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Cell determination strategies in the *Drosophila* eye

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SUMMARY

Cells in the *Drosophila* eye are determined by inductive signalling. Here I describe a new model of eye development that explains how simple intercellular signals could specify the diverse cell types that constitute the ommatidium. This model arises from the recent observation that the *Drosophila* homologue of the EGF receptor (DER) is used reiteratively to trigger the differentiation of each of the cell types — successive rounds of DER activation recruit first the photoreceptors, then cone and finally pigment cells. It seems that a cell's identity is not determined by the specific signal that induces it, but is instead a function of the state of the cell when it receives the signal. DER signalling is activated by the ligand, Spitz, and inhibited by the secreted protein, Argos. Spitz is initially produced by the central cells in the ommatidium and diffuses over a small distance. Argos has a longer range, allowing it to block more distal cells from being activated by low levels of Spitz; I have termed this interplay between a short-range activator and a long-range inhibitor 'remote inhibition'. Since inductive signalling is common in many organisms and its components have been conserved, it is possible that the logic of signalling may also be conserved.

Key words: *Drosophila*, eye development, cell signalling, EGF receptor, Spitz, Argos

INTRODUCTION

A cell often learns its developmental fate by interpreting signals in its environment. For example, it can be determined by signals emanating from neighbouring cells or tissues. These 'inductive' interactions are common in both vertebrates and invertebrates (for reviews see Davidson, 1991; Gurdon, 1992; McMahon, 1993; Bienz, 1996). Traditionally, induction has been seen as a property of groups of cells or whole tissues — for example, one germ layer inducing differentiation of another. However, there are examples of induction beween single cells (e.g. Sulston and White, 1980; Shah et al., 1996) and it seems likely that more will be discovered. Inductive interactions between individual cells have been much studied in the *Drosophila* eye and, as well as knowing many of the signalling molecules involved, we now understand some of the underlying logic of determination. The degree of conservation of these molecules is very striking and it seems likely that the developmental strategies in which they participate will also be widespread.

In this review, I emphasise some of the themes that emerge and will also describe a new model for the inductive determination of all the cells in the eye. Many other aspects of eye development are also being studied and have been recently reviewed. These include the function of long-range morphogens, the regulation of cell division, cell and tissue polarity, spacing, axonal pathfinding and signal transduction (e.g. Kunes and Steller, 1993; Krämer and Cagan, 1994; Thomas and Zipursky, 1994; Zipursky and Rubin, 1994; Bonini and Choi, 1995; Heberlein and Moses, 1995).

A description of eye development

The fly has a compound eye, comprising about 750 facets, or 'ommatidia' (Fig. 1A). Each ommatidium contains the same complement of cells: eight photoreceptor neurons, four lens-secreting cone cells and two primary pigment cells; there are also six secondary and three tertiary pigment cells, which are shared with neighbouring ommatidia (Fig. 1B). The eye develops from a monolayer epithelium — the eye imaginal disc. Until the third larval instar the cells in the disc proliferate but do not differentiate. Around the beginning of the third instar, a groove known as the morphogenetic furrow starts to sweep anteriorly across the disc, leaving rows of developing ommatidia in its wake (Ready et al., 1976). Ommatidial development is therefore progressive, with a gradient of increasing maturity extending posteriorly from the furrow (Fig. 1C). The detailed description of photoreceptor recruitment carried out by Tomlinson and Ready has been critical to the emergence of the eye as a powerful experimental system (Tomlinson, 1985; Tomlinson and Ready, 1987b).

Ahead of the furrow, the cells continue to proliferate, but they arrest in the G_1 phase of the cell cycle as the furrow approaches. In the furrow, a 'precluster' of cells destined to become the first five photoreceptors becomes recognisable; the cells that will form the final three photoreceptors, and the cone and pigment cells are not born until a few rows behind the furrow, where a final round of synchronised mitosis (the 'second mitotic wave') occurs (Ready et al., 1976). The eight photoreceptors are individually distinct, based on their invariant positions in the ommatidium, their patterns of gene expression and their spectral properties. They develop in a

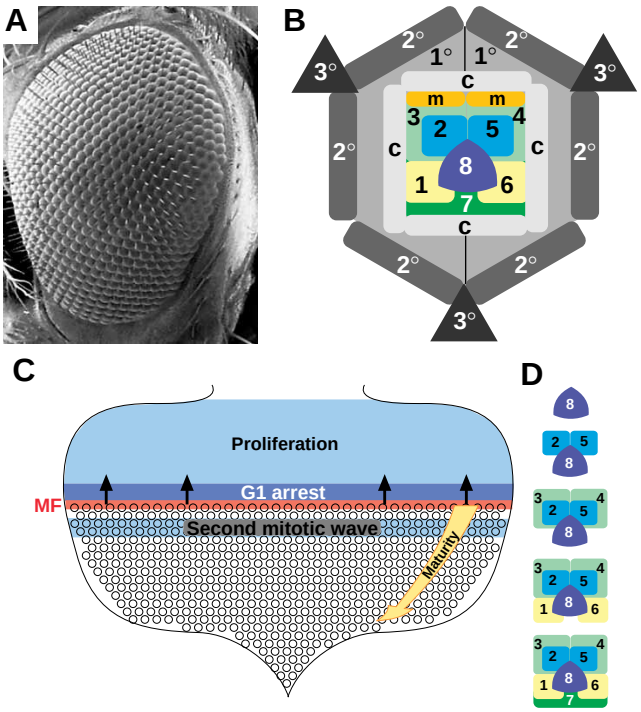

Fig. 1. The structure and development of the *Drosophila* eye. (A) The fly's eye is composed of a regular array of about 750 ommatidia; anterior to the left. (B) Each ommatidium has the same internal structure: this cartoon shows all the cells, but for simplicity the photoreceptors (1-8) are arranged in the layout that they have as they develop, rather than their adult positions. The 'mystery cells' (m) are only present in the third instar disc — they later leave the developing ommatidium. c, cone cell; 1°, 2°, 3°, primary, secondary and tertiary pigment cell, respectively. (C) The third instar imaginal eye disc, anterior at the top. The morphogenetic furrow (red) sweeps anteriorly, leaving developing ommatidia (small circles) in its wake. There is therefore a gradient of maturity of developing clusters, indicated by the yellow arrow. Cells ahead of the furrow (pale blue) are proliferating, but they enter G_1 arrest (dark blue) in front of the furrow and go through one more mitotic cycle posterior to the furrow. (D) The stereotyped pattern of differentiation of the photoreceptors, with clusters of increasing maturity from the top (see text).

stereotyped order (Fig. 1D): first R8 and then, as one moves posteriorly from the furrow, cells are added pairwise, R2 and R5, R3 and R4, and R1 and R6; R7 is the last photoreceptor to be added to each cluster (Tomlinson and Ready, 1987b). Soon after, the four cone cells join the ommatidium and, later still, — in the pupa — the pigment cells join, first the primaries and finally the secondaries and tertiaries (Cagan and Ready, 1989a). Until recently, nothing has been known about how the cone and pigment cells join the ommatidium; for instance, it was not known if an inductive mechanism is involved, or if these non-neuronal cells use different strategies.

For many years it was believed that each ommatidium was a clone of cells and that their fate was determined by lineage. This was wrong: all the cells have the same developmental potential and they acquire their fates by interacting with other cells (Ready et al., 1976; Lawrence and Green, 1979; Wolff and Ready, 1991b). However, ommatidia that have been isolated genetically or physically can develop normally, implying that the positional cues interpreted by cells must be generated within the ommatidium rather than being long-range (Leibovitz and Ready, 1986; Baker and Rubin, 1989). These features led Tomlinson and Ready (1987b) to propose a 'combinatorial induction' model of photoreceptor development in which a cell's fate is determined by the stereotyped set of contacts that it makes with previously determined cells — a process of 'recruitment' of undetermined cells into the growing ommatidium. This model implies that each photoreceptor subtype interprets its position by receiving a unique combination of signals from its neighbours, thereby learning its specific fate.

Development of the R7 photoreceptor

This view of specific signals triggering a cell's fate was supported by the analysis of the *sevenless* gene. *sevenless* was isolated in Benzer's laboratory in a screen for flies with abnormal UV phototaxis. R7 is the only photoreceptor sensitive to UV and *sevenless*$^-$ flies have no R7s, although eye development is otherwise normal (Harris et al., 1976; Campos-Ortega et al., 1979). Subsequent study of *sevenless* has led to R7 development being understood better than any other cell in the eye.

Mosaic analysis of mitotic clones is a powerful genetic technique that allows one to tell if a gene acts cell autonomously (i.e. on the reception side of an intercellular signal) or non-autonomously (i.e. on the signalling side) (for discussion of the technique's significance see Lawrence, 1992). Mosaic analysis of *sevenless* showed it to be autonomous — it acts only in R7, the cell that is missing in *sevenless*$^-$ mutants (Harris et al., 1976; Campos-Ortega et al., 1979; Tomlinson and Ready, 1987a). This conclusion was given molecular support when the gene was cloned and found to encode a receptor tyrosine kinase (RTK) (Hafen et al., 1987), suggesting that it is involved in receiving an inductive signal. RTKs form a very large class of cell surface receptors responsible for regulation of development and growth in all animals (see van der Geer et al., 1994).

Sevenless and R7 development have been extensively reviewed elsewhere (e.g. Dickson and Hafen, 1994; Simon, 1994; Zipursky and Rubin, 1994): here I describe only a few results that are specifically relevant. The ligand for Sevenless, Boss, was discovered by Zipursky and colleagues, and is a membrane-bound protein that is only expressed in R8 (Reinke and Zipursky, 1988; Hart et al., 1990). Although Sevenless is expressed in most ommatidial cells (Banerjee et al., 1987; Tomlinson et al., 1987), its activity is restricted to the presumptive R7 by a combination of two mechanisms: only those cells that contact R8 encounter the ligand and cells that have already acquired an earlier fate are no longer able to respond to Sevenless signalling (Basler and Hafen, 1989; Bowtell et al., 1989; van Vactor et al., 1991). A number of genetic modifier screens have been performed to look for members of the signal transduction pathway that link Sevenless to the subsequent changes of gene expression needed to induce R7. These, and related screens, pioneered by Simon et al. (1991), have proved spectacularly successful and have uncovered the complete signal transduction machinery. They were instrumental in demonstrating a now widespread observation, namely that the Ras/Raf/MAP Kinase pathway ('the Ras pathway' from now on) is the primary effector of RTK signalling (Fig. 2A).

However, Sevenless is not sufficient to form an R7. It is now

clear that, although it triggers the presumptive R7 cell to differentiate, that cell's identity is specified by other mechanisms. The first indication of this came from the ectopic expression of the *rough* gene. *rough* encodes a homeobox-containing transcription factor required for the determination of R2 and R5 (Tomlinson et al., 1988). When it is ectopically expressed in the presumptive R7 cell, it transforms it into a cell with the characteristics of R2 and R5, yet this cell remains dependent on Sevenless (Basler et al., 1990; Kimmel et al., 1990). Therefore Sevenless can trigger a cell to become a non-R7 photoreceptor. Moreover, Dickson et al. (1992) showed that a constitutively active form of Sevenless forces cells to become R7s only in a narrow band of ommatidia, corresponding to the region where R7s would normally form; prior to that other photoreceptors are triggered. They concluded that there is a 'prepattern' — cells learn what type of cell they will eventually become before their differentiation is initiated by Sevenless.

Determination of other photoreceptors

Inspired by the example of *sevenless*, a number of genetic screens were carried out to look for genes that determined specific photoreceptors other than R7. In the combinatorial induction model, there are predicted to be sufficient extracellular signals to give each distinct cell type a unique code (note that this does not mean that there needs to be a specific receptor for each, since combinations are predicted). Genes identified in these screens include *rough*, required for R2 and 5 (Heberlein et al., 1991); *sina*, also required for R7 (Carthew and Rubin, 1990); *seven-up*, required for R3, 4, 1 and 6 (Mlodzik et al., 1990; Hiromi et al., 1993; Kramer et al., 1995); *BarH1* and *BarH2*, required for R1 and 6 (Higashijima et al., 1992); *phyllopod*, required in R1, 6 and 7 (Chang et al., 1995; Dickson et al., 1995); and *lozenge*, which regulates at least *seven-up* and *Bar* expression (Daga et al., 1996). It is striking that the products of all these genes appear to be nuclear — they are probably transcription factors. No examples have yet been uncovered of signalling molecules, or cytoplasmic signal transduction candidates that show the subtype specificity predicted. The diverse approaches that have led to the identification of these genes suggest that the absence of signalling components is real, not an artefact of 'blind spots' in the screens. If so, an underlying assumption of the model — a combinatorial code of different signals — is not upheld.

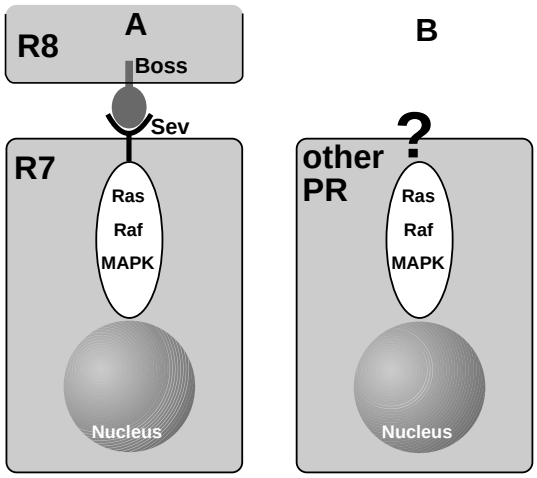

Fig. 2. Recruitment signals in photoreceptors. (A) Sevenless is a receptor tyrosine kinase that triggers the Ras pathway in the R7 cell. It is activated by a membrane-bound ligand, Boss, which is presented by the R8 cell. (B) The Ras pathway is also needed in all the other photoreceptors, but Sevenless is only required in R7 — what triggers the pathway in the non-R7 cells?

A puzzle arises from the dissection of the Sevenless signal transduction pathway — while Sevenless itself is only needed in R7, the Ras pathway is required in all photoreceptors (e.g. Simon et al., 1991). What is the presumptive missing receptor (or receptors) that triggers the pathway in the non-R7 photoreceptors (Fig. 2B)? It is now clear that it is the *Drosophila* homologue of the epidermal growth factor (EGF) receptor (DER), another RTK. DER acts in many different tissues and stages of development (Clifford and Schupbach, 1992; Raz and Shilo, 1992) — the first evidence that it functions in the eye was the discovery that the long-known *Ellipse* eye mutations were actually gain-of-function DER alleles (Baker and Rubin, 1989). Baker and Rubin showed that *Ellipse* eyes have a reduced number of ommatidia: in homozygotes there are only a few, scattered in a 'sea' of undetermined cells. The ommatidia that do form are usually normal. This led them to propose that DER activity inhibits the initial formation of the ommatidium and its function is in regulating ommatidial spacing, rather than in the determination of photoreceptors themselves (Baker and Rubin, 1992). They could not examine the phenotype of DER loss in the eye since it is an embryonic lethal and *DER*$^{-}$ clones do not survive, probably because DER is needed for normal cell proliferation.

Xu and Rubin (1993) later overcame this problem by making clones at a much higher frequency than possible by traditional X-ray methods, using the flp/frt system (Golic and Lindquist, 1989); this allowed them to study the fate of *DER*$^{-}$ clones in the imaginal disc. Small clones were found and they did not have the predicted phenotype (i.e. the opposite of gain-of-function *Ellipse* alleles). Instead they found that *DER*$^{-}$ cells could not become photoreceptors. Because the *DER*$^{-}$ clones were much smaller than controls and were known to die before adulthood, the cells might not have differentiated because of a general reduction in cell viability, rather than because DER was specifically required. However, even 1-cell clones, induced just before the last cell division, never became photoreceptors. They concluded that DER was essential for the formation of all photoreceptors.

The inconsistency between *Ellipse* mutations, which indicate no role for DER in photoreceptor recruitment per se, and *DER*$^{-}$ clones, showing the opposite, meant that the function of DER remained unclear. A more recent approach using a dominant negative form of DER has clarified its role in cell determination in the ommatidium (Freeman, 1996). By expressing a truncated form of the receptor, it is possible to block its normal function. This allowed DER activity to be removed from the developing eye after cell proliferation was complete (Freeman, 1996), so that any role of DER in cell division was irrelevant. It was found (Fig. 3) that DER was required for the initial determination of all the photoreceptors, including R7 (but note that R8 was not tested), thus supporting the conclusions of Xu and Rubin (1993). Furthermore, DER is also required for the determination of cone and pigment cells, implying that it is necessary for the formation of all cells

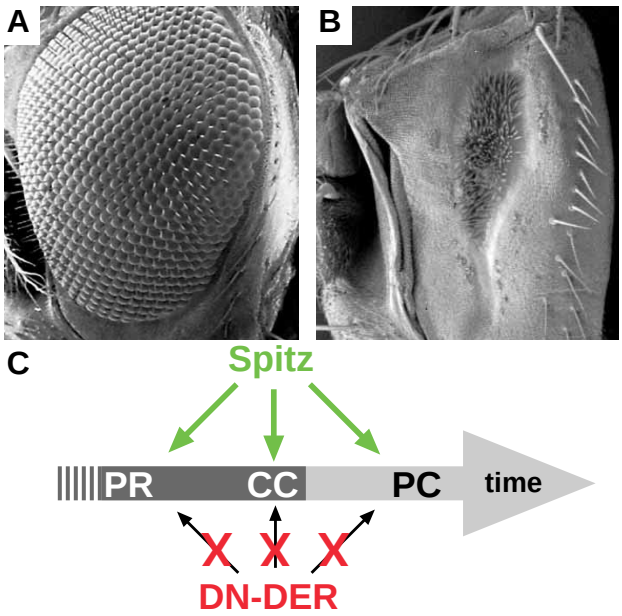

Fig. 3. The *Drosophila* EGF receptor in the eye. Widespread expression of DN-DER in the developing eye abolishes the whole eye, bar a few bristles; (A) wild-type eye, (B) GMR-Gal4 × UAS-DN-DER eye (Freeman, 1996). (C) DER is required reiteratively for the determination of all the cells in the ommatidium: first photoreceptors (PR), later cone cells (CC), and finally the pigment cells (PC). Consistent with this, activation of DER by its ligand Spitz is able to trigger the development of all these cell types. The identity adopted by a cell upon DER activation is dependent on the time in development that the signal is received.

in the ommatidium. This universal need for DER suggested that all ommatidial cells are determined similarly, and implies that neurons and other cells do not use distinct mechanisms.

If DER is the receptor that triggers the determination of these cells, it should not just be necessary for their recruitment, but also sufficient. This turns out to be the case. When DER is activated in uncommitted cells, it can trigger them to adopt all the different ommatidial fates. Importantly, a cell's fate upon DER activation is dependent on its developmental stage: near the furrow, cells become outer photoreceptors; further back, in more mature ommatidia, they become R7s; later still they become cone cells. In pupae, DER activation in the eye leads to the formation of first primary, and then later secondary and tertiary pigment cells.

Spitz and Argos

DER's role as the trigger for the differentiation of all the cells in the ommatidium is supported by two earlier results. First, Spitz, the main activating ligand of DER (Rutledge et al., 1992; Schweitzer et al., 1995b), is required for the formation of all the photoreceptors except R8 (Freeman, 1994b; Tio et al., 1994; Tio and Moses, 1997). The *spitz* gene also interacts genetically with *DER* mutations in the eye, suggesting that the ligand and receptor are necessary for photoreceptor development. Second, *argos* mutations affect all ommatidial cell types similarly: its loss leads to the over-recruitment of photoreceptor, cone and pigment cells, while its over-expression leads to a reduction in their number (Freeman et al., 1992b; Freeman, 1994a). *argos* encodes a secreted protein with an EGF-like motif, making it structurally related to Spitz (Freeman et al., 1992b; Kretzschmar et al., 1992; Okano et al., 1992). It has recently been shown that Argos is an extracellular inhibitor of DER (Schweitzer et al., 1995a), a previously unknown kind of RTK regulation. Interestingly, *argos* expression is dependent on DER activation, forming a negative feedback loop (Golembo et al., 1996b): upon DER activation, *argos* is expressed and this leads to DER inactivation. This mechanism has important regulatory implications that are discussed below.

If DER is a key trigger of determination in the eye, we need to understand how its activating ligand, Spitz, is regulated. Like TGFα, one of the ligands for the human EGF receptor, Spitz is produced as a transmembrane protein, with an extracellular EGF motif (Rutledge et al., 1992). Proteolytic cleavage releases the extracellular portion of the protein and only this cleaved form is active as a DER ligand (Freeman, 1994b; Schweitzer et al., 1995b; Freeman, 1996). Thus the presence of ligand is not controlled by its expression, but by subsequent cleavage. Indeed, like DER, Spitz is expressed in most tissues during fly development (Rutledge et al., 1992), including all developing photoreceptors (Tio et al., 1994), but its processing is tightly controlled. There is accumulating evidence that the products of the

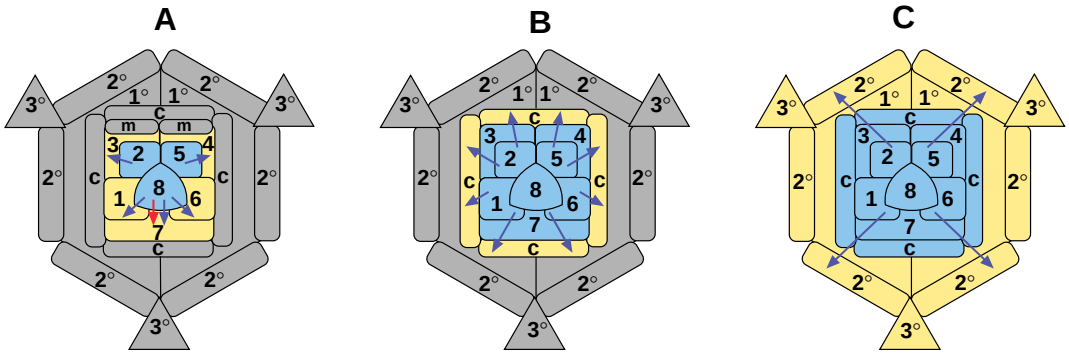

Fig. 4. A model of ommatidial development. Ommatidia of increasing maturity are shown from left to right; (A) the recruitment of photoreceptors, (B) cone cells and (C) pigment cells. Blue cells are the source of Spitz and they are able to recruit the neighbouring yellow cells at each stage, but are unable to trigger the more remote grey cells, which are inhibited by Argos. Argos is expressed by each cell as it is determined. Because of its long range of action (see text), it may be more accurate to think of it usually forming a 'sea' of inhibitor surrounding all the developing ommatidia. However, isolated ommatidia (in *Ellipse* mutants) usually develop the correct number of cells, implying that Argos contribution from neighbouring ommatidia is not essential. R7 needs to be triggered by Boss (red arrow) as well as Spitz (blue arrow). See text for discussion of the model.

rhomboid and *Star* genes regulate the cleavage of Spitz, and their expression prefigures the activation of DER in several tissues. Despite earlier uncertainty, Rhomboid and Star are required in the signalling rather than the receiving cells (Golembo et al., 1996a), as expected if they regulate the production of DER's ligand. The expression of both in the eye is initially limited to only the first three photoreceptors R8, R2 and R5 (Freeman et al., 1992a; Heberlein et al., 1993), suggesting that these cells are the early source of ligand in the ommatidium.

A model of ommatidial determination

Two other points need to be made before describing a model of ommatidial development that is based on the reiterative use of DER (Freeman, 1996). First, the ommatidium is formed of roughly concentric rings of cells: the photoreceptors are surrounded by cone cells, which are surrounded by pigment cells (see Fig. 1B). Second, the two extracellular proteins that affect DER activation, Spitz and Argos, have different ranges of action in the eye. Mutant clones of *argos*$^-$ cells can be rescued by surrounding wild-type cells up to a distance of about 10-12 cell diameters (Freeman et al., 1992b). In the same test, the range of the activating ligand, Spitz, is much less: it diffuses within an ommatidium, but has no rescuing ability between ommatidia (Freeman, 1994b). This sets a maximum Spitz range of three or four cell diameters, which is therefore much shorter than that of the inhibitor, Argos.

The model (Fig. 4) begins by postulating that in the early ommatidium, active Spitz is produced by the three earliest and centrally located cells, R8, R2 and R5. This then activates DER in the neighbouring cells, which are recruited as R3, R4, R1, R6 and R7. R7 also needs Sevenless to be activated by Boss. As each cell starts to respond to DER activation, it expresses Argos, which diffuses further than Spitz, thus blocking more remote cells from responding to the activating ligand. Argos is, however, unable to block cells that are exposed to a high level of Spitz, or cells that have already started to differentiate. In more mature ommatidia, the source of Spitz expands, caused by an expansion of the expression of Rhomboid and Star, to include all the photoreceptors; this overcomes the activation block in the next concentric ring of cells, by now destined to become cone cells. Argos still blocks the outer ring of cells from responding. Later still, in the pupa, I assume that the Spitz source expands further, overcoming the final Argos block, and allowing DER in the outermost cells to be activated — now causing them to become pigment cells.

This model is likely to be an oversimplification of ommatidial determination, but it encapsulates all current evidence. Support for its main points is as follows. First, as described above, DER is a necessary trigger for the determination of all ommatidial cells (Xu and Rubin, 1993; Freeman, 1996). Second, the initial source of Spitz is R8, R2 and R5, although all cells destined to become photoreceptors must be exposed to the cleaved ligand (Freeman, 1994b; Tio et al., 1994). Third, Star and Rhomboid, required for Spitz processing, are only expressed in R8, R2 and R5 throughout the early stages of eye development. However, their expression domain does expand later, thereby potentially expanding the Spitz source (Freeman et al., 1992a; Heberlein et al., 1993; Kolodkin et al., 1994). Fourth, Argos is indeed expressed in each ommatidial cell as it starts to differentiate (Freeman et al., 1992b; Kretzschmar et al., 1992; Okano et al., 1992).

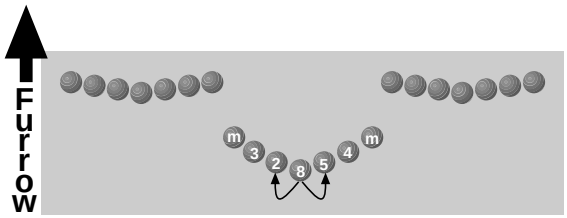

Fig. 5. Cluster initiation in the morphogenetic furrow. A small region of the morphogenetic furrow, anterior at the top. Early clusters are V-shaped, suggesting a possible explanation of why these photoreceptor cells are not recruited simultaneously. R8 might initially signal to R2 and R5, and they in turn signal to R3 and R4. This sequential process would allow time for a change in cell state, causing the distinct identities of R8, R2 and R5, and R3 and R4. The mystery cells do not normally show any signs of neuronal determination and leave the cluster a little later; in *argos*$^-$ mutants they do become photoreceptors (Freeman et al., 1992b). R1, R6 and R7 are born later, after the second mitotic wave.

The most obvious gap in my proposal is that it does not directly explain how the specificity of cell type arises, other than to emphasise that the developmental history of a cell is critical, not the signal that triggers it. This will be discussed more fully below. Another over-simplification is the treatment of all the photoreceptors as equivalent and simultaneously recruited. In fact, as described above, at least five classes of photoreceptor can be distinguished, and each pair is added in a stereotypical sequence (Tomlinson and Ready, 1987b). A possible explanation is that the determination of the first five cells in the precluster occurs before the ommatidium has rounded up into its characteristic bulls-eye. At this early stage (see Fig. 5), the cells form a V-shaped line with R8 at the apex, flanked by R2 and R5, which themselves are flanked in turn by R3 and R4, and the mystery cells (Wolff and Ready, 1991a). This linear array could account for the sequential determination of cells in the precluster. Thus R8 signals first to its immediate neighbours, R2 and R5, which in turn relay the signal on to the more distal cells, R3 and R4. The precursors of R1, R6 and R7 are born later (after the second mitotic wave), so do not form part of the precluster.

Since Rhomboid and Star are both key regulators of DER signalling — being involved in Spitz production — how does their function in the eye fit with this DER-centred model? Star is required only in R8, R2 and R5 (the only cells in which it is expressed early), but its absence from these cells prevents any photoreceptors from forming (Heberlein and Rubin, 1991; Heberlein et al., 1993). This fits the model well, since those three cells are the source of Spitz. Rhomboid's expression pattern in the eye is very similar, also fitting well with the model. Its expression in R2 and R5 is dependent on the transcription factor, Rough (Freeman et al., 1992a). In *rough*$^-$ eyes, the earliest stages of recruitment appear normal (R8 still makes Rhomboid and can thus process Spitz) but later stages are disrupted, leading to ommatidia with rather variable loss of photoreceptors (Tomlinson et al., 1988). This is exactly the predicted phenotype if loss of Rhomboid in R2 and R5 prevents them from processing Spitz: R8 cannot produce enough Spitz to recruit all the photoreceptors. However, this interpretation is inconsistent with one earlier experiment: Rhomboid is apparently not required for normal photoreceptor recruitment

(Freeman et al., 1992a). This result is also at odds with the requirement for Rhomboid in all other tissues where DER functions. Given these strong arguments, I now think it possible that the result from these apparently *rhomboid*$^-$ clones was wrong (the wild-type clones that I found may have been the product of rare, X-ray induced, double recombination events), and now plan to redo the experiment using the flp/frt system. The prediction is that genuine *rhomboid*$^-$ clones will not appear in adult eyes due to complete failure of recruitment (as occurs in *Star* mutant cells).

Remote inhibition by Argos

An important feature of my model of ommatidial development is that Argos inhibits the activation of cells at a distance from the source of Spitz. This occurs because Argos is expressed in cells in which DER is activated and is able to diffuse further than Spitz. (It should be noted that we know nothing about the physical mechanisms of Spitz and Argos action at a distance: I use the term 'diffusion' for simplicity, but take it to cover possibilities ranging from passive diffusion to various relay mechanisms. Note also that it is possible that Argos spreads efficiently enough to produce a 'sea' of inhibitor surrounding all the ommatidia.) This process of 'remote inhibition' is distinct from lateral inhibition, mediated by the receptor Notch and its ligand Delta, in which only cells abutting the Delta-expressing cells are inhibited (Simpson, 1990). The molecular reason for this distinction is that Delta is a membrane-bound ligand (Vässin et al., 1987; Kopczynski et al., 1988), whereas Argos is diffusible. In the eye, Argos converts the response to a gradient of Spitz (formed by the ligand diffusing away from its central source) into a simple on or off decision: cells close to the Spitz source are activated, those more distal are blocked.

This kind of interaction between two diffusible ligands, a short-range activator and a long-range inhibitor, also provides other possibilities for modulating signalling. For example, remote inhibition by Argos can also be used to stabilise a gradient of Spitz acting as a morphogen. This is what appears to occur in the ventral ectoderm of the embryo: Argos stabilises a gradient of Spitz activity from which at least three different fates are read (Golembo et al., 1996b).

A useful way of understanding the role of Argos is to imagine it replacing the 'sink', usually postulated in theoretical treatments of gradients (for a discussion of gradient models see Slack, 1991). Without a sink, the activating ligand will tend to accumulate with time at increasing distance from the source, thereby progressively flattening the gradient. This is exactly what occurs in the posterior of *argos* mutant eyes. The over-recruitment of cone cells in *argos*$^-$ eyes is much more dramatic in the earliest forming, posterior, ommatidia, than in those developing later in the anterior (Freeman et al., 1992b). This is because the early ommatidia have to mark time for many hours after cone cell recruitment is complete before pigment cells are added in the pupa, simultaneously across the whole eye. During this delay Spitz slowly accumulates above its threshold level in cells increasingly remote from its source and, in the absence of Argos, all these cells become recruited as extra cone cells — indeed every cell seems to become a cone cell in the most posterior region, implying that Spitz eventually diffuses across the whole field.

What does Sevenless do?

If the model is correct, why are both DER and Sevenless needed in R7? The answer is not certain, but we can rule out various explanations. The first possibility is that the two receptors activate different pathways, both of which are needed to become an R7. However, this is not the case: they both activate primarily the Ras pathway (Simon et al., 1991; Fortini et al., 1992; Diaz-Benjumea and Hafen, 1994), and the two receptors are apparently interchangeable (Freeman, 1996). Another possibility is that the duration or strength of activation of the two receptors is different (remember that Boss is a membrane-bound ligand and Spitz is diffusible) and that this kinetic difference accounts for the difference between R7 and the other photoreceptors. This idea is based on a similar proposal that has been made in rat PC12 cells, which show a differential response to the activation of two different RTKs (Qiu and Green, 1992; Traverse et al., 1992; Marshall, 1995). However, this is not what happens in the eye since altering the kinetics of signalling does not affect the fate of an ommatidial cell (Freeman, 1996). Perhaps the most plausible explanation is that R7 requires two separate bursts of Ras activation — an early one induced by Spitz and DER, and a later one induced by Boss and Sevenless. In support of this, the presumptive R7 does indeed show several early signs of differentiation prior to, and independent of, Sevenless function. These include the expression of the *prospero* gene (Kauffman et al., 1996) and the enhancer trap line H214, both of which are switched on in the presumptive R7 before Sevenless is active and are still expressed in *sevenless*$^-$ mutants (Mlodzik et al., 1992).

What determines the fate of cells in the eye?

Although I propose that DER triggers all the cell types in the ommatidium, it is nevertheless possible that there are additional specific receptors that generate the combinatorial code of the Tomlinson and Ready model (1987b). For example R7 needs Sevenless and DER — although since the former does not actually confer the R7 subtype on cells (see above), it does not fit the model very clearly. Perhaps the other cell types are also determined by additional specific signals. The main problem with this idea is that there are no other candidates for subtype-specific signals, despite extensive screens to search for them. Instead, a different type of mechanism could specify cell identity in the scheme that I have described. Thus, upon activation by DER, a cell would start to differentiate towards the fate appropriate to its developmental stage, indicating that it is the age or developmental history of a cell that is responsible for determining its ultimate fate (Fig. 6). This implies that a cell passes through a series of 'states', each representing a potential fate. Each cell state presumably derives from the subset of transcription factors that are present and which can be activated by the Ras pathway. As described above, there are several good candidates for nuclear proteins that control photoreceptor subtype (Dickson, 1995 for review), although their exact role remains to be clarified. The interplay between them and their relationship with the Ras pathway, will probably define the molecular basis of the cell states that specify fate in the eye.

How might a cell measure its history or age? Several possible mechanisms can be imagined and they can be

separated into two classes of model. One class invokes an intrinsic property of the cell. For example, a cell could have an internal clock that produces the change of state at fixed times; its fate upon DER activation would depend on the time on the clock. The idea of an intrinsic mechanism of this kind was proposed by Reh and Cagan (1994). As in the model I have proposed, they postulated that cells in the eye could be determined by a non-specific signal triggering their differentiation; they then speculated that specificity would be regulated by an internal clock, perhaps measuring time since the last mitosis. The second class of model relies on extrinsic signals: a cell could sense the sequential determination of its neighbours. For example, upon determination, a cell might produce a diffusible signal that tells all its neighbours to move on to the next of a pre-programmed series of potential fates. Thus all undifferentiated cells would 'ratchet' through a series of states until they are triggered to differentiate by DER activation. Note that this kind of signal can be non-specific: it can be the same each time, as it only needs to tell cells to change state. If such a signalling system is used, it would have an initially central source in the ommatidium and be expressed in each cell type as it becomes determined. It is even conceivable that Spitz itself could provide both functions — the trigger and the ratchet — a low level inducing a change of state and a high level triggering differentiation.

Remaining questions

Even if my model is correct in outline, it highlights many remaining questions — beyond the issue of how specificity is determined. Reiterative activation of the EGF receptor is responsible for triggering the differentiation of each of the cell types successively, but this does not preclude DER having other functions in the developing eye, and the indications are that it also acts in cell proliferation and ommatidial spacing (Baker and Rubin, 1992; Xu and Rubin, 1993). A related issue is whether R8, the first photoreceptor to form, requires DER activation. R8 appears to require the Ras pathway and DER for its differentiation (Simon et al., 1991; Xu and Rubin, 1993), but paradoxically not the presence of Spitz for its initial formation (Tio and Moses, 1997). It is possible that other ligands activate DER in R8, although none has yet been identified. There are at least two other activating ligands in other parts of the fly: Gurken and Vein (Neuman-Silberberg and Schupbach, 1993; Schnepp et al., 1996). It is also possible that R8 is triggered by low levels of constitutive DER signalling. This first cell must in any case be determined in a different way from the later ones, since there is no earlier cell to recruit it and it is the only photoreceptor to depend on the proneural gene, *atonal* (Jarman et al., 1994, 1995). This suggests that a proneural mechanism, of the kind that determines sensory organs in other parts of the fly, may also act in the furrow to establish ommatidia (for review of events in the morphogenetic furrow see Thomas and Zipursky, 1994; Bonini and Choi, 1995).

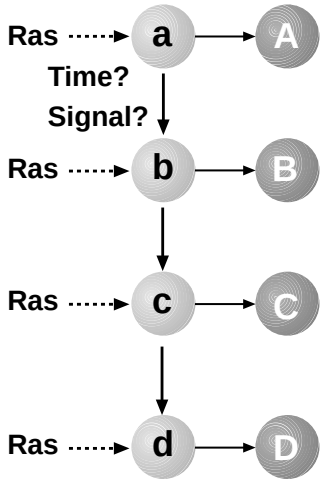

Fig. 6. How a cell's identity could be specified. Since there seems to be no specificity built into the recruitment signal, I propose that cells 'know' what they will become at the point that they are triggered by Ras activation. A cell in state 'a' will become type A, but if it is not triggered it will move successively through a number of preprogrammed states. We do not know what is responsible for 'ratcheting' the cell from one state to the next — it could be an intrinsic clock or a series of pulses of an external signal. The series of states through which all cells must pass could be dependent on a series of mutually inducing transcription factors.

There are also a number of other genes that are involved in ommatidial recruitment, but which do not fit the current model. Most significant are the genes involved in Notch signalling, principally *Notch* itself and *Delta*. Their roles are clearly crucial since they are required at each stage of determination for the recruitment of the correct number of photoreceptors, cone and pigment cells (Cagan and Ready, 1989b; Baker and Zitron, 1995; Parks et al., 1995). In their detailed analysis of Notch in the eye, Cagan and Ready (1989b) proposed that Notch establishes periods when cells are receptive to inductive signalling. Ectopic activation of Notch also gives phenotypes that suggest a role in timing of determination (Fortini et al., 1993). Thus there is good evidence that the Notch signalling system does somehow affect timing of inductive signalling in the eye, and understanding the relationship between it and the DER pathway is now an important goal. Another gene whose function must be accommodated is *fat facets* (Fischer-Vize et al., 1992), which encodes a ubiquitin-specific protease (Huang et al., 1995). Intriguingly, it is needed in cells outside the ommatidium to regulate the number of photoreceptors recruited (Huang and Fischer-Vize, 1996). This implies that the view of a one-way flow of information, from the developing ommatidium out to the sea of potential recruits, is an over-simplification.

The logic of eye development

As well as identifying the signalling pathways that are responsible for inductive signalling, we need to understand the underlying logic of how they act. I have described how, in the fly eye, some of this developmental logic is becoming apparent. It seems a reasonable guess that these principles will turn out to be conserved across evolution, like the signalling molecules themselves. For instance, the concept of reiterative use of a signal is important because it provides an answer to a problem apparent in many systems: how can relatively few signal transduction pathways generate the complete diversity of an organism? The genetic analysis possible in the fly's eye should allow us to understand how a cell's state changes to allow this diversity of response to a single signal.

Another feature of eye development that may be widespread

is the use of diffusible inhibitory molecules in mechanisms such as remote inhibition. Argos is critical in allowing the diffusible ligand, Spitz, to maintain a graded activity. Since diffusible factors are common in the development of vertebrates and invertebrates, remote inhibition seems likely to recur. Indeed, similar inhibition occurs in the Bmp4/dpp signalling system in *Xenopus* and flies. Graded activity of Bmp4/dpp is a key step in pattern formation (Ferguson and Anderson, 1992; Wharton et al., 1993; Graff et al., 1994; Suzuki et al., 1994; Schmidt et al., 1995), and this gradient is established by the production of diffusible antagonists — chordin/sog and noggin (for review see Ferguson, 1996). In this case, the antagonists act by sequestering the activating ligand, not by interacting with the receptor (Piccolo et al., 1996; Zimmerman et al., 1996), as occurs with Argos and DER, so the two systems are formally distinct. Nevertheless, they have in common the use of diffusible inhibitors to establish and/or maintain graded signals.

R7 uses two different RTKs to activate the Ras pathway, probably at different times. It is not clear if this is a quirk of eye development, or if the use of multiple receptors to regulate intricate signalling requirements will occur elsewhere. However, this example does emphasise the importance of time in regulating development. This is well illustrated by the often-reported result that precocious activation of Ras in the presumptive cone cells causes them to become ectopic R7s (Basler et al., 1991; Dickson et al., 1992; Fortini et al., 1992; Gaul et al., 1992; Rogge et al., 1992; Brunner et al., 1994). The role of time is perhaps the key difference between the model that I have proposed for eye development and the combinatorial induction model proposed by Tomlinson and Ready (1987b). In the earlier view, it was the precise set of contacts made by a cell that specified its fate. In the current model, although the cells do adopt stereotyped positions in the ommatidium, it is their developmental history that determines whether they differentiate as photoreceptors, cone or pigment cells.

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