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# Hairy and Emc Negatively Regulate Morphogenetic Furrow Progression in the Drosophila Eye

**Nadean L. Brown,* Carol A. Sattler,† Stephen W. Paddock,* and Sean B. Carroll***
*Howard Hughes Medical Institute
and Bock Laboratories
†McArdle Laboratory
University of Wisconsin
Madison, Wisconsin 53706

## Summary

**The initial steps of pattern formation in the developing Drosophila eye involve the coordination of cell cycles, changes in cell shape, and the specification of the R8 photoreceptor cell. These events begin several cell rows ahead of the morphogenetic furrow and are positively regulated by secreted signaling proteins and the proneural HLH transcription factor atonal (ato). Two HLH regulatory proteins that function to suppress neuronal development in other tissues, extra macrochaetae (emc) and hairy (h), are expressed ahead of the morphogenetic furrow. While neither h nor emc is required for photoreceptor cell determination, in *emc*$^{-}$*h*$^{-}$ clones the morphogenetic furrow and differentiated eye field advance up to eight ommatidial rows ahead of adjacent wild-type tissue. This indicates that morphogenetic furrow progression and neuronal differentiation are negatively regulated by a combination of anteriorly expressed HLH regulatory proteins.**

## Introduction

The compound eye of Drosophila is made up of 800 ommatidia arranged in a hexagonal array and develops from the larval eye imaginal disc. The reiterated pattern of ommatidia is established within the morphogenetic furrow (MF) of the eye disc with the differentiation of neuronal photoreceptor cells (Ready et al., 1976). The MF is a dorsal–ventral indentation that traverses the eye disc from posterior to anterior during the third larval instar. Anterior to the MF, undifferentiated cells are randomly arranged and actively divide, while posterior to it, cellular differentiation and the ordered assembly of ommatidial founder cells commence (Ready et al., 1976; Tomlinson and Ready, 1987). Development in this field proceeds in a continuous temporal sequence, as each ommatidial row posterior to the furrow is approximately 1.5 hr more mature than the row directly anterior to it (for reviews, see Banerjee and Zipursky, 1990; Wolff and Ready, 1993). Changes in cell shape are coordinated with cell cycle synchronization and with the initiation of both photoreceptor cell organization and differentiation within the MF. This suggests that the timing of these processes may be regulated at multiple points.

The best-understood process of Drosophila eye development is the neuronal differentiation of photoreceptor cells that begins within the MF. Within the developing ommatidium or precluster, photoreceptor cell R8, which resides in a central position within each ommatidium and differentiates first (Tomlinson and Ready, 1987; Wolff and Ready, 1991), initiates an inductive cascade of differentiation of the photoreceptor cells R2–R5. Regularly spaced preclusters of five cells form within the MF and exit the posterior edge of the furrow, where the remaining undifferentiated cells undergo a synchronous round of mitosis. Cells born from this cell division will give rise to photoreceptor cells (R1, R6, and R7) and nonneuronal accessory cells. Some of these cells also appear to be recruited and induced to differentiate by cues sent from neighboring photoreceptor cells (reviewed by Zipursky and Rubin, 1994). The R8 photoreceptor founder cells have been hypothesized to arise similarly to sense organs in other Drosophila imaginal discs (Baker et al., 1990) that require the helix-loop-helix (HLH) transcriptional regulatory proteins encoded by the *achaete–scute* complex (Campuzano and Modolell, 1992; Ghysen et al., 1993; Jan and Jan, 1993). Indeed, Jarman et al. (1994) have demonstrated that *atonal* (*ato*), which encodes another HLH transcription factor (Jarman et al., 1993), is a proneural gene for photoreceptor cell development. *ato* function is required for R8 selection and for photoreceptor cell formation, and ato protein is expressed specifically within the MF and the differentiated photoreceptor cell R8.

Other HLH proteins also function in the development of sense organ patterns and are expressed within the developing eye. The embryonic segmentation HLH gene *hairy* (*h*) is expressed in a dorsal–ventral stripe in several rows of eye disc cells immediately anterior to the MF (Carroll and Whyte, 1989). *h* mutations affect sensory organ number and pattern and proneural gene expression in the developing wing and leg (Botas et al., 1982; Moscoso del Prado and Garcia-Bellido, 1984a, 1984b; Skeath and Carroll, 1991). No requirement for *h* function in eye development has been demonstrated, despite its provocative and evolutionarily conserved expression pattern (Brown et al., 1991; Orenic et al., 1993). Proper regulation of this gene, however, is essential for the formation of a normal eye, since ectopic h expression causes structural abnormalities and alterations in cell fate (Brown et al., 1991). *extra macrochaetae* (*emc*) lacks the HLH basic DNA-binding domain and negatively regulates *achaete*, *scute*, or *daughterless* (*da*) by dimerizing with and sequestering these positive regulators (Van Doren et al., 1991) so they are unable to bind to DNA. *emc* is expressed in a roughly complementary expression pattern to *achaete* and *scute* in the wing imaginal disc and is regulated independently of these genes (Cubas and Modolell, 1992; Van Doren et al., 1992). Interestingly, Drosophila embryonic cells undergoing morphogenetic events such as gastrulation have recently been shown to express *emc* mRNA (Cubas et al., 1994; Ellis, 1994).

Cells located within or posterior to the MF in the eye disc supply the inductive information necessary to propagate morphogenesis and the movement of the furrow anteriorly. Two secreted signaling molecules, *hedgehog* (*hh*) and *de-*

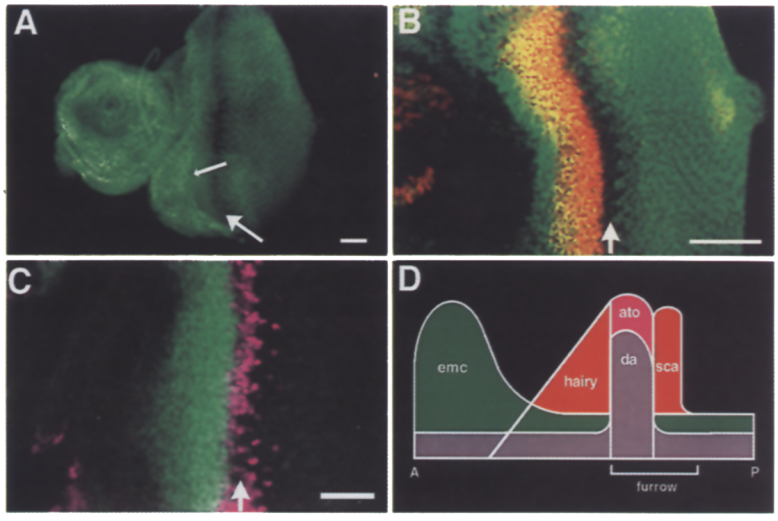


Figure 1. Spatiotemporal Expression of HLH Proteins

Confocal images of wild-type eye discs with the following antibodies: (A), rabbit anti-emc (green); (B), double label with rabbit anti-emc (green) and mouse anti-h (red); and (C), double label with mouse anti-h (green), rabbit anti-ato (pink).

(A and B) Extra macrochaetae protein is expressed nonuniformly by all cells of the eye disc, with a higher stripe of expression very anterior in the eye disc (small arrow in [A]) and lowest expression within the MF. The highest level of emc is found in cells anterior to those prefurrow cells that express h protein (B). This high level of emc protein appears to grade downward across several cell rows as h expression grades upward, as delineated in yellow and orange in the merged image in (B).

(C) The expressions of h and ato proteins border one another along the anterior edge of the MF. The h protein is expressed by all cells just prior to their entry into the MF. In the adjacent, posterior row of cells, strong expression of the proneural ato protein is first observed.

(D) Schematic diagram summarizing spatial domains of HLH protein expression anterior to and within the MF. The position of proneurally determined R8 cells is denoted by the peak of *sca* β-galactosidase expression. The spatial relationships between h and sca proteins can be found in the work of Ma et al. (1993). Anterior is to the left in all panels. Large arrows mark the relative position of the furrow in each panel.

Scale bar in (A)–(C), 50 μm.

*capentaplegic* (*dpp*) (Heberlein et al., 1993; Ma et al., 1993) are required for furrow movement and eye development. *hh*, which is expressed by cells posterior to the MF, is continuously required for the progression of the furrow and induces the expression of *dpp* within the MF. *dpp* appears to act as a positive inducer of furrow movement by signaling cells anterior to the MF. Several lines of evidence suggest that while cells anterior to the furrow are undifferentiated, they do respond to signals emanating from the MF or behind it. For example, several genes are activated or expressed in the region ahead of the furrow (Carroll and Whyte, 1989; Bonini et al., 1993). Importantly, in *hh* mutants one pattern of gene expression, the stripe of h protein expression, changes in cells ahead of the furrow (Ma et al., 1993).

In this paper, we examine the function of two negative regulators (*emc* and *h*) of proneural determination in the developing Drosophila eye. We find that emc protein expression is clearly modulated across the developing eye disc, in an anterior-to-posterior gradient ahead of the furrow and at very low levels within the furrow. Clonal analysis indicates that *emc* has a very minor role in photoreceptor cell organization. While single-mutant *h* or *emc* clones exhibit no or only subtle phenotypes, in double-mutant clones of *emc* and *h*, MF progression, neuronal development, and *ato* expression advance dramatically. These results demonstrate that these two HLH proteins act together to regulate the rate at which furrow progression and neuronal development proceed.

## Results

We have carefully analyzed the spatial domains of HLH protein expression within the temporal sequence of eye development. These protein patterns reflect a very dynamic landscape in the anterior region of the developing eye. Here, we focus on the expression and function of the emc and h proteins; additional information on the expression, function, and interactions between da and ato will be presented elsewhere (N. L. B. et al., unpublished data).

### Emc, H, and Ato Protein Expression Is Modulated across the Developing Eye

Emc protein is present in all eye disc cells but is expressed nonuniformly (Figure 1A). The highest level of emc expression (Figure 1B, green) is within the nuclei of cells in a dorsal–ventral stripe anterior to the domain of h expression (Figure 1B, red). The lowest level of emc expression occurs within the MF (large arrow in Figure 1A). Cells that express the proneural ato protein (Figure 1C, pink) within the MF form a continuous stripe that resolves rapidly to expression within the nuclei of R8 cells at the posterior edge of the furrow (Figure 1C; Figure 4 in Jarman et al., 1994). In eye discs doubly labeled with anti-ato (Figure 1C, pink) and anti-h (Figure 1C, green), we find the anteriormost expression of ato borders precisely the posterior edge of h expression, suggesting that the first cells to express ato protein are within the MF.

The expression domains of these HLH proteins demarcate regions of the eye disc that are at different temporal stages of development (Figure 1D). At the anterior edge of the prefurrow, emc protein is expressed at its highest level in a dorsal–ventral stripe and precedes that of h, which is expressed in a graded manner by all cells just before they enter the MF. Interestingly, it is within this first row of cells at the anterior side of the MF that ato (and another HLH protein, da [N. L. B. et al., unpublished data]) is first observed. Ato and da expression by all cells within the MF and within the developing R8 cell (Figure 1C; N. L. B. et al., unpublished data) suggests that the time of action for both of these genes is prior to R8 proneural

Table 1. *emc*$^{1}$ Clonal Analysis of Normally Constructed Mosaic Facets

| Photoreceptor Cell | Number of $w^{+}emc^{+}$ per Number Scored | $w^{+}emc^{+}$ (%) |
|---|---|---|
| R1 | 60/125 | 48 |
| R2 | 51/125 | 41 |
| R3 | 44/125 | 35 |
| R4 | 74/125 | 59 |
| R5 | 53/125 | 42 |
| R6 | 79/125 | 63 |
| R7 | 59/125 | 47 |
| R8 | 55/125 | 44 |

Phenotypically normal mosaic facets, 125; mutant mosaic facets, 8.

determination (marked by *scabrous* [*sca*] expression; Figure 1D).

These spatial relationships between HLH proteins are suggestive of regulatory roles or interactions during eye development. For example, the juxtaposition of ato or da expression with h expression and the expression of emc and h in the prefurrow could be due to, or reflect, regulatory interactions among HLH proteins. To examine these possibilities further, we have focused on the function of *emc* and *h* by examining eye development in patches of eye tissue lacking either one or both of these proteins.

### Loss of *emc* Function Has Only Subtle Effects on Photoreceptor Cell Development

Since the proneural antagonist *emc* gene is expressed in a dynamic pattern in the prefurrow of the eye disc, we examined *emc* function in clones by using the severe hypomorphic allele *emc*$^{1}$. This allele was utilized because stronger alleles such as *emc*$^{FX119}$ are cell lethal (Garcia Alonso and Garcia-Bellido, 1988; N. L. B., unpublished data) and because *emc*$^{E12}$, the only available amorphic allele, is a cell lethal as well as a deficiency that removes at least ten chromosomal bands (Garcia Alonso and Garcia-Bellido, 1988; Cubas et al., 1994). In both adult ($w^{-}$ cells) and imaginal disc ($lacZ^{-}$ tissue) *emc*$^{1}$ clones, we observed the occasional presence (6%; see Table 1) of either extra ectopic photoreceptor cells or photoreceptor cells with extra rhabdomeres in both wholly mutant and mosaic ommatidia (small arrows in Figures 2A and 2B). Improperly oriented but otherwise normal ommatidia were also rarely observed (arrowhead in Figure 2A).

Photoreceptor cells in normally constructed mosaic ommatidia were scored to investigate possible *emc* function in the eye. There is no requirement for *emc* function demonstrated by any photoreceptor cell (Table 1). The inappropriate neuronal cells seen in clones are most likely caused by loss of *emc* function in cells not normally fated to become photoreceptor cells (such as mystery cells). Since only subtle and infrequent phenotypes are seen in *emc*$^{-}$ clones, and because no photoreceptor cell specifically requires *emc*, it appears that this gene has only a minor function in normal eye development.

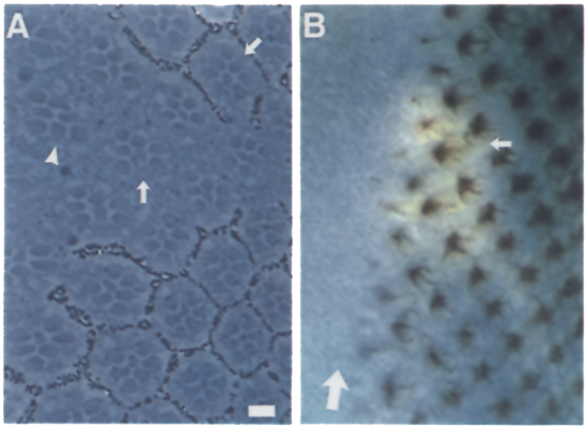


Figure 2. Loss of *emc* Has Only Subtle Effects on Eye Development

(A) Phase-contrast light micrograph of a tangential section through an adult eye containing both mutant and mosaic *emc*$^{1}$ ommatidia. Wild-type photoreceptor cells are pigmented, and homozygous *emc*$^{1}$ cells are unpigmented. Arrows denote either wholly mutant or mutant mosaic ommatidia with either an extra photoreceptor cell or a photoreceptor cell with two rhabdomeres. Rarely, ommatidia were found improperly oriented with respect to dorsal–ventral axes, anterior–posterior axes, or both (arrowhead).

(B) Third instar eye disc containing both mutant and mosaic *emc*$^{1}$ ommatidia, which have been labeled with both X-Gal (blue) and 22C10 (brown). Wild-type photoreceptor cells contain a transposable element that allows ubiquitous expression of *lacZ* from the α-tubulin promoter. The arrow points to an extra inappropriate neuronal cell present within the clone. Anterior is to the left in both panels, and the large arrow marks the position of the furrow.

Scale bar in (A), 11.8 μm; in (B), 15.8 μm.

### Negative Regulation of Neuronal Development and MF Progression

While clones of cells lacking either *h* (Brown et al., 1991; Figure 3A) or *emc* (Figures 2A, 2B, and 3B) function alone have no or only subtle phenotypes, we were provoked to investigate the phenotype of double-mutant $emc^{-}h^{-}$ clones for two main reasons. First, both *emc* and *h* function as negative regulators of proneural development in the wing disc (Botas et al., 1982; Moscoso del Prado and Garcia-Bellido, 1984a; Orenic et al., 1993). Second, the intriguing spatial patterns of expression for both proteins precede proneural determination within the MF. Two different alleles of *h*, $h^{C1}$ (a null allele), and $h^{IL79K}$ (an embryonic lethal allele with some antimorphic activity), were utilized along with *emc*$^{1}$ to produce $emc^{1}h^{C1}$ and $emc^{1}h^{IL79K}$ mutant chromosomes. Eye discs containing double-mutant clones were triple labeled with anti-Myc to mark the position of the clones, 22C10 to examine photoreceptor cell development (Zipursky et al., 1984), and rhodamine–phalloidin, a cell shape marker, to reveal the position of the MF (see Experimental Procedures).

Both $emc^{1}h^{C1}$ and $emc^{1}h^{IL79K}$ clones exhibited identical striking phenotypes in which differentiated photoreceptor cells arose in much more anterior positions than in adjacent wild-type tissue (Figures 3C and 3E; 22C10 expression is in pink, Myc epitope expression in green). Photoreceptor cell clusters within the double-mutant tissue (lacking Myc staining) appeared to be more temporally

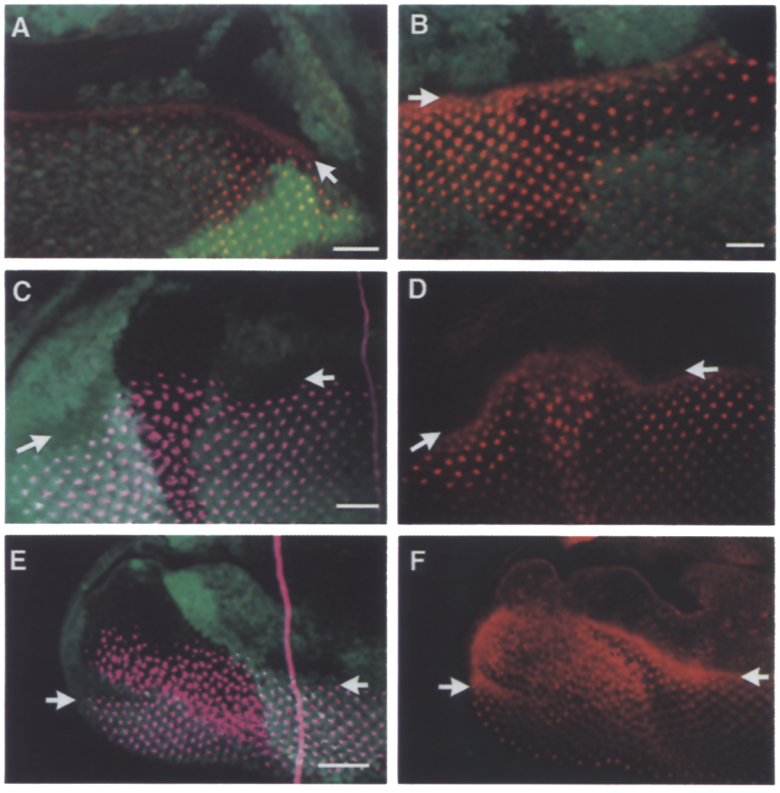


Figure 3. Derepression of MF Progression in *emc*$^{-}$*h*$^{-}$ Double-Mutant Clones

Confocal images of triple-labeled eye discs containing clones of mutant tissue. Discs were labeled with anti-Myc (green), rhodamine–phalloidin (red), and 22C10 (pink). Large arrows mark the position of the furrow in wild-type tissue. Anterior is up in all panels.

(A) Third instar eye disc containing both mutant and mosaic $h^{C1}$ tissue. This eye disc contains one large clone that spans the MF at two positions (cells that do not express the Myc epitope). Rhodamine–phalloidin (which binds to actin filaments and highlights cell shape) staining demonstrates that cell shape at and around the furrow is normal in this clone. 22C10 labeling of this disc (data not shown) shows normal neuronal development within the homozygous $h^{C1}$ clone.

(B) Eye disc containing one large $emc^{1}$ mutant clone that crosses the furrow. The position of the furrow (marked by phalloidin staining) within *emc*$^{-}$ tissue is normal. This disc was also labeled with 22C10, which illustrates occasional extra neuronal cells posterior to the MF within the clone (see Figure 3B; data not shown) but otherwise shows normal photoreceptor cell development.

(C and D) Mosaic eye disc containing an $emc^{1}$ $h^{IL79K}$ clone that spans the MF. Both neuronal photoreceptor cell development (C) and furrow progression (D) are accelerated anteriorly as compared with adjacent wild-type tissue. *emc*$^{-}$*h*$^{-}$ clones always had a curved appearance (as opposed to an abrupt, linear shape), suggesting that mutant cells may affect wild-type behavior.

(E and F) An eye disc containing a large $emc^{1}h^{IL79K}$ clone. Neuronal development is greatly advanced in contrast with the neighboring wild-type tissue (E). At least eight ommatidial rows are present more anterior to the position of differentiated R8 cells in adjacent normally developing ommatidia (arrows in E). Additionally, the furrow in the doubly mutant region of the disc has advanced ahead of its position in wild-type tissue (F).

Scale bar in (B) and (C), 25 μm; in (A) and (E), 50 μm. The specimens in (D) and (F) are the same as those in (C) and (E), respectively.

advanced in their development as well (Figures 3C, 3E, 4D, and 4E). This phenotype could arise if *emc* and *h* function together to regulate the timing of neuronal differentiation such that it does not occur anterior to the MF. In this case, loss of *emc* and *h* would lead to differentiation of competent cells within the prefurrow. Alternatively, these two genes might regulate an earlier step in eye development, such as MF movement, in which case the position of the furrow would be accelerated.

To distinguish between these possibilities, the position of the MF in eye discs containing double-mutant clones was examined. Phalloidin staining (shown in red) demonstrated that the position of the MF itself is shifted anteriorly within the double-mutant clones, well ahead of adjacent wild-type tissue (see Figures 3D and 3F). In the largest double-mutant clone obtained, at least eight rows of ommatidia form within the clone ahead of normal neighboring tissue (see Figures 3E and 3F; 22C10 expression is shown in purple, phalloidin staining in red). In addition, the expression of the proneural ato protein was also accelerated in double-mutant clones (Figures 4A and 4B; ato is shown in pink). This demonstrates that both neuronal development and the movement of the MF are progressing at a faster rate within the *emc*$^{-}$*h*$^{-}$ mutant clone than in wild-type tissue. Since the position of the MF and the temporal program of photoreceptor cell development are normal in single-mutant clones of $h^{C1}$ (red line in Figure 3A), $h^{IL79K}$ (data not shown), and $emc^{1}$ (red line in Figure 3B), which span the MF ($n \geq 5$ for each genotype), we conclude that *emc* and *h* function together to regulate the timing of furrow progression and photoreceptor development.

The phenotype of *emc*$^{-}$*h*$^{-}$ clones depended upon their position and size (Table 2). For example, the width of furrow-spanning clones had to be >3 ommatidia to perturb either furrow movement or neuronal development (Table 2). In addition, clones ahead of the furrow had no discernible effect on eye development unless they were adjacent to or partially spanned the MF. Double-mutant clones adjacent to the furrow contain ectopic neuronal cells separate from the normal eye field at their anterior edge (see pink cells at the edge of the *myc* clone in Figure 4C); however, the position of the furrow within these clones is normal, i.e., not advanced (data not shown). It appears from these results that the negative regulation of neuronal development by *emc* and *h* may be a separate function from their negative control of furrow movement.

This negative regulation is almost certainly influenced by diffusible factors produced by cells in the furrow. This may explain how narrow furrow–spanning clones can be rescued nonautonomously (i.e., by wild-type cells; Table 2). Further support for the idea that wild-type tissue may affect mutant cell behavior stems from the observation

Table 2. *emc⁻hairy⁻*[a] Double-Mutant Disc Clones

| Position of Clone in Eye Disc | Relative Size of Clone[b] | Number of Clones Analyzed | Phenotype(s) |
|---|---|---|---|
| Anterior to MF | S, M, L | 4 | No effect on furrow position or photoreceptor cell development |
| Anterior to MF[c] (immediately) | M | 5 | Ectopic neuronal cells<br>Furrow position normal |
| Span across MF[d] (>3 ommatidia wide)[e] | S, M, L | 21 | Furrow progression and neuronal development accelerated |
| Span across MF[d] (1–2 ommatidia wide)[e] | M | 5 | No effect on furrow position or photoreceptor cell development |
| Posterior to MF | S, M, L | 29 | Ommatidial composition and spacing abnormal<br>Furrow position normal |

[a] Data compiled from both *emc¹h^C1* and *emc¹h^L79K* clones.
[b] Clones were compared with each other to assign a size value of small (S), medium (M), or large (L).
[c] These clones appear to border but not overlap the MF.
[d] Clones spanning across the MF also include mutant tissue in adjacent anterior and posterior regions of disc.
[e] Width of clones is defined as number of wholly mutant ommatidia present in the clone in the dorsal–ventral dimension at the MF.

that in double-mutant clones that cross the MF, ommatidial development is accelerated across only a portion of the prefurrow and does not travel all the way to the anterior margin of the eye field (see the anterior portion of clones in Figures 3C and 3E). Lastly, *emc⁻h⁻* clones anterior to the furrow only exhibit ectopic neuronal cells when the clone is immediately adjacent to the MF (Figure 4C); perhaps this is because they lie within the range of diffusible substances produced within the MF.

Clones located posterior to the MF within the field of differentiated ommatidia had both abnormal spacing (see spacing of pink clusters in the *myc* clone in Figure 4D) and abnormal numbers of photoreceptor cells (extra pink cells in ommatidial clusters in Figure 4E). Confocal optical sections through eye discs containing strictly posterior *emc⁻h⁻* clones suggested that mutant ommatidia were also arranged abnormally in the apical–basal plane (N. L. B., unpublished data). At the border of double-

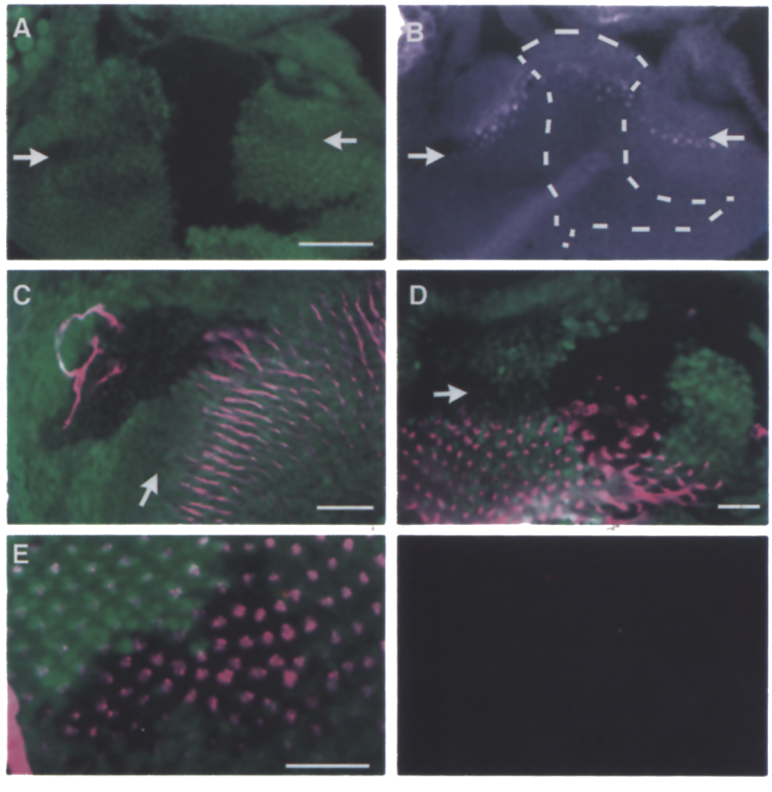


Figure 4. Ectopic and Accelerated Neuronal Development in *emc⁻h⁻* Clones

Confocal images of eye discs containing double-mutant clones and labeled with anti-Myc (green), 22C10 (pink), or anti-ato (purple). Large arrows mark the position of the MF in wild-type tissue, and anterior is to the left in (C) and at the top in all other panels.

(A and B) *emc¹h^C1* clone labeled with anti-Myc (A) and anti-ato (B). In this large clone, the expression of the proneural ato protein is advanced ahead of its position in adjacent wild-type tissue. The dotted line in (B) marks the position of the edge of the clone.

(C) *emc¹h^C1* clone immediately anterior to and just overlapping the MF. In the most anterior region of the clone, ectopic neuronal development has occurred. This basal optical section through the eye disc clearly demonstrates the presence of inappropriate 22C10-positive cells that appear to be clustering together. Note that the differentiated mutant photoreceptor cells within the MF (arrow) arise ahead of neighboring wild-type cells.

(D) *emc¹h^L79K* double-mutant disc clone centered around the furrow. Within the mutant tissue photoreceptor cell clustering and ommatidial spacing (as highlighted by 22C10 expression) are abnormal.

(E) *emc¹h^C1* mosaic eye disc containing a double-mutant clone located posterior to the MF. The position of the furrow is off the top edge of this panel. Ommatidia are spaced abnormally within the clone and at the interface between wild-type and mutant ommatidia. The composition of mutant ommatidia appears abnormal as well (also see [D]) and may reflect spacing defects, abnormal neuronal differentiation, aberrant cell positioning, or a combination of these features.

Scale bar in (C)–(E), 25 μm; in (A), 50 μm ([B] shows the same specimen as [A]).

mutant and wild-type tissue (where green Myc-expressing cells and unstained cells meet in Figure 4E), the interface of normal and $emc^{-}h^{-}$ ommatidia was irregular. The phenotypes observed within posterior $emc^{-}h^{-}$ clones suggest that this portion of the developing eye disc has developed at a different rate than the adjoining wild-type tissue.

## Discussion

The developing Drosophila eye has become a powerful model for the study of such diverse processes as cell cycle synchronization (Thomas et al., 1994), receptor-mediated signal transduction (reviewed by Zipursky and Rubin, 1994), cell polarity and rotation (Choi and Benzer, 1994), proneural determination (Jarman et al., 1994), and morphogenesis (Heberlein et al., 1993; Ma et al., 1993). All of these events are components of a developmental program deployed in temporal sequence within the eye imaginal disc. Two manifestations of this program are the progression of the MF and the invariant sequence of photoreceptor cell differentiation within each ommatidium. We have shown that the expression of the three HLH proteins emc, h, and ato reveals a dynamic landscape of HLH gene expression anterior to and within the MF (see summary diagram in Figure 1D). Despite their subtle or absent single-mutant phenotypes, *h* and *emc* are necessary for normal eye development. These two proteins act in concert to regulate negatively the rate of both MF progression and neuronal differentiation of photoreceptor cells.

### The Rate of Furrow Progression Is Regulated

The premature neuronal development and advanced position of the MF within $h^{-}emc^{-}$ double-mutant clones were entirely unexpected because of no or only slight single-mutant phenotypes. The manner in which *emc* and *h* may regulate neuronal development and furrow progression is not clear. Emc can bind in vitro to several proneural proteins, such as achaete, scute, and da, and form nonfunctional DNA-binding heterodimers (Van Doren et al., 1991, 1992), while h has been recently shown to bind to regulatory sequences in the *achaete* gene (Ohsako et al., 1994; Van Doren et al., 1994). Since *emc* and *h* employ different mechanisms of gene regulation, these two genes may regulate furrow progression and neuronal development by acting on the same target (perhaps redundantly) or on different targets (in which case the double-mutant phenoype might be a synergistic effect).

### What Are the Targets of Negative Regulation?

Five cellular processes are regulated as cells enter the MF: the cell cycle is synchronized, cell shape changes are coordinated, changes in gene expression occur, cell fates are specified, and neuronal differentiation is initiated (see Figure 5). It is likely that one or more of these processes are negatively regulated by *emc* and *h*. In the double-mutant clones, ato expression shifts anteriorly (Figure 4B), suggesting that *h* and *emc* may regulate ato to prevent proneural determination (and subsequently neuronal differentiation) from occurring prematurely ahead of the furrow. In addition, because $emc^{-}h^{-}$ prefurrow cells can differentiate ahead of adjacent wild-type cells, they may enter the G1 phase of the cell cycle inappropriately. This suggests that *emc* and *h* may directly or indirectly regulate the expression of anteriorly expressed cell cycle genes such as *roughex* or *string*. This idea is supported by recent evidence that the vertebrate *emc* homolog Id (which also lacks a basic DNA-binding protein domain) is required for the G1 progression of cultured 3T3 fibroblast cells (Peverali et al., 1994).

Another possible target for *emc* and *h* is the regulation of the posterior-to-anterior induction signal (Figure 5). This could be accomplished either by preventing inappropriate *dpp* expression in cells anterior to the furrow or by regulating the expression of genes that act in the reception or interpretation of the *dpp* signal. In this scenario, the expression pattern of either *dpp*, its receptor, or some other downstream gene may spread anteriorly in $emc^{-}h^{-}$ clones. Lastly, prefurrow cells within double-mutant clones must prematurely change shape when the rate of furrow movement is accelerated. It is not known whether cell shape changes occur independently or as a consequence of prefurrow events such as cell cycle synchronization or the reception of the *dpp*-inducing signal. Until more is known about the relative independence of cell shape changes, it may be difficult to test whether *emc* and *h* regulate this particular process directly. It should be noted that in *ato* mutants, the MF still exists (Jarman et al., 1994; N. L. B.,

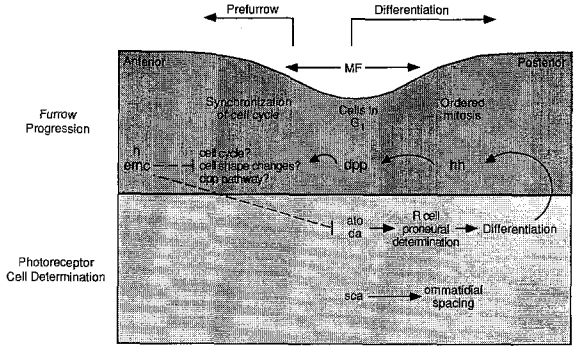


Figure 5. Model for Negative Regulation and Positive Signaling of Furrow Progression

Schematic of developing eye imaginal disc and the regulatory pathways guiding neuronal development and MF movement. Anterior is to the left, and the furrow is moving toward the left. The secreted molecule *hh* is produced by differentiated photoreceptor cells and induces the expression of *dpp* within the MF. *dpp*, in turn, diffuses anteriorly (most likely to cells immediately anterior of the MF that are already competent to respond to the *dpp* signal) to participate in furrow propagation. Prefurrow cells enter the furrow and initiate photoreceptor cell proneural determination, which requires both the *ato* and *da* gene products (Jarman et al., 1994; N. L. B. et al., unpublished data). Negative regulation of furrow progression and photoreceptor cell neuronal development is provided by the combined function of the *emc* and *h* HLH transcription factors. The target(s) of this negative regulation may be genes that participate in the synchronization of cell cycle, changes in cell shape, the anteriorly directed *dpp* signaling pathway, the proneural genes *ato* and *da*, or a combination of these.

unpublished data), suggesting that proneural determination and cell shape changes are separable processes.

## Eye Morphogenesis

Two general models for the progression of eye development have been proposed. One contends that cells within the prefurrow possess an inherent competence to become ommatidia without inductive instruction from posterior, differentiated cells (Bodenstein, 1953; Nowel and Shelton, 1980; Lebovitz and Ready, 1986). In a simple view of this model, a prefurrow factor would be expressed in a decreasing anterior-to-posterior gradient across the eye. Cells immediately ahead of the furrow would then respond to a certain level of this factor and become poised to enter the MF and execute their developmental program across the eye. Supporting evidence for this model has come from Lebovitz and Ready (1986). They transplanted and cultured eye disc fragments lacking the MF and the posterior patterned region of the disc in larval hosts. Recovered disc fragments contained differentiated photoreceptor cells in a normally arranged ommatidial pattern. Although the emc protein pattern would fit with the anterior factor of this first model, the inability of mutations in this gene to affect normal eye development globally suggest that this is not the case.

In the second model, differentiated cells posterior to the furrow induce and instruct undifferentiated cells anterior to the MF (White, 1963; Shelton and Lawrence, 1974; Ready et al., 1976), and this is supported by studies of *hh* and *dpp* function in the eye (Heberlein et al., 1993; Ma et al., 1993). Additional evidence for the second model has recently come from the examination of ectopic *hh* expression in the developing eye disc. Heberlein et al. (unpublished data) have observed that when prefurrow cells are induced to express *hh*, ectopic *dpp* expression and ectopic furrow formation and progression occur, suggesting that *hh* function is sufficient to coordinate the progressive development of the eye. Interestingly, both ato and h protein expression are induced by ectopic *hh* (Heberlein et al., unpublished data); i.e., both positive and negative (h) regulators of eye development are activated by the *hh* signal.

The data presented in this paper suggest a picture of eye development that combines some features from each model. The observation that two prefurrow genes, *emc* and *h*, function together to regulate the timing of furrow progression and neuronal development strongly implicates the regulation of prefurrow cell processes in the progressive development of the eye. The identification of posteriorly produced signaling molecules (*hh* and *dpp*) and the results presented here demonstrate that cells on both sides of the MF (as well as those within it) execute portions of a continuous developmental program rather than cells on one side or the other of the furrow solely controlling this program. That is, the progressive development of the eye is regulated by a circular pathway (Figure 5) instead of being strictly anterior or posterior in directionality. The minimal role of genes expressed in the prefurrow appears to include the regulation of the rate of furrow propagation and neuronal differentiation, as well as cell cycle synchronization.

## Experimental Procedures

### Drosophila Stocks

Flies homozygous for $P(w^+)62A$ were a gift from T. Laverty, of the laboratory of G. Rubin. $emc^1$ flies were provided by J. Posakony, $h^{IL79K}$ flies were provided by E. Wieshaus, and $h^{C1}$ flies were provided by D. Ish-Horowicz. The X-linked heat-shock promoter–FLP recombinase insertion was described by Golic and Linquist (1989). Flies containing the third chromosome FRT (for FLP recombinase target) insertion at chromosome band 80B (*80-w*$^+$), or the cell-marking Myc epitope and 80B FRT insertions (*3L 80-πM*) were obtained from the Bloomington Drosophila Stock Center. $emc^1h^{C1}$ and $emc^1h^{IL79K}$ double *cis*-mutant stocks were made by using standard recombination methodology. Flies containing the X-linked heat-shock promoter–FLP recombinase insertion $w^{1118}P(hs\ FLP1)$; and the α-tubulin/β-galactosidase (α-tub/β-gal) transgene 3L were the gift of T. Orenic.

### *emc* Mosaics

Heterozygous flies for adult mosaic analysis were generated by crossing $w^-$; $emc^1$/*TM3 Sb* males to $w^{1118}$; $P(w^+)62A$ females and X-irradiating (1200 R) 24- to 36-hour-old progeny with a Philips X-ray machine. Clones were produced at a frequency of 1 in 150 flies. Imaginal disc mosaic analysis was performed by generating $w^-$; $emc^1$ *FRT/TM6 Tb* flies and crossing males to $w^{1118}P(hsFLP1)$; *P(α-tub/β-gal) 3L FRT* females. FLP recombinase was induced by subjecting first instar progeny to a 60 min heat shock. Third instar eye discs were fixed and stained with X-Gal to screen for the presence of mutant clones. Those discs containing clones were then stained with 22C10 (see below for details).

### Generation of $emc^1$, $h^{C1}$, $h^{IL79K}$, $emc^1h^{C1}$, and $emc^1h^{IL79K}$ Clones Spanning the MF

Virgin females of the genotype *80πM* (Xu and Rubin, 1993) were mated separately with males of these five genotypes. First instar progeny were subjected to 4000 R of γ-irradiation to induce mitotic recombination (see Blair, 1992, for details). Tb$^+$ third instar larvae were heat-shocked to induce the Myc epitope tag, dissected, fixed, and stained with the anti-Myc and 22C10 antibodies and rhodamine-conjugated phalloidin. Clones were generated at a frequency of 1:20 for each genotype, and clones that crossed the MF were observed at an approximate frequency of 1 in 200 discs.

### Immunohistochemistry

The expression of β-galactosidase in imaginal discs was assayed by an activity stain using the substrate X-Gal (Ghysen and O'Kane, 1989). After overnight staining with X-Gal at 25°C, imaginal discs were rinsed in 0.1 M phosphate buffer (pH 7.2) and immediately blocked for 1 hr by use of the modified staining protocol of Brown et al. (1991). Antibody staining of X-Gal-stained discs with 22C10 was performed with the same protocol. Eye imaginal discs were stained with antibodies against emc, ato, h, 22C10, or Myc epitope tag by use of the buffers and incubations described by Carroll and Whyte (1989) and the multiple labeling strategy outlined by Paddock et al. (1993). Double-labeling experiments using two monoclonal antibodies were carried out by using sequential overnight incubation at 4°C (for example, primary antibody number 1 overnight, biotinylated secondary antibody, streptavidin–fluorescein isothiocyanate [FITC] teritiary antibody, primary antibody number 2 overnight, fluorochrome-conjugated secondary antibody; see Paddock et al., 1993, for details). Polyclonal antibodies against ato (1:5000) and emc (1:1000) were the gift of A. Jarman and Y. N. Jan. The monoclonal antibody 22C10 (1:200) was provided by the laboratory of S. Benzer. The anti-human c-Myc hybridoma line 1–9E10.2 was obtained from the American Type Culture Collection, and hybridoma culture supernatant was used at a dilution of 1:5. Polyclonal (1:200) and monoclonal (1:5) anti-h antibodies have been described previously (Brown et al., 1991; Paddock et al., 1993). Rhodamine–phalloidin (Molecular Probes, Incorporated) was included with the last antibody incubation step of disc clone experiments at a concen-

tration of 250 nM. Following an incubation of 3 hr at 4°C, disc complexes were washed as described by Carroll and Whyte (1989). Confocal images were collected with a Bio-Rad MRC600 laser scanning confocal microscope (Bio-Rad Microscience Division, Hercules, CA) and Adobe Photoshop 2.5 (Adobe Systems, Mountain View, CA) using a Macintosh Quadra 950 microcomputer and methods described previously (Paddock et al., 1993).

### Histology

Adult Drosophila heads were fixed and embedded in Eponate resin as described by Brown et al. (1991). Sections of 0.5 μm were mounted in Polymount medium (Polysciences) and viewed under phase-contrast optics on a Zeiss Axiophot microscope.

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**Note Added in Proof**

The data referred to throughout as Heberlein et al., unpublished data, are now in press: Heberlein, U., Singh, C. N., Luk, A. Y., and Donohoe, T. J. (1995). *hedgehog* coordinates growth and differentiation in the *Drosophila* eye. Nature, in press.