of the presumptive photoreceptor cells during this initial phase of precluster formation, we fate-mapped the R2, R3, R4, R5 and R8 photoreceptors using mosaic analysis. We induced clones of white^- (w^-) cells in eyes and when the clone border passed through an ommatidium we scored which of the photoreceptor cells belonged to the clone and which did not. Presumptive photoreceptor cells that arise from adjacent cells are likely to be contained within the same clone, whereas those that arise from cells further apart are more likely to derive from different clones and hence have a higher frequency of mosaicism. A total of 224 mosaic ommatidia were scored and the lineage relationship between each pair of cells was determined (Table 1). This fate mapping suggests that although the presumptive...
R3 and R4 cells are adjacent in the precluster, they derive from cells that are initially most distant from each other, at the ends of the lines of cells from which the precluster will form. This initial separation could, in principle, facilitate the ability of the presumptive R3/R4 pair to detect differences in the abundance of factor X activity (Fig. 1C; see Discussion).

The role of Fz in establishing ommatidial chirality

Ommatidia mosaic for fz activity

Ommatidia in an fz mutant eye occur as three different shapes interspersed in an apparently random fashion (Zheng et al., 1995). These are the blue and red chiral forms described above, as well as pseudo-symmetrical ommatidia in which the R3/R4 pair appear to occupy symmetrical positions; we color code this last form black. A previous analysis of fz mosaics suggested that the activity of Fz in the R3/R4 pair is important in determining which becomes R3 and which R4 (Zheng et al., 1995). That is, the presence of fz+ in only one cell of the pair frequently directed that cell to become the R3 cell and the other the R4. However a sizeable fraction (7%) of the ommatidia were of the opposite configuration, with the R4 cell carrying fz+. In their analysis, Zheng et al. (1995) separated the ommatidia into data groups depending on their orientation. The orientation results from the rotation of the ommatidia (Tomlinson and Ready, 1987) and although rotation correlates with chirality in wild-type eyes, its relationship to chirality in mutants is complex. The rotation correlates with chirality in wild-type eyes, its relationship to chirality in

From the lineage linkage between the cells we infer their initial proximity. The higher the number of times a pair of cells are found in the same clone the more likely they are to have arisen in proximity. The most distantly related pair of cells are R3/R4 and the most closely related are R2/8 and R5/8. From this we infer that R2 and R5 arise in close proximity to the central cell R8 and that these three cells form the core of the precluster. We note that R4 is as closely related to R8 as it is to R5, and R3 is as closely related to R8 as it is to R2. This is inconsistent with a simple linear array of cells; rather it suggests that the precluster begins life as a stretched-out group of cells with R2/8/5 at the core and the most distant cells being R3 and R4.

Table 1. Lineage relationship between cells of the precluster

<table>
<thead>
<tr>
<th>Pair</th>
<th>Times Found in Same Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>2/3</td>
<td>123</td>
</tr>
<tr>
<td>2/4</td>
<td>105</td>
</tr>
<tr>
<td>2/5</td>
<td>125</td>
</tr>
<tr>
<td>2/8</td>
<td>150</td>
</tr>
<tr>
<td>3/4</td>
<td>84</td>
</tr>
<tr>
<td>3/5</td>
<td>92</td>
</tr>
<tr>
<td>3/8</td>
<td>121</td>
</tr>
<tr>
<td>4/5</td>
<td>120</td>
</tr>
<tr>
<td>4/8</td>
<td>112</td>
</tr>
<tr>
<td>5/8</td>
<td>147</td>
</tr>
</tbody>
</table>

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chirality and cell genotype, and ignoring orientation. We separated ommatidia into three classes – those that made the wrong chiral choice, those that made the right choice and those that remained symmetrical. Given the random distribution of red, blue and black ommatidia in \( \text{fz}^- \) eyes, we infer that mutant ommatidia adopt their chiral shape randomly. Hence, for those mosaic ommatidia that showed the correct chirality we do not know whether they succeeded in doing so because they retained the ability to make the correct choice, or whether they lacked this ability but made the correct choice by chance. However, for those mosaic ommatidia that showed the inappropriate chiral form, it is clear that they lacked the ability to decode the polarizing signal. Clones of cells marked by the \( w^- \) marker and homozygous for the amorphic \( \text{fz}^{H51} \) or \( \text{fz}^{KDA} \) mutations (referred to subsequently as just \( \text{fz}^- \)) were induced in the developing eye and the chiral forms were assessed.

Mosaicism in incorrect chiral forms

311 incorrect chiral ommatidia were scored at the mosaic interface. In those ommatidia in which the mosaic boundary separated the R3/R4 pair (193/311), the R4 cell was invariably mutant and the R3 wild type (Fig. 2A). Since we are considering here only the class of ommatidia which have inappropriate chirality, it follows that the presumptive R3 cell in each of these ommatidia chose incorrectly to develop as an R4 cell. This result allows us to define a focus for \( \text{fz} \) gene activity specifically in the presumptive R3 cell. Of the remaining 118/311 ommatidia in this class, some (64) were mutant for both the R3 and R4 cell; however, the remainder (54) were wild type for both cells. Indeed, we find that all photoreceptors within an ommatidium can be wild type, even though the ommatidium makes the wrong chiral choice. These results argue for another focus of \( \text{fz} \) gene function outside the R3/R4 pair and the photoreceptor group generally (see below).

Mosaicism in correct chiral forms

276 correct ommatidia were scored at the mosaic interface. Of these, 182 showed mosaicism between R3/R4, of which 179 had the R3 cell wild-type for \( \text{fz} \) and the R4 mutant. The remaining three were of the opposite configuration, with R4 cell carrying \( \text{fz}^+ \). We attribute the presence of these three ommatidia to persistence of the \( \text{fz}^+ \) transcript or protein in mutant presumptive R3 cells, which endow it with residual \( \text{fz} \) gene function. The frequency of such ‘perdurance’ effects should depend on when the clones of \( \text{fz}^- \) cells are induced, possibly accounting for why our frequency of ‘aberrant’ R3/4 mosaics is lower than that of Zheng et al. (1995).

Mosaicism in symmetric chiral forms

Five symmetric ommatidia were scored at the mosaic interface. Four of these were mutant for both members of the R3/R4 pair (the one exception probably resulting from perdurance, as we suggest above for the rare mosaics that have the correct chiral form but are mutant for the R3 cell).

Combining the data sets we can make the following three statements. (1) When mosaicism exists between the R3/R4 pair, the cell that is wild type will become the R3 cell and the one that is mutant will become R4. (2) Ommatidia do not adopt the symmetrical (black) form when mosaicism exists between R3/4. (3) Ommatidia can adopt the incorrect chiral form, even when both members of the pair are wild type. We note that in this last instance, chirally incorrect ommatidia that are wild type for the R3/R4 can occur at any position along the border of the clone, but are found predominantly at the polar side of the clone. These reflect a non-autonomous effect of neighboring \( \text{fz}^- \) cells described by Zheng et al. (1995), which we consider further in the Discussion. The following experiments are all focused on the requirement for \( \text{fz} \) in the presumptive R3 cell.
Normal development of ommatidia in which Fz is expressed only in sevenless expressing cells

The enhancer of the sevenless (sev) gene drives transcription in a number of cells during ommatidial development, but at the early stage of photoreceptor differentiation when chirality is established, this enhancer is active in only the presumptive R3/R4 pair and in the ephemeral mystery cells (Basler et al., 1989; Bowtell et al., 1989). We therefore engineered a transgene in which a single sev enhancer was coupled to the sev promoter to express fz in the sevenless expression pattern. Eye development appears normal in flies carrying a single copy of this 1Xsev-fz transgene. Moreover, this transgene fully rescues the roughening phenotype of the eye in fzt- animals. By contrast the bodies show the frizzled phenotype (as expected if the transgene is active only in the eye). When we sectioned these eyes we observed a normal chirality pattern except for rare (<5%) incorrectly chiral ommatidia (Fig. 2D). Thus, it appears that the restricted expression of fz in just the presumptive R3/R4 pair and the mystery cells is sufficient to allow developing ommatidia to adopt the correct chirality.

Mosaic analysis of sev-fz ommatidia

In the experiments described above, the fz coding sequence was expressed under the control of a single sev enhancer and the relatively weak sev promoter. However, when fz is expressed using the sev expression vector derived from Basler et al. (1989), in which two sevenless enhancers are placed in front of the stronger hsp70 promoter, the resulting eyes show a dominant frizzled-like phenotype in which blue, red and black ommatidia are randomly intermixed within the eye (Tomlinson et al., 1997). We performed mosaic analysis with this 2Xsev-fz transgene first in an fzt- mutant background. Mosaic ommatidia in which both the R3 and R4 cells lacked the transgene, or carried the transgene, appeared to choose chirality randomly, generating correct, incorrect and symmetric forms with similar frequency to ommatidia in fzt- mutant eyes. However, the presence of the transgene in only one of the two members of the R3/4 pair caused that cell to become R3 and the other R4 (264 ommatidia mosaic for R3 and R4 were scored and in 263 the R3 cell had the 2Xsev-fz transgene; Fig. 2C). The presence or absence of the transgene in other cells did not affect chiral choice. Since the only Fz proteins in the cells of this experiment were derived from the transgene, we infer that the sevenless enhancer is most likely active during the time window in which ommatidia chirality is determined. We then performed mosaic analysis with the 2Xsev-fz transgene in an fzt- background and found that ommatidia with inappropriate chirality arise at the mosaic interface. In all such incorrectly patterned ommatidia, either one or both members of the R3/R4 pair contained the 2Xsev-fz transgene. Moreover, whenever the R3 and R4 pair are mosaic in such chirally incorrect ommatidia (N=41), we found that the R3 cell always carried the sev-fz transgene (Fig. 2B). Indeed, we found that the R3 cells also invariably carried the sev-fz transgene in chirally correct ommatidia that were mosaic for the R3/R4 pair (N=50). Finally, we found that ommatidia in which both members of the R3/R4 pair lacked the transgene invariably adopted the correct chiral form. Since this mosaic analysis was performed in an fzt- background, it follows that when one of the R3/4 pair carried the transgene, that cell chose the R3 fate because it expressed more Fz protein and presumably experienced a higher level of Fz activity. Thus it appears that the presumptive R3 and R4 cells can assess differences in their relative levels of Fz activity.

The role of Notch in establishing ommatidial chirality

By manipulating Fz activity in the above experiments, we found that the presumptive R3 and R4 cells have the capacity to detect relative differences in their levels of Fz activity. Moreover, we found that such differences are sufficient to commit these two cells to follow alternative cell fates, with the cell having higher activity becoming an R3 cell while the cell with lower activity becomes an R4 cell. This behavior is reminiscent of the phenomenon of lateral specification observed between the presumptive AC/VU pair of cells in the nematode, as well as between neuroblasts and surrounding ectodermal cells in proneural clusters in insects (Doe and Goodman, 1985; Greenwald, 1998). Lateral specification depends in these systems on ligands of the Delta/Serrate/Lag-2 (DSL) family, which activate the Lin12/Notch (N) class of receptors. We therefore examined the possible roles of DSL-N signaling in establishing ommatidial chirality.

Notch reveals a role for N in establishing chirality

Clones of cells devoid of N activity do not survive in the developing eye, precluding an assessment of frank loss of N gene function in the chirality read-out mechanism. We therefore examined the consequences of reduced N gene function by placing Nts third instar larvae at the restrictive temperature (31°C) for 8 hours, before returning them to the permissive temperature (18°C). Many effects of these temperature shifts on differentiation in the eye have been documented (Foster and Suzuki, 1970; Shellenbarger and Mohler, 1978; Cagan and Ready, 1989) and eyes from such temperature-shifted animals typically carry a large dorso-ventral scar formed by those cells in the morphogenetic furrow at the time of the temperature shift. Ahead of the scar, in the tissue insensitive to the treatment, normal dorsal and ventral domains of ommatidia exhibiting red and blue chirality occur separated by an apparently normal equator (Fig. 3A). However, immediately behind the scar, inappropriate chiral types are found, with a preponderance of ommatidia exhibiting the symmetrical form (Fig. 3A). Thus, a reduction of N gene function suffices to compromise the ability of the R3/R4 pair to choose alternative R3 and R4 fates.

In principle, the symmetrical ommatidia observed in Nts eyes could reflect the choice of both members of the presumptive R3/R4 pair to differentiate as the same type of photoreceptor, either R3 or R4, or possibly a photoreceptor of hybrid R3/R4 type. Fanto and Mlodzik (1999) and Cooper and Bray (1999) have reported that they can recognize R3/R3 symmetrical ommatidia and distinguish them from R4/R4 ommatidia, based on their morphological characteristics. They also report that symmetrical ommatidia observed in Nts eyes are of the R3/R3 type. However, using the same criteria described by these authors, we find symmetric ommatidia of both apparent R3/R3 and R4/R4 types in Nts eyes. The situation is further complicated by our finding that departures from chirality are frequently apparent in some planes of section, but not others. For example, Fig. 3F shows a series of sections through a ‘symmetrical’ ommatidium (marked with an asterisk). At one
level this ommatidium appears as one chiral form (red, in this case), lower down it appears symmetrical (black), and still farther down it appears as the other chiral form (blue). Thus, there can be significant complexity to interpreting the identity of the two members of the R3/R4 pair in symmetric ommatidia. As a consequence, we refer to all ommatidia that show symmetrical forms at any plane of section as ‘symmetrical’ and do not attempt to distinguish between putative R3/R3 or R4/R4 classes of symmetric ommatidia.

Activating the Notch transduction pathway directs presumptive R3/R4 cells to adopt the R4 fate

To investigate further a role for N in establishing chirality we expressed a truncated, constitutively active form of the Notch intracellular domain (N\textsubscript{intra}\textsuperscript{Δ}) under sevenless enhancer control (sev-N\textsuperscript{intra}\textsuperscript{Δ} transgene). Ommatidia in which all cells carry the sev-N\textsuperscript{intra}\textsuperscript{Δ} transgene show severely disrupted patterns of photoreceptor differentiation (see also Fortini et al., 1993) and chirality cannot be assessed. However, when we examined ommatidia which are mosaic for the presence of the transgene in an otherwise wild-type background, we found that they could form the normal complement of eight photoreceptors, provided that both the R1 and R6 cells did not carry the transgene. When such normally composed ommatidia were mosaic for the R3/R4 pair, we observed that the cell carrying the sev-N\textsuperscript{intra}\textsuperscript{Δ} transgene invariably developed as the R4 photoreceptor, generating correct chirality in 25 cases and incorrect chirality in 28 cases (Fig. 3B). Ommatidia in which both members of the R3/R4 pair carried the transgene were wild type, invariably showed normal chirality.

Repressing the Notch transduction pathway directs presumptive R3/R4 cells to adopt the R3 fate

Since we could not examine N\textsuperscript{−} clones in mosaics, we engineered a transgene that would dominantly inhibit the N transduction pathway and used this transgene in mosaic eyes to selectively reduce N transducing activity in one of the two cells of the presumptive R3/R4 pair.

Suppressor of Hairless (SuH) encodes a DNA binding protein that is thought to upregulate the transcription of downstream target genes in response to activation of N (Schweisguth and Posakony, 1992; Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). We therefore expressed a chimeric form of Su(H) protein, Su(H)-EnRep, which bears a repressor domain from the Engrailed protein fused to the C terminus and partially suppresses the Notch signal transduction pathway (H.-M. Chung and G. Struhl, unpublished). Eyes of flies carrying a sev-Su(H)-EnRep transgene contain a high preponderance of symmetrical ommatidia indicating, as in the case of the N\textsuperscript{Δ} experiment, that compromising the N transduction pathway interferes with the ability of the presumptive R3/R4 cells to resolve into distinct R3 and R4 photoreceptors. We then performed mosaic analysis and found that when only one of the pair carried the transgene, that cell invariably developed as an R3 photoreceptor, while the remaining member of the pair became the R4 (Fig. 3C; N=26). Thus, when N signal transduction is selectively compromised in one of the two cells of the R3/R4 pair, that cell is directed to the R3 fate while directing the remaining cell to adopt the R4 fate; this is the opposite effect to that observed with the sev-N\textsuperscript{intra}\textsuperscript{Δ} transgene.

Requirement for the Notch ligand Delta in establishing ommatidial chirality

Since we were able to drive either of the two presumptive R3/R4 cells within an ommatidia to become the R3 or R4 photoreceptor by reducing or increasing Notch transducing activity in that cell, we infer that these cells choose alternative cell fates through a Notch-dependent process of lateral specification. In this process, the two cells ‘compete’ to become R3, the winning cell sending a signal that activates Notch in the loser, thereby directing the loser to become an R4 and blocking its ability to send back the same signal. If this model is correct, then eliminating the ability of one of the two cells to express the Delta pathway in the other, and hence bias the outcome of the competition. Another prediction is that if both cells are unable to express N ligands, then neither would be able to inhibit the other. Under this condition, the presumptive R3/R4 pair might not be able to resolve into distinct R3 and R4 photoreceptors leading to the formation of a symmetrical ommatidium. Alternatively, the cell-fate choice of each cell might be made in a stochastic fashion, or be biased by interactions with other ligand-expressing cells in the vicinity.

Two well-established ligands for Notch are encoded by the Delta (Dl) and Serrate (Ser) genes. We therefore induced the formation of Dl\textsuperscript{−} and Ser\textsuperscript{−} mutant clones in the eye and assessed the effects of such clones on the chirality of mosaic ommatidia. Ser\textsuperscript{−} clones generally appear wild type and only very rarely caused perturbations in chirality (Fig. 3E). By contrast, Dl\textsuperscript{−} clones induced early during eye development were associated with large abnormal patches of mutant tissue, with only rare mosaic ommatidia containing normal cohorts of eight photoreceptors forming at the interface with the wild-type tissue (data not shown). To obtain a higher frequency of mosaic ommatidia with normal photoreceptor cell complements, we induced clones later in eye development, during the larval third instar. Many of the resulting mosaic ommatidia exhibited normal photoreceptor cell complements.

In those normally composed ommatidia in which the R3/R4 pair was mosaic for Dl gene function (Fig. 3D), the mutant cell invariably developed as R4, whereas the wild-type cell developed as R3, irrespective of the location of the ommatidium within the eye (N=49). By contrast, all of the normally composed mosaic ommatidia in which both the R3 and R4 cells remain wild type showed normal chirality (N=12). Thus, eliminating the ability of either the presumptive R3 or R4 cell to express Dl appears to direct the reciprocal cell fate choices made by both cells, causing the ommatidia to adopt the wrong chiral form when the presumptive R3 cell is deficient.

A more complex situation emerges when we consider those normally composed ommatidia in which both the R3 and R4 cells are mutant for Dl (N=16). Six of these were symmetrical,
as would be expected if the presumptive R3 and R4 cells normally signal to each other using the N pathway in order to make the correct cell fate choices. However, ten succeded in adopting asymmetric types, and, for reasons we do not understand, all of these were of the incorrect form. To investigate a possible role for Ser in chiral choice when both ommatidia are mutant for Dl, we induced Dl-Ser- double mutant clones. These clones behaved as Dl clones alone, Fig. 3F, with the exception that ommatidia containing both R3 and R4 mutant cells were rare. This prevented us from assessing a role for Ser in the chirality choice when R3 and R4 are both mutant for Dl.

Asymmetries in R8 position within the ommatidium
R8 is the central cell in the developing ommatidium and about 36 hours after preclusters form, the cell body of R8 moves eccentrically between those of R1 and R2 to achieve an asymmetric position on the R3 rather than the R4 side of the adult ommatidium (Tomlinson, 1985). In the fz analyses described above, the R8 cell position usually (>95%) correlated with the R3/R4 asymmetry. However, we frequently observed an uncoupling of the R8 position from the R3/R4 asymmetry in sev-N intral, sev-Su(H)-EnRep and Dl- mosaics, which alter N signaling. In these cases, R8 cells were usually found between R5/R6, occasionally found between R1/R6, and in rare cases observed between other cells.

Epistasis experiments: N is downstream of Fz
The presumptive R3 and R4 cells normally choose their correct fate depending on relative differences in Fz and N activity: higher Fz activity promotes the R3 fate and higher N activity promotes the F4 fate. To determine whether these signaling
activities occur sequentially and in a functionally dependent fashion, we performed epistasis experiments in which we biased the activity of one receptor while compromising the activity of the other.

Differential Fz activity does not influence ommatidial chirality in eyes compromised for N signal transduction

As noted above, we cannot analyze the consequences of eliminating N activity in mosaics by removing N gene function in somatic clones, as these do not survive. However, we have observed that compromising N signal transduction, as in \( N^{ts} \) and sev-Su(H)-enRep eyes, is sufficient to cause most ommatidia to become symmetrical. To test whether this loss of chirality reflects a failure of the mutant ommatidia to respond appropriately to differences in Fz activity, we induced clones of \( f^{c-} \) cells in flies carrying the sev-Su(H)-enRep transgene and then scored the chirality of the ommatidia which were mosaic for \( f^{c-} \) cells. In \( f^{c-} \) eyes a similar outcome occurred. Of 33 ommatidia scored 32 carried the sev-Ntrans gene in the R4 cell and not R3, and in the remaining case, both R3 and R4 carried the sev-Ntrans gene. Hence, absence of \( f^{c-} \) gene function does not compromise the ability of an activated form of N to drive a cell to the R4 fate (Fig. 4A).

Altering N gene dosage can control ommatidial chirality in \( f^{c-} \) mutant eyes

Both epistasis experiments place the N signaling pathway downstream of Fz and suggest a model in which DSL-N signaling is essential to amplify an initial difference between the presumptive R3 and R4 cells achieved by differential Fz activation. Accordingly, Fz signal transduction might provide only a modest bias, which operates in a manner equivalent to stochastic differences in Lag-2/Lin-12 signaling between the presumptive AC/VU cells in the nematode. We tested this possibility by removing \( f^{c-} \) gene function (and thereby removing the normal bias) and then varying N gene dosage in the R3/R4 pair such that one cell had two wild-type copies of N and the other three. Under these conditions, we find that the cell with three copies invariably becomes the R4 cell and the other becomes the R3 (Fig. 4C; \( N=48 \)). Thus, in the absence of Fz activity, a relatively small bias in the level of wild-type

![Image](https://via.placeholder.com/150)
DISCUSSION

The *Drosophila* eye is composed of several hundred ommatidia, which occur in either of two chiral forms. Strikingly, all of the ommatidia in the dorsal half of the eye adopt one chiral form, while all of the ommatidia in the ventral half adopt the other form. Previous studies suggest that each ommatidium chooses its chirality in response to a signal, 'factor X', which is graded in the equatorial/polar axis in each half of the eye (Wehrli and Tomlinson, 1998; Papayannopoulos et al., 1998; Cho and Choi, 1998; Dominguez and De Celis, 1998). To do so, cells within the ommatidium must be able to detect the direction of change in the activity of factor X, a vectorial property, and to modify their behavior accordingly to generate the appropriate chiral form.

The mechanisms by which ommatidia sense the vector of factor X activity and adopt the appropriate chiral form are addressed here. Three main findings are presented.

First, the findings of previous work (Zheng et al., 1995) are extended to confirm that the focus for the chirality choice maps to the presumptive R3 and R4 cells. Possibility that the presumptive R3 and R4 may meter factor X expression is sufficient to dictate reciprocal cell fate choices by the presumptive R3 and R4 cells.

We note that varying the gene dosage of *N* in one member of the R3/R4 pair of otherwise wild-type flies generally had no effect on ommatidial chirality (Fig. 4D), except for the rare occurrence (<2%) of ommatidia of symmetric type.

We show that the resulting disparity in *N* signal transduction is both necessary and sufficient to specify the reciprocal R3 and R4 cell fates and to determine the chirality of the ommatidia.

Sensing the gradient of factor X: the role of Frizzled in the acquisition of ommatidial chirality

We present two models for how Fz activity within the R3/R4 pair might bias the N-DI interaction. In the first model, factor X activity positively regulates Fz activity, whereas in the second it negatively regulates Fz activity. As noted in the Introduction, we have adopted a convention of factor X activity being high at the equator and low at the poles. However, our results can be equally well explained if factor X has the opposite distribution, high at the poles and low towards the equator. In this scenario the positive and negative influences of factor X on Fz activity in the two models will be reversed.

The scalar model (Fig. 5A)

In this model, a difference in the levels of Fz activity between the two cells of the R3/R4 pair determines which cell will become R3 and which will become R4. Because the presumptive R3 cell lies closer to the equator than the presumptive R4, it will detect higher levels of factor X activity and consequently will have a higher level of Fz activity. The higher levels of Fz activation then bias the subsequent N-DI interaction so that the cell with greater Fz activity becomes a dedicated DI signaling cell while it partner with less Fz activity becomes a dedicated DI receiving cell. For example, the level of Fz activity in each cell could govern the activity or level of expression of a component of the N-DI signaling mechanism, such as N itself or DI. Even a small difference in signaling capacity between the two cells would then bias the N-DI system of feed-back regulation causing the cell with initially higher N transducing capacity to become a dedicated DI receiving cell (and hence R4), while the remaining cell becomes a dedicated DI sending cell (R3). In Fig. 5C-D, we show how experimentally induced changes in Fz activity, which reverse the relative difference in Fz activity between the presumptive R3 and R4 cells, might cause a corresponding reversal in direction of N-DI signaling and the R3/R4 cell fate decision. Similar models to this have been proposed by Fanto and Mlodzik (1999) and Cooper and Bray (1999).

An issue raised by this model is whether the presumptive R3 and R4 cells have the capacity to meter accurately what are likely to be small differences in their absolute levels of factor X. Consider that up to 15 ommatidia can form along the equatorial-polar axis of each half of the eye, with the presumptive R3 and R4 cells located next to each other in each cluster and separated by several cell diameters from their counterparts in neighboring clusters. More than 75 cells are arrayed in each half of the eye along the Eq/Pl axis and any two neighboring cells at any position within that array would need to faithfully decode the factor X gradient. However, the results of our fate-mapping analysis and prior histological studies suggest that the presumptive R3 and R4 cells are initially located at a distance from each other, separated along the Eq/Pl axis by the remaining three cells of the precluster (the presumptive R2, R8 and R5 cells). This raises the possibility that the presumptive R3 and R4 may meter factor X abundance when they are located at opposite ends of these
suggest that Fz signaling generally mediates the establishment of cell polarity without requiring an N-Dl feedback amplification step. Hence, the involvement of N-Dl signaling in establishing ommatidial chirality may reflect a special attribute of this system, perhaps to allow the polarity of just two cells, the presumptive R3/R4 pair, to be used as a cue to control the pattern of a much larger ensemble of cells, the ommatidium.

Comparing and interpreting differences in the relative levels of Frizzled activity: the roles of Delta and Notch

How might a small initial difference in Fz activity bias lateral specification mediated by Notch? Building on the mosaic studies of Seydoux and Greenwald (1989), Heiztler and Simpson (1991) showed that even a relatively small difference in N signal transducing capacity between competing cells, such as a 3:2 ratio in the dosage of the wild-type N gene, is sufficient to bias the outcome of the competition. Here, we reach a similar conclusion for the retinal epithelium of fzmutants, where we find that a 3:2 ratio in gene dosage between the two members of the R3/R4 photoreceptor pair invariably determines the fate choice of each cell. We therefore infer that even a relatively slight differential in N signal production or N signal transducing capacity is sufficient to control the R3/R4 cell fate decision. The role we ascribe to Notch in establishing ommatidial chirality is analogous to the role of Lin-12 in mediating the AC/VU decision in the nematode gonad. However, the two situations differ in that the outcome of the AC/VU decision depends on the amplification of a stochastic advantage achieved by one of the two cells, whereas in the Drosophila eye the competition is biased, owing to the differential activity of Fz.

Symmetric ommatidia in the absence of Fz signal transduction

When Fz activity is absent throughout eye development, the Fz-dependent bias should be eliminated and each cell of the presumptive R3/R4 pair should have an equal chance of becoming either the signaling cell (R3) or the receiving cell (R4). Under these conditions, the choice of which cell becomes R3 and which becomes R4 would be determined by a stochastic variation, which gives one of the two cells a slight advantage that is then amplified by the N-Dl feedback mechanism. This explains why ommatidia in fzmutants ‘choose’ their chirality randomly when both cells have the same N gene dosage, but non-randomly when there is a 3:2 differential in N gene dosage. However, in fzmutant eyes, approximately one third of the ommatidia are symmetrical, indicating that the R3/R4 distinction was not resolved. One possible explanation is that the interaction between the R3 and R4 cells may be limited to only a few hours and this may be too short to ensure that a stochastic variation will arise and be amplified by the N pathway in all fzmutant ommatidia. Timing may not be as critical in wild-type ommatidia, because the bias from Fz signaling is sufficient to ensure an appropriate resolution during this relatively brief interval.

The R3/R4 cell fate choice

Although we consider the R3 and R4 photoreceptors to define alternative cell fates, we note that these cells can only be
distinguished at present by two characteristics. The first is that
they transiently express different components of the N-DI
signal transduction pathway, such as the Enhancer of split
(E(spl)) gene (expressed by the presumptive R4 cell) and DI
(expressed by the presumptive R3 cell). These differences in
gene expression are observed before any other manifestation
of a difference between the two cells, and have led others to
propose that Fz signaling controls ommatidial chirality by
directly regulating DI and E(spl) transcription (Fanto and
Mlodzik, 1999; Cooper and Bray, 1999). However, such
Non-autonomous effects of fz- clones

fz mutant clones in the wing have long-range, non-autonomous effects, disrupting planar cell polarity in distally located cells many cell diameters beyond the border of the clone (Vinson et al., 1989). A similar, long-range effect on ommatidial patterning has been reported in the eye (Zheng et al., 1995). These data suggest that Fz is not only involved in reading a polarizing signal, but is also involved in propagating it (Vinson and Adler, 1989; Zheng et al., 1995). However, restricting our analysis to the effects of fz- clones on ommatidial chirality, we find evidence for only short-range non-autonomy. The examples of non-autonomy we observe are those situations in which both the R3 and R4 cells are wild-type for fz gene function and yet the ommatidium adopts the incorrect chirality. Such ommatidia frequently occur along the polar border of the clone.

We suggest that this local non-autonomy may reflect a role for Fz- and possibly N-dependent signaling between the presumptive R3/R4 cells and the pair of ‘mystery’ cells with which they appear to compete for entry into the precluster (Tomlinson and Ready, 1987). These cells are present as the precluster unit forms, but leave the cluster, preventing us from analyzing their effects on chirality in fz mosaic eyes. If interactions between the mystery cells and the presumptive R3/R4 pair depend on signals mediated by Fz and N, then in fz mosaic, the presence of a mutant mystery cell next to one of the two presumptive R3/R4 cells could alter N signaling between the two members of the pair, reversing the normal bias and yielding an ommatidium of incorrect chirality.

Thus, although our evidence reveals a second focus for Fz activity outside the presumptive R3/R4 pair, this focus can be accommodated by invoking additional Fz-dependent interactions between members of the pair and adjacent mystery cells, which may form a larger ‘equivalence group’ in the early precluster. This possibility is also supported by our finding that a sev-fz transgene can rescue chirality in otherwise fz mutant eyes. The sev promoter is active in only the R3/R4 pair and the mystery cells prior to the time the R3/R4 decision is made, indicating that fz is required only in these cells to ensure the correct decoding of the factor X gradient. Conversely, this finding suggests that Fz is not required in any of the remaining cells which lie in between neighboring clusters, providing evidence against a second and distinct role for Fz in propagating the factor X signal in the eye.

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Planar polarity in the Drosophila eye

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