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Regulation of EGF receptor signaling establishes pattern across the developing *Drosophila* retina

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SUMMARY

Developing epithelia use a variety of patterning mechanisms to place individual cells into their correct positions. However, the means by which pattern elements are established are poorly understood. Here, we report evidence that regulation of *Drosophila* EGF receptor (DER) activity plays a central role in propagating the evenly spaced array of ommatidia across the developing *Drosophila* retina. DER activity is essential for establishing the first ommatidial cell fate, the R8 photoreceptor neuron. R8s in turn appear to signal through Rhomboid and Vein to create a patterned array of 'proneural clusters' which contain high levels of phosphorylated ERKA and the bHLH protein Atonal. Finally, secretion by the proneural clusters of Argos represses DER activity in less mature regions to create a new pattern of R8s. Propagation of this process anteriorly results in a retina with a precise array of maturing ommatidia.

Key words: *Drosophila melanogaster,* Ommatidium, DER signalling, Retina, Patterning, Argos, Spitz, Vein

INTRODUCTION

Most tissues are composed of myriad cell types, each placed in its correct position within the epithelium. Several mechanisms could account for this precision: for example, lineage-based information could pre-program each cell, and cell migration could be used to target each cell to its appropriate niche. Lineage mechanisms, however, require an enormous amount of *a priori* information, and cell migration depends upon extensive guidance cues provided by other cells. Instead, most developing epithelia make use of local cell-cell communication to direct cell fates, a mechanism which ensures coordination of development between neighboring cells. However, reliance on local communication presents two problems regarding patterning: how is the first cell type established in its correct position if all cells begin equal and multipotent, and how are pattern elements coordinated to form a correct macropattern?

Early work with mathematical and experimental models has suggested that pattern might be initiated in epithelia through a combination of diffusible activators and inhibitors (Turing, 1952; Locke, 1960; Lawrence, 1969; Nardi and Kafatos, 1976; Meinhardt, 1977; Richelle and Ghysen, 1979; Oster and Murray, 1989). By combining a short-range activator with a long-range inhibitor, differences might be introduced into an otherwise homogeneous sheet, permitting the emergence of 'pioneer' cells which could then propagate this pattern through further signaling. However, the molecules that underlie this process have yet to be identified. In this paper, we demonstrate how the progressive regulation of the *ras* signal transduction pathway leads to reiteration of pattern elements by a self-renewing mechanism during early cell type specification in the developing *Drosophila* retina.

In contrast, most recent work on epithelial patterning has focused on 'prepatterns' inherited from the syncitial blastoderm, whose lack of cell membranes permits diffusion and direct action by transcription factors (reviewed by Martinez-Arias, 1989; St. Johnston and Nüsslein-Volhard, 1992). In this view, regions of competence within the epithelium are established first, with individual cell fates emerging later. For example, evidence from work in *Drosophila* has led to the suggestion that cell fate initiation and positioning require a two step process: the epithelium is commonly partitioned into discreet regions of competence inherited from early embryonic stages; each of these regions is then further narrowed to one or a few cell types. In the developing nervous system, regions of neuronal competence known as 'proneural clusters' are thought to give rise to one or a few neuronal cells. Proneural clusters can be recognized in part by their expression of a class of 'proneural' basic helix-loop-helix (bHLH) transcription factors. The positions of these clusters within the embryonic nervous system are established as cells respond to broad cues present in the developing embryo (Skeath et al., 1992). In addition, position within the embryo appears to set the coordinates within imaginal discs (Neumann and Cohen, 1997). However, these static embryonic cues do not appear to be sufficient for fine patterning of tissues such as the developing retina, where the placement of ommatidia is dependent upon factors induced transiently within the morphogenetic furrow (MF) as it

sweeps across the larval eye (Heberlein et al., 1993; Ma et al., 1993).

The adult *Drosophila* retina is composed of a precisely patterned array of unit eyes known as 'ommatidia' (Fig. 1A). The emergence of these ommatidia during development occurs in a highly organized and stereotyped fashion. Throughout embryonic and most of larval life, cells in the eye imaginal disc proliferate and remain developmentally multipotent. In the mature larva, cells undergo cell cycle arrest in G_1, and their nuclei drop basally in a coordinated fashion to form a groove known as the morphogenetic furrow (MF; Fig. 1B). The MF first appears near the posterior edge of the eye disc and is 'pushed' anteriorly through a mechanism requiring *hedgehog*-mediated signals provided by maturing ommatidia (Heberlein et al., 1993; Ma et al., 1993). Within the MF, a patterned array of proneural clusters is established and eventually the first cell type emerges, the photoreceptor neuron R8. As a result, the eye disc contains a continuous gradient of developmental maturity in which anterior ommatidia are less mature than those found in more posterior regions. Once R8 emerges, a chain of local inductive cues is used to recruit the remaining 19 cells required to build a complete ommatidium. Lineage-derived information does not play an important role in determining retinal cell fates (Ready et al., 1976; Lawrence and Green, 1979).

Atonal is necessary for R8 specification (Jarman et al., 1994), and thus the expression pattern of Atonal reflects which cells are competent to differentiate as R8s. Atonal is required to specify this first cell fate and missexpression can promote the R8 fate in at least some retinal cells (Jarman et al., 1994; Dokucu et al., 1996). Since each ommatidium requires an R8 for further photoreceptor recruitment, the expression pattern of Atonal provides the earliest indication of the establishment of the ommatidial pattern in the eye. This expression can be divided into four steps (Fig. 1C,D; Stages as in Dokucu and Cagan, 1996). First, Atonal appears as an unpatterned stripe of weak expression at the anterior edge of the MF (*Stage 0*). Second, in slightly more posterior cells within the MF, the unpatterned stripe breaks into a series of well-spaced 10-15 cell 'proneural clusters' which express Atonal at high levels (*Stage 1*). This event represents the earliest evidence of pattern within the neuroepithelium. Within each proneural cluster, the nuclei of 2-3 cells have risen apically; this 2-3 cell group has been referred to as the 'R8 equivalence group' (*Stage 2*; Dokucu et al., 1996). Finally, one cell within the R8 equivalence group maintains Atonal expression for 3-4 rows and differentiates as the R8 photoreceptor neuron (*Stage 3*). As the remaining cells lose Atonal expression they gain expression of Rough and E(spl), two transcription factors which provide negative regulation to Atonal (Baker et al., 1996; Dokucu et al., 1996). Based on this evolving expression pattern, it has been proposed that pattern within the retina is first established when Atonal expression makes the transition from an unpatterned stripe to a patterned array of proneural clusters, and that cell fate initiation then occurs when this expression further narrows to a single cell (Jarman et al., 1995; Baker et al., 1996). As with other bHLH transcription factors, narrowing of Atonal expression is affected by alterations in the *Notch* signal transduction pathway (Baker et al., 1996; Dokucu et al., 1996), although the role of *Notch* signaling in selecting the position of the final neuron is unclear.

Another signaling pathway implicated in early patterning is the *ras* signal transduction pathway. Typically, surface signals are mediated through the small GTPase Ras, which in turn causes activation of a cascade of kinases and eventual phosphorylation and activation of ERK, a member of the MAP kinase family of serine/threonine kinases (reviewed by Marshall, 1995; Ferrell, 1996; Treisman, 1996). Prolonged activation of ERK can result in its nuclear translocation and direct phosphorylation of target transcription factors to influence their activity. Thus, phosphorylation of ERK is an indicator of *ras* pathway activity, and the recent generation of antibodies to phospho-specific forms of ERK provide a sensitive in vivo indicator of the local activity of *ras* signaling (Gabay et al., 1997a,b; Yung et al., 1997).

Activation of the *Drosophila* Ras ortholog Dras1 results in alteration of a variety of cell fates throughout ommatidial development (Fortini et al., 1992; Miller and Cagan, 1998). Dras1 is part of a signal transduction pathway that is triggered through activation of receptor tyrosine kinases such as DER, the fly ortholog of the mammalian epidermal growth factor (EGF) receptor family (Livneh et al, 1985; Wadsworth et al., 1985; Schejter et al., 1986; Perrimon and Perkins, 1997). Hyperactivation of DER results in early defects in ommatidial spacing in *Drosophila* and the EGF receptor can promote specific fates in the developing rat retina (Baker and Rubin, 1989; Lillien, 1995). Early removal of *DER* activity in the *Drosophila* retina results in loss of normal proliferation and neuronal differentiation (Clifford and Schupbach, 1989; Xu and Rubin, 1993). Genotypically normal ommatidia surrounding these *DER*$^{-}$ cells were found to contain ectopic neurons, suggesting that a *DER*-mediated signal may provide diffusible information to neighboring cells. In this paper, we explore the nature of this signal.

Four potential ligands are currently known for DER: Gurken, Spitz, Vein and Argos. Gurken is a TGFα ortholog required exclusively during oogenesis but which apparently plays no role in development of adult structures (Neuman-Silberberg and Schupbach, 1993). Spitz and Vein are most similar to TGFα and Neuregulin, respectively, and are required for DER activation at multiple steps in epithelial development (Rutledge et al., 1992; Schnepp et al., 1996). Loss of *spitz* function results in defects in a variety of tissues in the embryo, and it is also required later in wing and retinal development (Mayer and Nusslein-Volhard, 1988; Clifford and Schupbach, 1989; Rutledge et al., 1992; Freeman, 1994a; Tio et al., 1994; Golembo et al., 1996a; Tio and Moses, 1997; Simcox, 1997). Similarly, loss of *vein* results in a variety of developmental defects, including loss of proper muscle attachment sites and wing veination (Simcox et al., 1996; Simcox, 1997; Yarnitzky et al, 1997). The sequence of *vein* suggests it encodes a secreted factor, whereas Spitz is initially generated as a membrane-bound precursor that requires the presence of the transmembrane protein Rhomboid for its release as a long-range DER activator (Schweitzer et al., 1995b; Golembo et al., 1996a). The phenotypes associated with mutations in these factors are similar to those observed in *DER*$^{-}$ mutants, and genetic interactions further suggest *gurken*, *spitz* and *vein* encode activating ligands of DER. By contrast, the secreted molecule Argos appears to act in opposition to DER function: loss of Argos function gives rise to a phenotype opposite to loss of DER activity, and overexpression leads to an apparent loss of DER function (Freeman, 1994b; Schweitzer et al.,

1995a; Golembo et al., 1996b). Biochemical studies support the view that Argos acts in opposition to Spitz and Vein, apparently through differences in their EGF domains (Schweitzer et al., 1995a; Schnepp et al., 1998).

In this paper, we provide evidence that regulation of DER signaling plays a central role in establishing retinal pattern. We demonstrate that DER and Dras1 are regulators of Atonal expression and R8 specification during successive steps of early patterning. This signaling is mediated through a combination of activators and inhibitors of DER. Our evidence indicates that: (i) activation of DER signaling establishes R8s within a limited zone of R8 competence; (ii) emerging R8s produce Rhomboid and Vein, which work in parallel to maintain the R8 fate and to activate DER in surrounding R8-incompetent cells (the proneural cluster); and (iii) these surrounding cells respond to this signal by secreting the DER inhibitor Argos, which creates an 'R8 exclusion zone' and determines the position of R8s in the next row. Together, these results outline a chain of events which are required to establish and propagate pattern in the developing retina.

MATERIALS AND METHODS

Fly stocks and heat-shocks

Most fly stocks were maintained at room temperature on standard cornmeal-yeast-agar medium. Transgenic stocks containing heat-shock-inducible genes were kept at 18°C to minimize chronic expression. Induction of activated Dras1 (w; P[w$^+$, hs-Dras1^{Val12}]/CyO) (Fortini et al., 1992), activated DER (w P[w$^+$, UAS-λ-DER] 4.2); P[w$^+$, hs-GAL4]) (Queenan et al., 1997), or dominant-negative DER (P[w$^+$, hs-GAL4]/P[w$^+$, DER^{DN}]) (Freeman, 1996) was accomplished by a 1 hour heat-shock at 37°C followed by a rest period (see text) at 25°C. For induction of Rhomboid (w P[w$^+$ hs-rho] 1B/FM6) (Sturtevant et al., 1993), antisense Rhomboid (w; P[w$^+$hs-rhomboid antisense]18-1; P[w$^+$hs-rhomboid antisense]18-6) (Ruohola-Baker et al., 1993), or Argos (w; P[w$^+$ hs-argos]; P[w$^+$ hs-argos] #4) (Sawamoto et al., 1996) continuous heat-shock at 37°C for the length of the experiment was necessary due to the rapid turnover of these products. In all cases, Ore-R flies were subjected to the same heat-shock protocol and used as controls. N^{ts1} is described by Lindsley and Zimm (1992); *rho AA69* is described by Nambu et al. (1990).

Gamma-induced mitotic recombination

To observe the effects of homozygous loss of *rho^{del1}*, *vn$^{\gamma 3}$* and *rho^{7M}vn^{ry}* double mutants on R8 formation, heterozygous stocks were crossed to flies containing a P[*ubiquitin-green fluorescent protein,w$^+$*] insertion at 61EF (gift from Erica Selva). Larvae were exposed to 1200 rads gamma-irradiation during first or late second larval instar. Eye discs were dissected during third instar and stained with antibodies against Boss, Atonal, or phosphorylated MAP kinase as described below; mutant clonal patches were recognized by loss of GFP and twin spots containing two copies of GFP.

Immunohistochemistry

Two antibodies against phosphorylated MAP kinase were used: anti-dually phosphorylated ERK (α-dpERK; Promega) is a rabbit polyclonal antibody; a similar antibody from Sigma is a mouse monoclonal. Both antibodies were raised against a dually phosphorylated ERK peptide whose amino acid sequence is conserved between vertebrates and *Drosophila*. Both antibodies were used at a concentration of 1:500 with virtually identical results; they are both denoted α-dpERKA in the text. α-Atonal (gift from A. Jarman and Y. N. Jan) and α-Rhomboid (gift from E. Bier) are rabbit polyclonal antisera and were used at dilutions of 1:4000 and 1:2000, respectively. α-boss mAbNT1 (gift from S. L. Zipursky) and α-E(spl) mAb323 hybridoma supernatant (gift from S. Bray) are mouse monoclonal antibodies and were used at concentrations of 1:2000 and 1:1, respectively. α-Rough mAb, α-β-galactosidase A41, and α-Argos are mouse monoclonals that were obtained as a hybridoma supernatants from DSHB and used at a dilution of 1:10.

Eye imaginal discs were dissected from wandering third instar larvae. For most experiments, discs were dissected into PBS and transferred immediately to fixative; for antibody staining of Argos, which is secreted, larvae were dissected directly into 4% paraformaldehyde in PBS. For staining with α-Atonal, α-Rough, α-E(spl), α-β-gal and α-dpERKA, discs were fixed with PEMP (100 mM PIPES, pH 7.0, 1 mM $MgSO_4$, 2 mM EGTA, 4% paraformaldehyde) for 20 minutes; for staining with α-Boss, discs were fixed with PLP (2% paraformaldehyde, 35 mM Na_2HPO_4, pH 7.4, 75 mM lysine, 10 mM sodium metaperiodate) for 40 minutes; for staining with α-Rho, α-Argos, and α-dpERK, and for double stains with one of these antibodies, discs were fixed with 4% paraformaldehyde in PBS for 25 minutes. After fixation, discs were washed in PBS and permeabilized in 0.3% Triton X-100/PBS; tissue was incubated overnight at 4°C in Triton/PBS containing the appropriate antibody and 10% serum. For visualization, tissue was incubated in Triton/PBS/serum with the appropriate secondary antibody: anti-rabbit and anti-mouse IgG conjugated to Cy3 (Jackson Labs) were used at a concentration of 1 µg/ml; anti-rabbit and anti-mouse FITC (Jackson labs) were used at a concentration of 5 µg/ml; for HRP-DAB stains, the avidin-biotin complex method was used (ABC, Vector). Fluorescent discs were mounted in Vectashield (Vector); HRP-DAB-stained discs were mounted in Gel/Mount (Biomeda Corporation).

In situ hybridizations to detect *vein* mRNA were performed essentially as described by Tautz and Pfeifle (1989) using digoxigenin-labeled anti-sense RNA probes; sense probes served as negative controls. Digoxigenin was detected by antibody (Boehringer-Mannheim); discs were mounted in Gel/Mount. β-gal was detected after in situ hybridization of *rho^{AA69}* discs using standard antibody detection as described above.

Mounted discs were photographed on a Zeiss Axioscope microscope, or confocal images were obtained using a Molecular Dynamics MultiProbe 2001 confocal laser scanning system. Images were collected and analyzed by ImageSpace software (Molecular Dynamics) running on a Silicon Graphics Iris Indigo Workstation. Confocal projection images were created by the superimposition of sections using the lookthrough-extended focus feature of the ImageSpace software.

Western analysis and immunoprecipitations

Western analysis using the dpERKA antibody was performed on tissue homogenates from third instar eye/antennal discs and brains. To test if phosphorylation was important for recognition by αdpERKA, 50 disc/brain complexes were incubated in Schneider's medium with or without phosphatase inhibitors (50 nM okadaic acid and 100 µM sodium vanadate) for 30 minutes at RT before homogenization. Tissue was then briefly pelleted and the medium removed; homogenization of both samples was performed in 100 µl 2× Laemmli buffer containing phosphatase inhibitors (0.125 M Tris, pH 6.8; 0.1% SDS, 5% β-mercaptoethanol, 20% glycerol, 0.0025% bromophenol blue, 100 µM sodium vanadate, 50 mM β-glycerophosphate). Insoluble material was removed by centrifugation and 5 µl of supernatant was loaded per lane of a 4-15% acrylamide gradient minigel (Biorad). After separation, proteins were transferred to PVDF membrane (Millipore) in 25 mM Tris, 192 mM glycine, 20% methanol. The membrane was washed briefly in TBST (10 mM Tris-HCl, pH 8.0; 150 mM NaCl, 0.05% Tween 20), blocked in 5% powdered milk/TBST for 30 minutes at 4°C, and incubated with a 1:5000 dilution of anti-active MAP kinase (Promega) in TBST+0.5 mg/ml

BSA overnight at 4°C. Detection was accomplished using an HRP-conjugated anti-rabbit IgG and the Phototopesystem (New England Biolabs).

To test if the protein recognized by α-dpERK was ERKA, protein was immunoprecipitated by α-dpERK and subjected to western blot analysis using an antibody against ERKA (gift from L. Zipursky). Immunoprecipitation was performed as follows: 60 eye/antennal disc/brain complexes were incubated in Schneiders medium containing 50 nM okadaic acid and 100 µM $NaVO_4$ for 30 minutes. Complexes were briefly pelleted and lysed in 300 µl RIPA buffer (PBS + 0.1% SDS, 1.0% Triton X-100, 1.0% sodium deoxycholate, 1 mM $NaVO_4$, 50 mM β-glycerophosphate, 20 mM NaF). After a brief centrifugation (5 minutes at 13,000 rpm), 1 µg α-dpERK antibody was added to half the supernatant; the other half served as a no-antibody control. Incubation with primary antibody was carried out for 2 hours at 4°C; antibody was recovered by further incubation with 20 µl GammaBind Plus Sepharose beads (Pharmacia) for 2 hours at 4°C. Antibody-bound beads were washed 5× with RIPA; protein was eluted by heating to 65°C for 5 minutes in 20 µl 2× Laemmli buffer (above). 10 µl of eluted protein was loaded per lane of a minigel; analysis was as described.

RESULTS

The following experiments examine the role of DER regulation on R8 specification and patterning, and are summarized schematically in Fig. 7.

Changes in *ras* pathway signaling affect the pattern of Atonal expression and R8 specification

One mutation which has been demonstrated to alter the spacing of ommatidia is DER^{Elp}, a hypermorphic (gain-of-function) allele of *DER*. The eyes of DER^{Elp}/+ flies are rough and irregular: spacing between ommatidia is uneven and somewhat fewer ommatidia overall are present than in wild type (Baker and Rubin, 1989, 1992). This loss of ommatidia appears to be due to repression of Atonal expression within the MF: the initial stripe of Atonal (Stage 0) is unaffected, but expression is lost in the region where proneural clusters normally form (Stage 1; Fig. 1F; Jarman et al., 1995). Remarkably, Atonal expression reappears in a 1-3 cell group (Stages 2, 3), and the majority of R8s still form. In DER^{Elp} homozygotes, nearly all ommatidia fail to form (Baker and Rubin, 1989), presumably due to a more extensive loss of Atonal. These results suggest that high levels of *DER* activity can repress Atonal expression and thereby alter the spacing of R8 photoreceptors and developing ommatidia. They also suggest that emergence of the R8 equivalence group may not depend on prior formation of a proneural cluster or Atonal expression within it.

To further explore the role of DER signaling on R8 specification, an activated form of the downstream target Dras1 was employed (Fortini et al., 1992). Flies containing $Dras1^{Val12}$ fused to an inducible heat shock promoter were subjected to a 1 hour heat shock followed by a rest period at room temperature to assess the effects of transient, ubiquitous expression. Within 2 hours after the initiation of $Dras1^{Val12}$ expression, Atonal expression was strongly upregulated throughout the MF, leaving a broad unpatterned band of Atonal in the region where it is normally partitioned into proneural clusters (Stage 1; Fig. 1G). This expansion in Atonal resulted in the production of ectopic R8s, as assessed by Boss expression 10 hours after heat-shock (Fig. 1H). Boss is an R8-

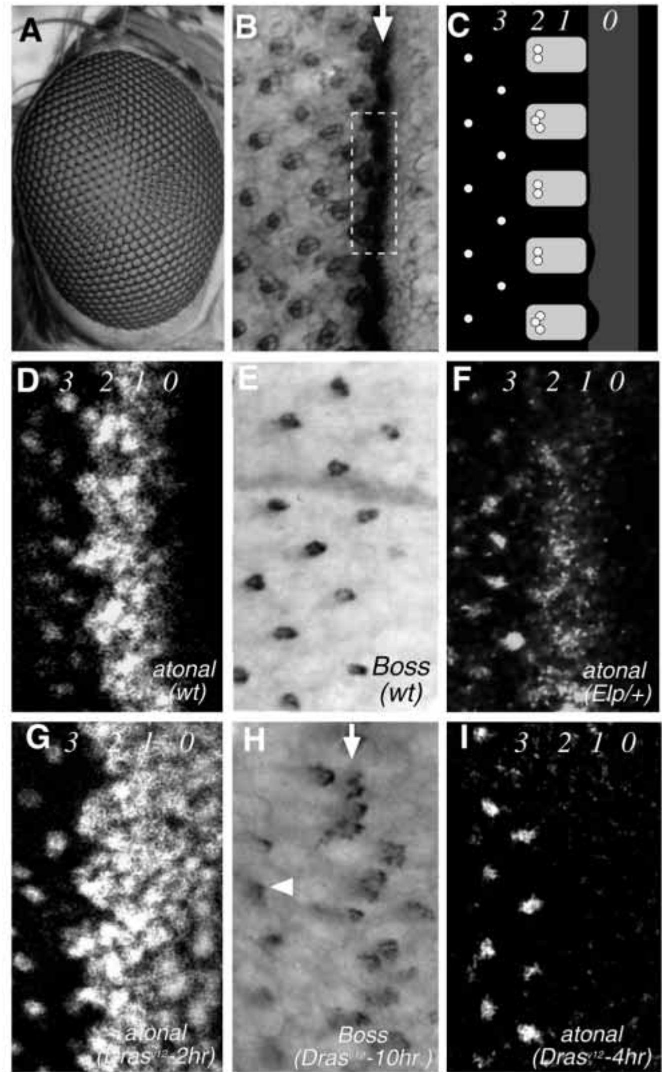

Fig. 1. Atonal expression is altered by *ras* pathway activation. (A) Scanning electron micrograph of a *Drosophila* eye; ommatidia are arranged in a precise pattern in which each row of ommatidia is out of register with the row next to it. (B) Cobalt sulfide stain of a portion of a mature third instar eye imaginal disc showing the pattern of developing ommatidia. Arrow indicates the MF, which can be seen as a dark dorsal/ventral stripe. Box includes approximate region of the MF shown in C and D. (C,D) Schematic (C) and antibody detection (D) of wild-type Atonal expression within the MF. In this and subsequent Figures, the top of most panels contain Stage labels to aid in orientation. Atonal is expressed at a low level by all cells at the anterior edge of the MF (‘0’ indicates Stage 0). Posterior to this, expression is elevated in proneural clusters (Stage 1) within which is an R8 equivalence group of 2-3 cells (Stage 2). A single R8 cell is selected from this group (Stage 3). (E) Detection of Boss protein with mAbNT1 shows the regular array of R8 cells posterior to the MF. (F) Antibody detection of Atonal in DER^{Elp}/+ discs, which lack upregulation of Atonal in proneural clusters (i.e. Stage 1 is missing). (G) 2 hours after expression of $Dras1^{Val12}$, Atonal expression in the proneural region (Stage 1) has broadened (compare with D). (H) 10 hours after $Dras1^{Val12}$ expression ectopic R8 neurons have formed (arrow), as assessed by Boss staining (compare with E); the row of ommatidia formed before $Dras1^{Val12}$ expression contains only single R8s (arrowhead), whereas more anterior cells fail to form R8s (data not shown). (I) 4 hours after $Dras1^{Val12}$ expression, Atonal has been eliminated in proneural clusters but remains in single cells (Stage 3).

specific protein which begins expression 6-8 hours after cells have left the MF; its ectopic expression 8-10 hours after heat-shock indicates the additional R8s were derived from cells within the MF at the time of heat-shock. Based on incorporation of the nucleotide analog BrdU, the presence of ectopic R8s was not due to cell proliferation within the MF, nor was any alteration in Atonal or Boss expression observed in wild-type flies receiving a similar heat shock regimen (data not shown). Interestingly, not all cells proved sensitive to R8 induction, suggesting that not all cells within the MF are competent to respond to Ras pathway signaling in this manner. These results indicate that strong Dras1 signaling can upregulate Atonal expression; however, only cells within a restricted zone are competent to respond to increased Atonal and *ras* pathway signaling by differentiating as R8s.

The upregulation of Atonal expression was followed by a ‘rebound’ downregulation. Loss of Atonal expression was observed 4-6 hours after transient expression of Dras1^{Val12}, leaving only a few cells near the posterior edge of the MF which still retained Atonal (Fig. 1I). Loss of Atonal was accompanied by an expansion in the expression of two inhibitors of Atonal function, Rough and E(spl) (data not shown), and a stable loss of R8 cells as assessed by Boss expression (see above). The arrest in addition of new R8s persisted for approximately 20 hours before reinitiating. This diminished Atonal staining is similar to that seen in *DER*^{Elp} and argues that strong or chronic *ras* pathway signaling may induce factors which feed back to shut down endogenous *DER*/*Dras1* activity (Golembo et al., 1996b).

One source of feedback repression appears to be Argos, a secreted inhibitor of DER. As Atonal expression and dpERKA were lost 4-6 hours after Dras1^{Val12} expression, expression of Argos was found to be dramatically upregulated (Fig. 6B,C; see below). This result is consistent with experiments in the embryo which indicated that Argos expression is upregulated by DER signaling (Golembo et al., 1996b). The importance of endogenous Argos in patterning DER activity will be explored below.

ERKA is activated in proneural clusters

Ubiquitous activation of Dras1 signaling eliminates pattern within the MF, yet endogenous Dras1 is expressed at high unpatterned levels throughout the MF (Katzen et al., 1991; Zak and Shilo, 1992). This raises the question as to which cells display active Dras1 signaling. One useful indicator of *ras* pathway activation is phosphorylation of the downstream target ERK: activation of the EGF receptor or Ras leads to phosphorylation of ERK on two closely spaced residues (Canagarajah et al., 1997). This activation can be assessed with an antibody specific for the phosphorylated form, and an α-dpERK antibody (Promega) recognizes doubly phosphorylated *Drosophila* ERKA (dpERKA; Fig. 2A). Remarkably, although ERKA is expressed in an unpatterned fashion throughout the eye disc (Biggs et al., 1994), presence of the activated form, dpERKA, is restricted to a repeating linear pattern within the MF (Fig. 2B; Gabay et al., 1997a). Based on co-localization with Atonal expression, ERKA is activated specifically within the proneural clusters (Stage 2; Fig. 2C) and is complementary to the expression patterns of Rough and E(spl) (data not shown). DpERKA was found to localize primarily to the cytoplasm, though occasional nuclear localization was also observed (data not shown).

Ubiquitous expression of Dras1^{Val12} resulted in broad expansion of the region of cells containing dpERKA within 30 minutes (Fig. 2D), a time course significantly faster than expansion of the Atonal region. All dpERKA was lost in a ‘rebound’ 2-3 hours later (Fig. 2E). No alteration in dpERKA pattern or intensity was observed in wild type control discs which received the same regimen of heat shocks (data not shown). Therefore, although Dras1 is normally present throughout the MF and is capable of activating ERKA, the *ras* pathway is only highly active within the proneural clusters.

DER activates the *ras* pathway in the MF

Activation of the *ras* pathway is thought to be mediated through receptor-tyrosine kinases (RTKs) such as the EGF receptor. To assess whether DER is the RTK responsible for activating the *ras* pathway in the proneural clusters, we examined the effect of transiently blocking DER function on proneural cluster formation (ERK phosphorylation and Atonal expression) and R8 specification (Atonal and Boss expression). Previous experiments that eliminated DER activity in retinal precursor cells blocked both proliferation and neuronal differentiation (Xu and Rubin, 1993). To separate DER’s proliferative effects from its role in R8 specification and patterning, we used flies containing GAL4 fused to an inducible heat shock promoter and a dominant negative form of DER (DER^{DN}) in a UAS construct (Freeman, 1996; see Material and Methods); DER^{DN} reduces DER function (Buff et al., 1998). Reduction of DER activity through ubiquitous expression of DER^{DN} transiently eliminated dpERKA and Atonal from proneural clusters (Fig. 3A,B). Loss of Atonal in the clusters correlated temporally with missing rows of Boss-expressing cells (Fig. 3C). When DER activity was blocked for long periods (e.g., 3 hours), Atonal expression in the most anterior region of the MF failed to upregulate or resolve into proneural clusters; that is, Atonal failed to make the transition from Stage 0 to Stage 1 (Fig. 3B). Maintaining the DER^{DN} block further broadened the stripe of low, unpatterned Atonal expression as the MF progressed anteriorly. These results indicate that DER activity is required to impose pattern on the initial band of Atonal expression, and that in its absence this band is unable to resolve into a repeating pattern of proneural clusters.

As an alternative to using DER^{DN} to block DER activity in the MF, we also examined the effect of ubiquitous expression of the secreted DER inhibitor Argos in *hs-argos* flies (Sawamoto et al., 1994). Similar to DER^{DN}, overexpression of Argos led to the immediate loss of phosphorylated MAP kinase in the proneural clusters (Stage 1; Fig. 3D); this was soon accompanied by loss of Atonal from cells in these clusters, but not from more posterior single cells (Stage 3; Fig. 3E). As with DER^{DN}, transient overexpression of Argos resulted in loss of 1-2 rows of R8 cells, as visualized by Boss (Fig. 3F). Thus, both methods of blocking DER function produced equivalent results: (i) loss of *ras* pathway signaling in the MF as assessed by the absence of dpERKA; (ii) loss of proneural clusters as assessed by loss of dpERKA and Atonal expression in the proneural clusters; and (iii) a block in R8 formation. Thus, *DER* activity appears to be required for formation of both the proneural clusters and R8s.

DER is expressed ubiquitously throughout the MF (Zak and Shilo, 1992). How, then, is its patterned activation achieved? To address this issue, we explored the role of two DER

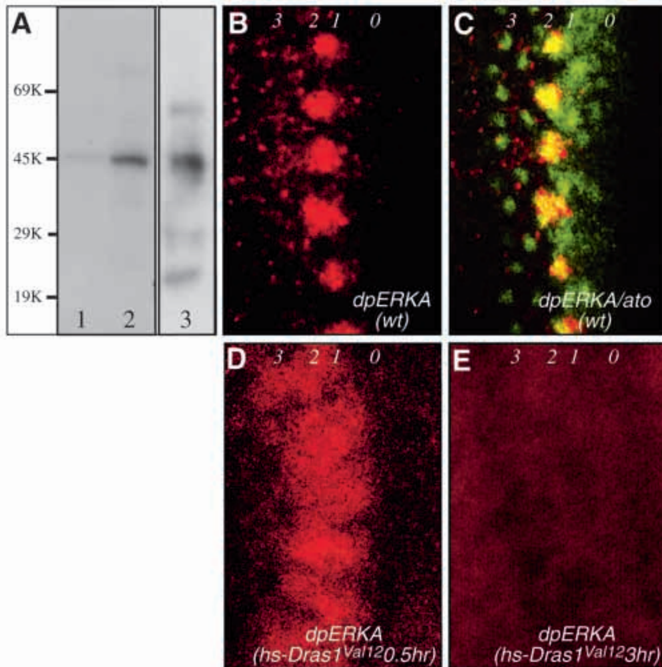

Fig. 2. ERKA is activated in proneural clusters. (A) αdpERKA recognizes the phosphorylated form of ERKA. Lane 1: western analysis shows αdpERKA recognizes a band at approximately 44 kDa, the size of ERKA. Lane 2: this band is intensified when tissue is treated with phosphatase inhibitors before homogenization. Lane 3: Immunoprecipitation from larval tissue with αdpERKA yields a band recognized by an antibody to ERKA. (B) ERKA is phosphorylated in groups of 6-10 cells within the MF. (C) dpERKA (red) colocalizes with Atonal protein (green) within the proneural clusters. (D) At early time points after expression of Dras1^{Val12} (30 minutes) ERKA phosphorylation expands throughout the proneural region. (E) At later times after Dras1^{Val12} expression (3 hour), ERKA phosphorylation is eliminated by a 'rebound' event.

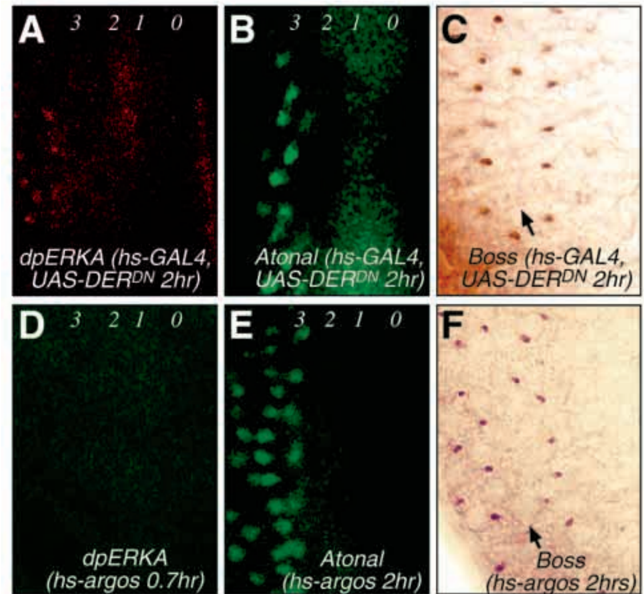

Fig. 3. *DER* activity regulates dpERKA, Atonal, and R8 specification. (A) Reducing DER activity through broad expression of DER^{DN} eliminates detectable dpERKA. (B) Expression of DER^{DN} eliminates patterning of Atonal in the proneural region (Stage 1). Atonal is expressed normally in R8s (Stage 3) and is also present throughout the anterior MF at the low level normally observed only at Stage 0. (C) The result is loss of R8s, as assessed by expression of Boss. The R8-specific epitope Boss normally first appears approx. 6 hours after R8 specification; each row of ommatidia thereafter is separated by approx. 2 hours of developmental time. When 2 hours of DER^{DN} overexpression was followed by 12 hours of rest at 25°C, Boss-expressing cells were often missing from lateral areas of maturing rows (e.g. arrow); these rows correspond to those forming during the period of DER^{DN} overexpression. (D-F) Similar results were obtained with broad, ectopic expression of Argos.

activators, Rhomboid and Vein, in localizing *ras* pathway activity in the MF.

Rhomboid demonstrates partial regulation of DER

Rhomboid is a seven membrane-spanning protein which enhances DER signaling and activation of ERKA (Bier et al., 1990; Sturtevant et al., 1993; Golembo et al., 1996a). It can activate DER signaling several cell diameters from the source of its expression, apparently by regulating release or activity of the DER ligand Spitz (Golembo et al., 1996a). An antibody specific for Rhomboid shows it to be expressed in cells near the posterior edge of each proneural cluster (Fig. 4A,B); a Rhomboid enhancer trap line confirms that expression begins in the 1- to 3-cell R8 equivalence group, based on the positioning of the group within the larger 'proneural cluster' and the apical position of its nuclei. Expression then quickly resolves to a single cell which can be unambiguously identified as R8 (Fig. 4C).

The previously demonstrated ability of Rhomboid to activate DER signaling at a distance suggests that cells of the R8 equivalence group could use Rhomboid to set the pattern of *DER*/*Dras1* activation across the proneural cluster. To test this possibility, we used *hs-rhomboid* flies to express Rhomboid throughout the MF. The result was similar to the effect of expressing Dras1^{Val12}: within 30 minutes of the initiation of ectopic Rhomboid expression, an unpatterned stripe of phosphorylated dpERKA emerged throughout the MF (Fig. 4D). This suggests that, indeed, expression of Rhomboid is sufficient to activate *ras* pathway signaling within the MF. The 'rebound' effect seen with ectopic Dras1^{Val12} was also observed with ectopic Rhomboid, but with a more rapid time course. 1-2 hours after initiating ectopic Rhomboid expression, all detectable dpERKA as well as Atonal expression in the proneural groups was lost (Fig. 4E,F); this was observed even if Rhomboid was expressed continuously during this period. In addition, the upregulation of Argos expression observed with ectopic activation of Dras1^{Val12} was also observed with ectopic expression of Rhomboid (compare Figs 4F and 6D; see below). Unlike experiments with Dras1^{Val12}, only a minor expansion of Atonal was observed with Rhomboid overexpression, presumably due to the brevity of *ras* pathway activation and its rapid subsequent down-regulation; Ras^{Val12} is able to produce activation for longer periods presumably because it acts intracellularly and downstream of Argos inhibition.

If Rhomboid signaling alone were responsible for directing DER activation within the MF, we would expect loss of Rhomboid function to result in a loss of ERKA

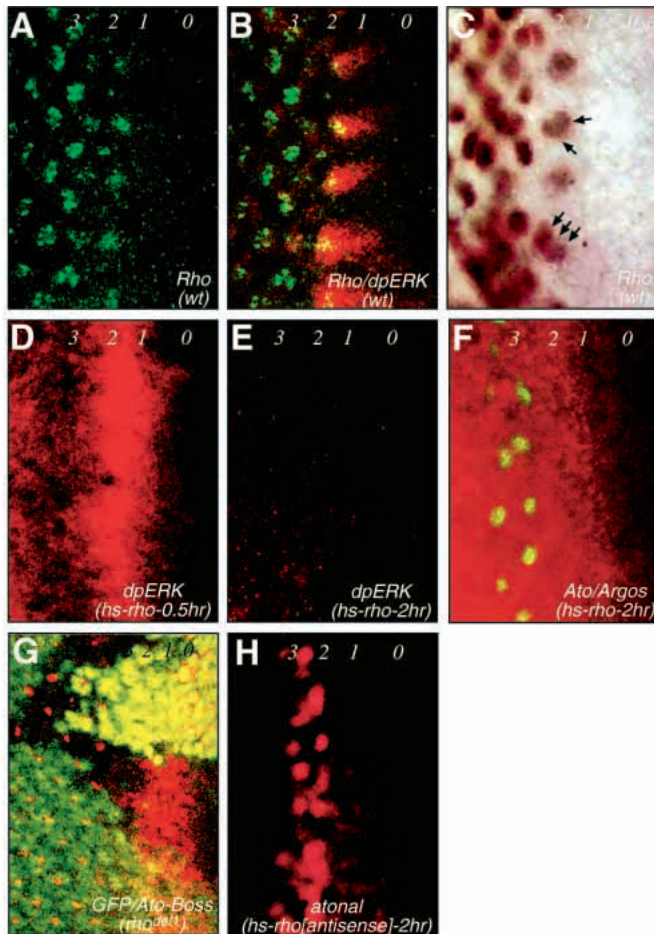

Fig. 4. Rhomboid is localized to R8 cells in the MF and can activate dpERK. (A,B) A Rhomboid-specific antibody (green) confirms that the earliest Rhomboid expression occurs near the posterior edge of the proneural cluster (αdpERKA red in B). (C) An enhancer trap which reproduces the expression pattern of Rhomboid indicates initial expression is in 1-3 cells (arrows). (D) Similar to activation of Dras1, ubiquitous expression of Rhomboid, via heat shock, results in a broadened activation of dpERKA; activation appears stronger than with $hs\text{-}Dras1^{Val12}$. (E) In addition, ubiquitous expression of Rhomboid also activated the 'rebound' effect but with a faster time course than that observed with λ-DER and $Dras1^{Val12}$; full loss of dpERKA was observed by 2 hours. (F) Expression of Atonal mirrored loss of ERKA: 2 hours after Rhomboid expression, Atonal was lost from all cells except already-differentiated R8s (green; Stage 3). Loss of ERK-A and Atonal was accompanied by increased Argos expression (red; compare to Fig. 6D). (G) A homozygous mutant patch of rho^{del1} is marked by absence of green fluorescent protein; red fluorescence in cells within the MF represents Atonal expression whereas red fluorescence posterior to the MF indicates R8 cells as identified by Boss expression. Lack of Rhomboid does not affect Atonal expression or R8 specification. (H) Ubiquitous expression of an antisense-Rhomboid construct resulted in transient loss of Atonal in the proneural clusters within 2 hours. This result may differ from that observed in G because of the high level of Argos present in the proneural clusters before expression of the anti-sense construct.

phosphorylation, Atonal expression and R8 specification. We blocked *rhomboid* activity in two ways: by creating patches of *rhomboid*⁻ homozygous tissue and by expressing an antisense construct. Gamma-irradiation of *rho*/+ heterozygotes was used to create patches of *rho*/*rho* homozygous mutant cells; these patches were recognized by the absence of a green fluorescent protein marker (GFP; see Materials and Methods). Loss of Rhomboid resulted in a diminution, but not complete loss, of phosphorylated MAP kinase within the MF (not shown). Consistent with this observation, proneural clusters retained high levels of Atonal expression (Fig. 4G), and Boss expression was normal (*n*=31 clones evaluated). These results are consistent with data indicating that loss of the Rhomboid target Spitz had little effect on R8 specification (Tio and Moses, 1997).

Similar conclusions were drawn when Rhomboid was eliminated through expression of a *rhomboid* antisense construct (Ruohola-Baker et al., 1993). Ubiquitous expression of *rhomboid* antisense eliminated detectable Rhomboid protein (not shown). Down-regulation of Rhomboid for 90 minutes resulted in transient loss of Atonal expression and dpERKA in proneural clusters (Fig. 4H). However, these losses were short-lived: even when antisense Rhomboid was expressed continuously, Atonal expression and dpERKA returned within 2-3 hours; R8 specification, as assessed by Boss expression, remained unaffected. Therefore Rhomboid, as with Spitz (Tio and Moses, 1997), is not sufficient to account for DER-mediated induction of the R8 fate. Together these results suggest that another ligand for DER may be present in the MF, and that this ligand may function redundantly with Rhomboid to activate *ras* pathway signaling, Atonal expression and R8 specification.

Rhomboid acts with Vein to regulate DER

Vein is a Neuregulin ortholog postulated to bind to and activate DER (Schnepp et al., 1996, 1998). Consistent with this view, removal of a single copy of *vein* in a DER^{Elp} mutant background (*vn*/+ DER^{Elp}/+) strongly enhanced the rough eye phenotype observed with DER^{Elp}/+ alone (data not shown; see below for further discussion of *DERElp*). *vein* mRNA is present at high levels throughout the anterior of second instar eye discs (Fig. 5A) where DER is thought to play a role in cell proliferation (Xu and Rubin, 1993). By the third larval instar, however, *vein* is restricted in the MF to single cells within the R8 equivalence group (Fig. 5B-D). Thus, at least one cell of the R8 equivalence group contains two potential activators of DER: Vein and Rhomboid.

To assess the role of Vein in R8 formation, we created early clonal patches homozygous for a *vein* null mutation. Few such patches were observed, although 'twin spots' (groups of cells containing two copies of the GFP marker and homozygous wild type for Vein, which are formed when mitotic recombination occurs) were common. This suggests that Vein may be required early for cell proliferation or survival, similar to the requirement previously observed for DER. Within the small mutant patches which did survive, Boss expression was normal (Fig. 5E; *n*=22 patches); thus loss of Vein alone, as with loss of Rhomboid, does not prevent R8 formation.

Our results suggest that neither Rhomboid nor Vein alone is essential for R8 differentiation. This is similar to what has been observed in the embryonic CNS, where neuroblast formation requires DER activity, but is only strongly affected if both *rhomboid* and *vein* activity are removed together (Skeath, 1998). To determine if Rhomboid and Vein also act in parallel

to specify R8 in the retina, *rho*$^{-}$*vn*$^{-}$ double mutant clonal patches were created by mitotic recombination. Patches were created later in second and third instar larvae to circumvent the requirement for Vein in early cell survival, and many of the resulting clonal patches (and their corresponding 'twin spots') contained only 4-8 cells. R8 specification was never observed in the interior of these patches (*n*=29 patches), although R8 cells were able to form along the periphery (Fig. 5F). In addition, often the pattern of ommatidia surrounding and anterior to the patch was altered. In rare *rho*$^{-}$*vn*$^{-}$ patches which crossed the MF, Atonal expression in the proneural clusters also appeared to be reduced (Fig. 5F); these large clones do not distinguish whether this loss is due to a direct requirement for *rho vn* function in proneural clusters or is a secondary consequence of a loss of more posterior, differentiated R8s.

These experiments suggest that Rhomboid/Vein-mediated DER activation has two roles: specification or maintenance of the R8 fate, and setting the pattern of proneural clusters. The observation that *rho*$^{-}$ *vn*$^{-}$ mutant clones produce disturbances in the spacing of more anterior ommatidia is reminiscent of defects observed in ommatidia surrounding *DER*$^{-}$ clones (Xu and Rubin, 1993) and suggests that the R8 neuron in one ommatidium might influence the positioning of R8s in neighboring and anterior ommatidia. By what mechanism might this influence arise? Above are presented experiments indicating *DER*/*Dras1* (through Rhomboid and presumably Vein) can activate expression of the secreted protein Argos. Therefore, we considered the potential for Argos to direct the pattern of emerging R8s through repression of DER.

Argos influences MF patterning

Argos is a secreted factor which can act several cell diameters from its source (Freeman et al., 1992; Freeman, 1997). It acts as a negative regulator of the DER pathway *in vivo* and can prevent autophosphorylation and activation of DER in tissue culture cells, leading to the suggestion that Argos directly binds DER (Schweitzer et al., 1995a; Golembo et al., 1996b; Sawamoto et al., 1996). Evidence for the presence of such a DER repressor in the MF was provided by a chimeric DER protein. λ-DER is a constitutively activated chimeric receptor in which the extracellular domain of DER has been replaced by the λ-repressor dimerization domain (Queenan et al., 1997). As described above, activation of DER through Dras1^{Val12} or Rhomboid resulted in an eventual 'rebound' loss of dpERKA and Atonal. By contrast, ectopic expression of λ-DER led to elevation of Atonal expression which persisted for at least 3 hours, even though – as with ectopic Rhomboid and Dras1^{val12} – Argos expression was also elevated in this time frame (Fig. 6A; compare with 6C). This result suggests that the rapid 'rebound' effect observed with Rhomboid requires a normal DER extracellular domain, and supports the view that it is mediated through a repressive ligand such as Argos.

Previous work in the embryo has found an upregulation of Argos transcription in response to DER signaling (Golembo et al., 1996b). Consistent with this observation, the highest levels of Argos expression in the MF were found in the regions of highest DER activity, the proneural clusters (Fig. 6D,E). Lower levels of the protein were observed between and anterior to these clusters, presumably due to diffusion from the proneural clusters into the surrounding tissue. The effects of Argos overexpression in the MF were presented above: Argos is capable of eliminating DER activity (as measured by ERKA phosphorylation) and Atonal expression in the proneural clusters (Fig. 3D,E). We find further that overexpression of Argos eliminates expression of the factors which localize DER activity to the cell destined to become R8: Rhomboid and Vein. A 90 minute heat-shock leading to overexpression of Argos eliminated Rhomboid protein from cells in the MF (Fig. 6F; *n*=3 experiments, approx. 40 eye discs). This is consistent with findings that down-regulation of the transcription factor CF2, a negative regulator of Rhomboid transcription, is induced by DER signaling (Hsu et al, 1996; Mantrova and Hsu, 1998). We also find that ectopic expression of Argos eliminates most or all *vein* RNA from cells in the MF (Fig. 6G; *n*=3 experiments, approx. 30 eye discs). Thus, Argos-mediated repression of *DER* pathway activity may normally contribute to the pattern of Rhomboid and Vein expression necessary for correct R8 specification.

To determine if Argos is necessary for setting the normal pattern of R8s, we examined Boss expression in the hypomorphic, partial loss-of-function mutant argos^{styP1}. Homozygous escapers of this line have rough eyes, due in part to the formation of ectopic ommatidia (Okano et al., 1992). Consistent with this, Boss-staining revealed that the pattern of R8 specification in these animals is disturbed: the spacing between R8s is variable and, most tellingly, R8s form aberrantly in positions between the normal ommatidial rows (Fig. 6H). These ectopic R8s were found in every eye disc of this genotype examined (*n*=24). This suggests that Argos produced by proneural clusters may normally diffuse anteriorly to repress DER activity (and Rhomboid and Vein expression), and the formation of R8s directly anterior to the cluster. In this model, R8s in the next row of ommatidia will be set at positions farthest from the site of Argos release, giving rise to the 'out-of-register' pattern of R8s found in wild type animals. Argos expression, in turn, is controlled by Rhomboid and Vein expressed in R8, indicating that each R8 has a role in patterning succeeding rows. It should be noted, however, that the disruption of ommatidial pattern observed when *argos* function is reduced is not very severe (see also Freeman et al., 1992), and suggests that one or more additional factors are likely to contribute to the regulation of Rhomboid and Vein transcription.

An evaluation of the *DER*^{Elp} phenotype

DER^{Elp} represents one of the first examples of a mutation that alters patterning in the retina: the eyes of *DER*^{Elp}/+ heterozygotes are rough and mispatterned and *DER*^{Elp}/*DER*^{Elp} homozygotes have greatly reduced eyes, which lack most ommatidia. In addition, proneural clusters appear to be absent, and Boss-expressing cells form less frequently and show aberrant patterning (Baker and Rubin, 1992; Jarman et al., 1995; data not shown). Genetic evidence indicates that the *DER*^{Elp} mutation is a hypermorphic (gain-of-function) allele (Baker and Rubin, 1989). The *DER*^{Elp} phenotype, then, is at odds with the data presented above which indicates that DER activation results in specification of more R8s (and ommatidia) rather than fewer. However, high levels of DER activity also lead to induction of the DER inhibitor, Argos (Golembo et al., 1996b; this paper). In fact, our data suggest that the patterning defects observed in *DER*^{Elp} retinae could result from the induction of ubiquitous and unpatterned Argos expression: *DER*^{Elp} heterozygotes show lower, rather than higher levels of

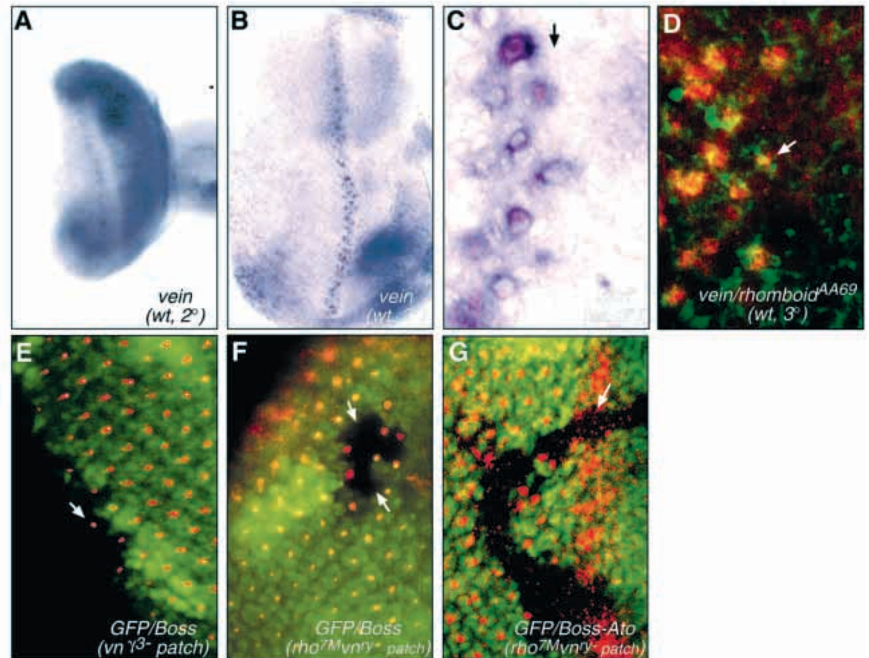

Fig. 5. Rhomboid and Vein together regulate R8 specification and spacing. (A) During the second larval instar, Vein mRNA is expressed throughout the proliferating cells of the anterior eye disc. This is consistent with a role in early proliferation. (B,C) In third larval eye discs, Vein expression is restricted to single cells in the MF (arrow in C), which correspond to differentiating R8 cells. (D) Vein RNA expression (green) overlaps with β-gal protein expression (red) in the Rhomboid enhancer-trap line *rho*^{AA69}. The in situ staining image was transformed and overlapped with β-gal expression electronically using Adobe Photoshop software. (E) Clonal patches homozygous for the null vein allele *vn*$^{\gamma 3}$ can be recognized by their lack of GFP expression (green). Although proliferation of *vn*$^{\gamma 3}$/*vn*$^{\gamma 3}$ cells is typically less than in the wild type 'twin spot' (bright green), R8s are unaffected as assessed by Boss expression (red, arrow). (F) A clonal patch doubly homozygous for*rho*^{7M}*vn*^{ry} is marked by the absence of GFP expression (green); R8 cells, as evidenced by Boss staining (red), are missing within the patch (arrows), although they do form along the periphery. (G) A clonal patch doubly homozygous for *rho*^{7M}*vn*^{ry} which crosses the MF; cells within this patch lack high levels of Atonal expression in the MF (arrow) and R8 specification, as assessed posterior to the MF by Boss expression (both shown in red). Thus while neither Rhomboid nor Vein alone is essential for R8 specification, mutations in both result in loss of proneural clusters and R8 cells.

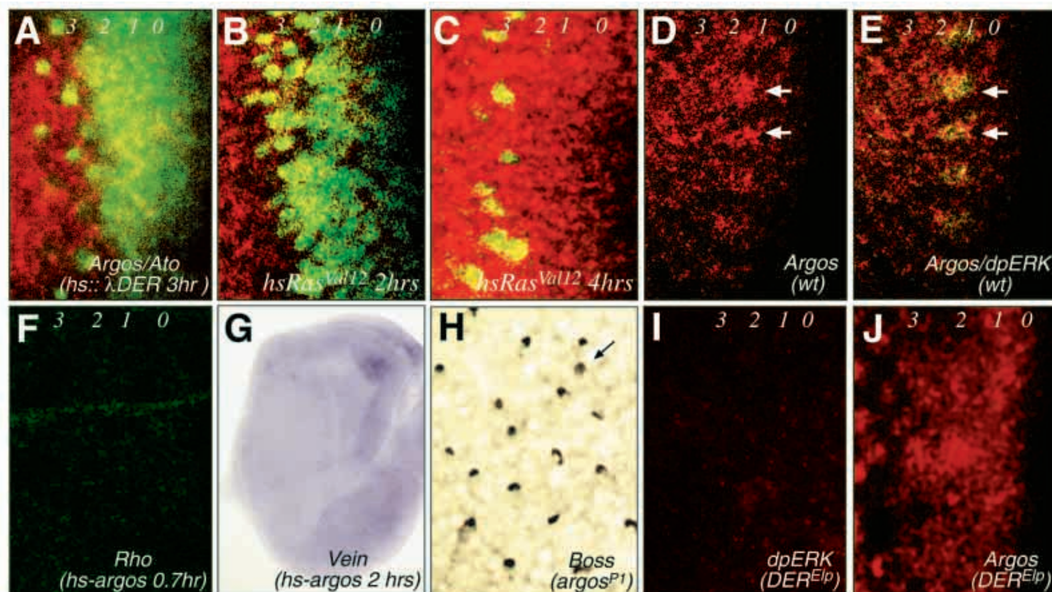

Fig. 6. Evidence of a role for Argos in patterning events within the MF. (A) Ubiquitous expression of the activated DER chimera λ-DER leads to sustained activation of Atonal (green); compare with Fig. 4F and 6C. This upregulation occurs despite the expanded expression of Argos (red), suggesting that Argos requires the extracellular domain of DER to mediate its activity. (B,C) Atonal down-regulation coincides with upregulation of Argos. (B) Ubiquitous expression of Dras1^{val12} leads to an initial upregulation of Atonal expression (C) As Atonal expression is lost, Argos expression is expanded. (D) Argos is expressed in diffusing stripes within the MF. (E) Co-staining with anti-dpERK (green) reveals that the stripes of highest Argos expression (red) within the MF correspond to the proneural clusters (arrows). (F) Overexpression of Argos in flies containing a P[hs-argos] construct eliminated Rhomboid protein within 40 minutes; compare to Fig. 4A. (G) Overexpression of Argos for 2 hours also eliminated *vein* mRNA expression in the MF, although a patch of staining in the anterior eye disc remained; compare with Fig. 5B. (H) Partial loss of Argos activity in *aos*^{P1} mutant flies is sufficient to mis-pattern ommatidia, as assessed by Boss expression. Note the formation of an ectopic R8 between normal rows of ommatidia (arrow). (I) ERK phosphorylation within the MF of *DER*^{Elp} heterozygotes is significantly lower than in wild type (compare to 2B). (J) Argos expression in *DER*^{Elp} heterozygotes is stronger and unpatterned when compared with expression in wild-type eye discs (see D).

dpERKA within the MF (Fig. 6I; compare to 2B) and Argos lacks the localization to proneural clusters present in wild-type animals (Fig. 6J, compare to 6D). Our data indicate that upregulation of Argos should result in loss of R8s, and this is observed in *DER*^{Elp} mutants.

DISCUSSION

As with many neural tissues, the fly retina requires the establishment of a precise pattern of cells to function optimally. In the case of ommatidial development, the first cells – the R8 photoreceptors – must be established at sufficient distance from one another to enable each ommatidium to recruit the twenty cells it will need for its mature function. The data presented in this paper provides evidence that the combination of activators (Rhomboid and Vein) and an inhibitor (Argos) can act together to delimit *DER* activity in a spatially and temporally restrictive manner to achieve correct ommatidial pattern. Eliminating DER activity – through transient expression of DER^{DN} or Argos, or by elimination of Rhomboid and Vein – blocks R8 specification and proneural cluster formation. Expanding DER activity, e.g. through Dras1^{Val12}, broadens Atonal expression, directs ectopic R8 specification, and expands expression of Argos. Consistent with this, reducing Argos activity results in poor ommatidial spacing and formation of ectopic R8s. The secreted nature of these regulators – Argos, Vein, and the Rhomboid-target Spitz – allows them to establish discreet zones across the epithelium in which *DER* activity typically is activated and then repressed. These factors are woven together by their own dependence on *DER* activity for their expression, providing the necessary cross-talk for a rolling, reiterative mechanism. Together, they play an important role in shaping the pattern of R8s within the MF.

A model for patterning

Differentiation of the R8 neuron

Our results suggest a model for the patterning of ommatidia within the retina (Fig. 8). We propose that patterning and R8 specification is set as cells respond regionally to regulation of DER activity. Beginning at the anterior edge of the MF, DER expression is upregulated and is expressed at levels that may be high enough to allow for low-level spontaneous activity (Zak and Shilo, 1990, 1992; Baker and Rubin, 1992; Tio et al., 1994; Schweitzer et al., 1995). Our results indicate that within the MF some cells become competent to respond to *DER*/*Dras1* signaling by differentiating as R8 photoreceptors (Figs 1H, 7C); the nature of this change in competence is not yet understood but may involve delayed expression or activation of a novel factor. Once competent, these cells respond to DER signaling by establishing a row of R8 equivalence groups (Fig. 8, top panel). Cells of this group express Rhomboid and Vein, a required step in maintaining the R8 fate. Once the R8 equivalence group is established, other factors including *Notch* signaling (Cagan, 1993; Powell and Cagan, unpublished data) and Rough (Dokucu et al., 1996) are required to select a single R8 from the group.

Positioning the emerging R8 neuron

In addition to their role in R8 differentiation, the production of Vein and Rhomboid/Spitz in the proneural clusters suggested

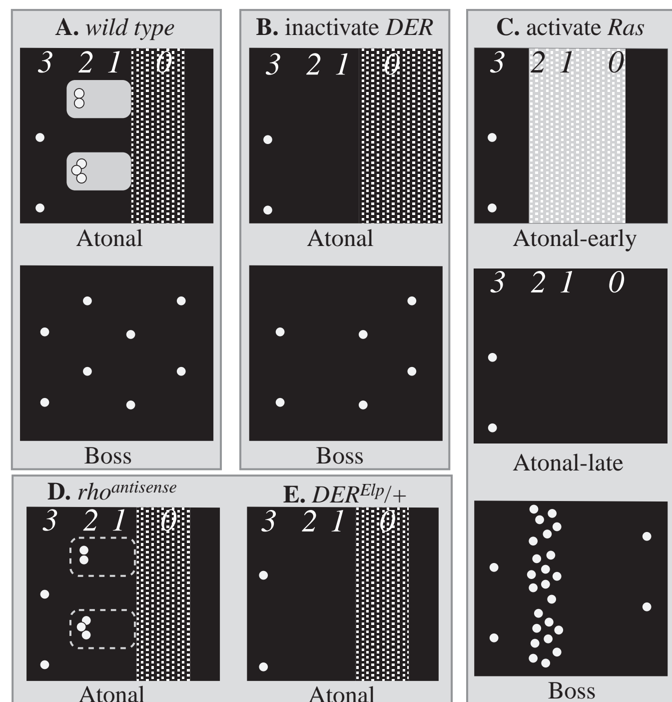

Fig. 7. Schematic summary of results. Atonal or Boss expression is indicated. (A) 2 panels. In wild-type eye discs, Atonal expression matures from low unpatterned expression (Stage 1), to high expression in patterned proneural clusters (Stage 2); R8 equivalence groups are within the proneural clusters (Stage 2) and give way to single R8 neurons (Stage 3). See Fig. 1C. The result is a stereotyped patterned array of R8s as assessed with Boss expression. (B) 2 panels. Inactivation of *DER* activity – through DER^{DN}, ectopic Argos, or *rho vn* clones – leads to loss of expression at Stage 2 and eventual loss of R8s. In DER^{DN}, the unpatterned expression of Atonal at Stage 1 expands as the MF travels anteriorly but fails to upregulate, suggesting that the presence of the proneural clusters requires *DER* function. (C) 3 panels. Ectopic activation of *Ras* activity through *hs-Dras1*^{Val12} leads to an initial, dramatic expansion of the proneural cluster (*top panel*) followed by a 'rebound' down-regulation of Atonal expression (*middle panel*). The result is ectopic R8s in a region defined as the 'R8 competence zone' and a loss of R8s in younger, more anterior cells (bottom panel). Two interesting special cases are presented in D and E. (D) Blocking *rhomboid* activity results in transient down-regulation of Atonal in the proneural clusters, although Atonal expression in the R8 equivalence group is retained. This indicates that Rhomboid, expressed earliest in cells of the R8 equivalence group, has anterior effects on Atonal expression presumably through Spitz. (E) In *DER*^{Elp}/+ heterozygotes, expression of Atonal at Stage 2 is mostly lost, and R8s demonstrate mild spacing defects.

that these diffusible factors may play a role in patterning. Based on our evidence (Figs 4H, 5F,G), we propose that R8's release of Vein and Spitz (via Rhomboid) activates DER in surrounding cells (Fig. 8, middle panel). This local activation of DER has two effects: upregulation of Atonal (Figs 1G, 6A) and upregulation of Argos (Figs 4F, 6A,C). Upregulation of Argos, in turn, blocks expression of Rhomboid and Vein in other cells within and directly anterior to the proneural group (Fig. 6F,G), thereby creating an 'R8 exclusion zone'. We propose that creation of these exclusion zones is necessary to

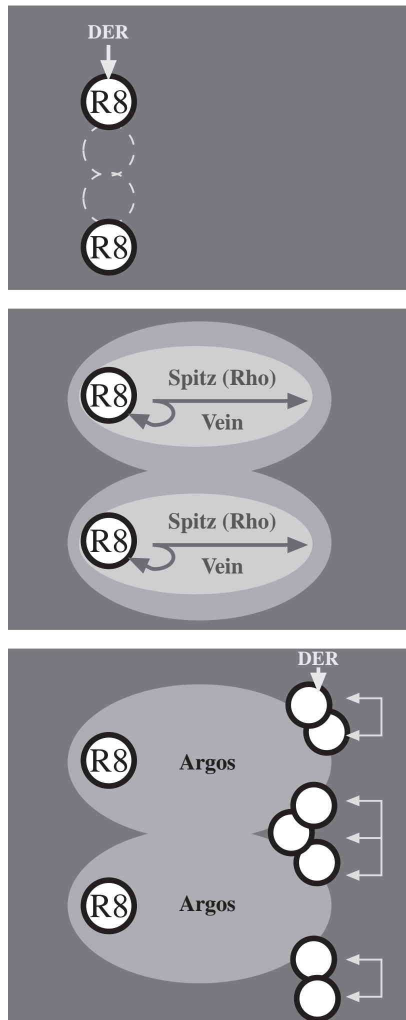

Fig. 8. A model for the role of *DER*/*Dras1* signaling in early patterning of the retina. This schematic represents proposed events within the MF; see Discussion for details. (Top panel) Activation of DER directs specification of the R8 equivalence group (not shown) and eventually R8 (labeled) within an 'R8 competence zone'; more anterior cells are not yet competent to respond to DER signaling. Expression of Argos from the previous row (not shown) prevents ectopic R8s (dotted circles) from forming. (Middle panel) R8 expresses Rhomboid and Vein, activating the *DER*/*Dras1* pathway at a distance to establish the proneural cluster and expression of Argos (light gray) which also diffuses (dark gray). Lateral inhibition of DER by Argos prevents the emergence of ectopic R8s within the same row, and anterior inhibition by Argos spaces R8s by establishing an 'R8 exclusion zone'. (Bottom panel). As R8 competence moves anteriorly with the sweep of the MF, cells outside the R8 exclusion zone are permitted to respond to DER signaling by establishing new R8 equivalence groups (white circles). Signaling through pathways such as *Notch* (arrows), single R8s are established. Rhomboid and Vein are expressed in the newly forming R8s and the process is repeated, propagating the pattern anteriorly.

prevent ectopic R8s (Fig. 6H). As R8 competence progresses anteriorly to cells beyond the R8 exclusion zone, new R8 equivalence groups would be permitted to form in the niches between the exclusion zones (Fig. 8, bottom panel).

As demonstrated in Fig. 8 (bottom panel), this localized Argos signaling should result in the arrays of R8s in neighboring rows being formed 'out-of-register' to each other, and this is indeed the case. In addition, loss of Argos should result in the emergence of ectopic ommatidia, and this has been observed as well (Okano et al., 1992; this paper). Therefore, the spacing between ommatidia and their overall pattern appears to depend on the number of cell diameters across which Argos normally diffuses. An analogous role for Argos in embryonic ectoderm (Golembo et al., 1996a,b) and subsequent steps of ommatidial maturation (Freeman, 1997) have been proposed. It has been estimated that Argos can exert its effects up to five cell diameters from its source (Freeman et al., 1992); neighboring proneural clusters, representing two sources of Argos, are typically separated by less than eight cell diameters.

Rhomboid and Argos

Ectopic Rhomboid proved a very rapid activator of *ras* pathway activity: the presence of dpERKA was dramatically expanded in as little as 20 minutes. In addition, loss of Rhomboid, either through expression of an antisense construct or in mutant clonal patches, greatly diminished dpERKA expression in the surrounding 10-15 cells of the proneural cluster. These results are consistent with the view that Rhomboid directly promotes release of a diffusible factor such as Spitz (Schweitzer et al., 1995a; Golembo et al., 1996a; Gabay et al., 1997). Spitz itself is expressed throughout the MF (Tio et al., 1994; Tio and Moses, 1997) and is apparently inactive in its uncleaved form (Schweitzer et al., 1995b). The presence of Rhomboid in members of the R8 equivalence group provides a mechanism to localize the source of Spitz activity, thereby delimiting the extent of the proneural cluster.

Activation of ERKA by Rhomboid, DER, or Dras1^{Val12} is also sufficient to induce expression of Argos (Golembo et al., 1996b; this paper). Upregulation of Argos expression by ectopic Rhomboid occurs very rapidly (within 1 hour), suggesting direct regulation by the *ras* pathway. Transcription targets of dpERKA such as Pointed (Brunner et al., 1994; O'Neill et al., 1994), a target of DER (Morimoto et al., 1996), could relay this signal. In keeping with the model that Argos is normally required to repress ommatidial formation between proneural clusters, reduction of *argos* activity leads to the emergence of small, ectopic ommatidia between normal ommatidia (Okano et al., 1992; this paper). However, complete loss of *argos* does not eliminate all pattern (Freeman et al., 1992). This suggests that other partially redundant factors could also contribute to setting the ommatidial pattern. Candidates for such factors include Bulge and Soba, which exhibit genetic interactions with Argos and produce similar eye phenotypes (Wemmer and Klämbt, 1995), and Scabrous, a secreted regulator of Notch (Baker et al., 1990; Powell and Cagan, unpublished data).

Does R8 establish the proneural cluster?

Previous work on specification of neurons has focused on the role of proneural cluster maturation. In this view, the neuron is created as *Notch*-mediated signals narrow expression of the bHLH transcription factor to a single cell. However, as soon as the proneural cluster can be recognized by Atonal expression, the R8 equivalence group as well as R8 itself can easily be

distinguished within it both by nuclear movements (Dokucu et al., 1996) and Rhomboid and Vein expression (Figs 4A-C, 5C,D). In addition, blocking Rhomboid function transiently eliminates both Atonal expression and dpERKA, the two factors which currently define the cluster; eliminating both Rhomboid and Vein further eliminates R8 specification itself. Finally, a single copy of the allele DER^{Elp} produces an eye with a nearly normal complement of ommatidia (Baker and Rubin, 1989), yet the proneural clusters are largely absent and Atonal first appears at a high level in the R8 equivalence group (Fig. 1F). These results support the surprising view that the R8 neuron (or its precursor) establishes the proneural cluster and not vice versa. In this model, the major role of the proneural cluster in the retina is to mediate patterning through the production of Argos and possibly other inhibitory factors. The presence of smaller equivalence groups has been suggested for other tissues in the fly (Goriely et al., 1991; Skeath and Carroll, 1992; Doe, 1992; Seugnet et al., 1997).

One consequence of the presence of Atonal in the proneural region would be to make cells competent to differentiate as R8s, and indeed blocking *Notch* activity results in differentiation of all cells within this region as R8s (Cagan and Ready, 1989a; Baker et al., 1990; Baker and Zitron, 1995). This supports the view (Muskavitch, 1994) that the role of Notch through most of the proneural region is strictly to maintain the block in neuronal (R8) differentiation. Interestingly, loss of *Notch* function results in ectopic R8s that first appear at the posterior of the proneural region and then rapidly expand anteriorly (Cagan and Ready, 1989a; Baker et al., 1990; Baker and Zitron, 1995; R. Cagan, unpublished results), further indicating that progressive rows of cells gain R8 competence as they mature following cell cycle arrest.

Conclusion

Several important questions remain to be determined. One is how this process is initiated near the anterior edge of the retinal neuroepithelium. Loss of *atonal* activity allows MF initiation but blocks its propagation, suggesting the two processes require different mechanisms (Jarman et al., 1995). Another is how events, once initiated, are coordinated with such precision across the retinal neuroepithelium. One clue comes from the order of ommatidial emergence: initiation occurs at the center ('equator') of the eye disc and then continues stepwise toward the periphery (Wolff and Ready, 1991; Baker et al., 1996; Dokucu et al., 1996). This center-out order of patterning is likely to be significant, as it simplifies the coordination of proneural cluster initiation. Recent evidence suggests that pathways involving *frizzled* and *mirror* help establish this boundary or provide patterning information from the equator outward (Zheng et al., 1995; Tomlinson et al., 1997; Strutt et al., 1997). Determining the link between these molecules and the *ras* pathway represents an important challenge. Secretion of Argos may contribute to this center-out progression: the Argos secreted between more central clusters may also contribute to refining the borders of their more peripheral neighbors.

It will be especially interesting to determine if regulation of the *ras* pathway plays a similar role in other patterning events. Mechanisms of differentiation in *Drosophila* tissue have often proved to be conserved in their vertebrate counterparts. The retina is one of the more dramatic examples of this: the establishment of retinal competent tissue requires orthologous molecules in both (Halder et al., 1995a,b). In addition, EGF receptor signaling can alter cell fates in the rat retina (Lillien, 1995) and the Atonal ortholog Xath5 promotes ganglion cells in the *Xenopus* retina (Kanekar et al., 1997). Interestingly, the antibody which recognizes phosphorylated ERKA in *Drosophila* also reacts with ERKs that are phosphorylated in immature ganglion cells of the zebrafish retina (data not shown). Although this activation does not indicate the nature of ERK's role in retinal patterning, it is intriguing that the same pathway is activated so early. One challenge will be to determine whether this phosphorylation event reflects a similarity in developmental mechanisms between these two distantly related species.

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