

Induction and autoregulation of the anti-proneural gene *Bar* during retinal neurogenesis in *Drosophila*

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Summary

Neurogenesis in *Drosophila* eye imaginal disc is controlled by interactions of positive and negative regulatory genes. The basic helix-loop-helix (bHLH) transcription factor Atonal (Ato) plays an essential proneural function in the morphogenetic furrow to induce the formation of R8 founder neurons. Bar homeodomain proteins are required for transcriptional repression of *ato* in the basal undifferentiated retinal precursor cells to prevent ectopic neurogenesis posterior to the furrow of the eye disc. Thus, precise regulation of Bar expression in the basal undifferentiated cells is crucial for neural patterning in the eye. We show evidence that Bar expression in the basal undifferentiated cells is regulated by at least three different pathways, depending on the developmental time and the

position in the eye disc. First, at the time of furrow initiation, Bar expression is induced independent of Ato by Hedgehog (Hh) signaling from the posterior margin of the disc. Second, during furrow progression, Bar expression is also induced by Ato-dependent EGFR (epidermal growth factor receptor) signaling from the migrating furrow. Finally, once initiated, Bar expression can be maintained by positive autoregulation. Therefore, we propose that the domain of Bar expression for Ato repression is established and maintained by a combination of non autonomous Hh/EGFR signaling pathways and autoregulation of Bar.

Key words: Bar homeodomain protein, Atonal, Hedgehog, Retinal neurogenesis, *Drosophila* eye

Introduction

A fundamental question in development of the nervous system is how neurogenic regions are determined and how neurons are generated from the presumptive neural tissues. Neural patterning in *Drosophila* is established by spatiotemporal regulation of proneural and anti-proneural genes. Proneural genes promote generation of specific neurons from a population of uncommitted cells, whereas anti-proneural genes inhibit this process. Clusters of photoreceptor cells in the *Drosophila* compound eye develop in a highly stereotypic and repetitive fashion from the retinal epithelium. Thus, *Drosophila* eye has been used as an excellent model for studying genetic basis of neural patterning and cell fate specification.

The adult eye consists of about 800 unit eyes or ommatidia, each of which harbors eight photoreceptor (R1-R8) cells (Ready et al., 1976; Wolff and Ready, 1993). Retinal neurogenesis takes place in a specific region of the developing eye called the morphogenetic furrow, which is generated at the posterior margin of the disc and progresses anteriorly (Heberlein et al., 1993; Ma et al., 1993; Dominguez and Hafen, 1997; Borod and Heberlein, 1998; Greenwood and Struhl, 1999; Curtiss and Mlodzik, 2000). The bHLH transcription factor Ato plays a key role in the furrow to initiate retinal neurogenesis (Jarman et al., 1994). Ato expression is dynamically regulated in the furrow (Frankfort and Mardon,

2002). Ato is expressed as a stripe pattern across the disc in the anterior region of the furrow (stage 1). Posterior to the stripe, Ato expression is restricted to about 20 cell groups called intermediate groups followed by R8 equivalence groups of two or three cells (stages 2/3). Finally, Ato expression is restricted to evenly spaced single cells that become R8 founder neurons (stage 4). After R8 selection, Ato-positive (Ato⁺) R8 cells sequentially recruit other photoreceptors from surrounding undifferentiated cells to form clusters of eight photoreceptors (Ready et al., 1976; Wolff and Ready, 1993).

Interestingly, positions of cell nuclei in the developing eye imaginal disc are dynamically regulated during retinal differentiation (Wolff and Ready, 1993). Prior to the furrow initiation or in undifferentiated region anterior to the furrow, nuclei of cells are randomly distributed throughout the depth of the disc epithelium. As the furrow progresses anteriorly, nuclei of the cells within the furrow sink and stay in the basal region of the disc. Posterior to the furrow, nuclei of cells that become photoreceptor precursors migrate apically, while those of undifferentiated cells stay in basal region of the disc, resulting in the formation of two distinct layers of nuclei (Fig. 1A). Consistent with this nuclear migration during retinal differentiation, nuclear positions of Ato-expressing cells near the furrow are also tightly regulated depending on its expression stages. The nuclei of Ato-expressing cells locate basally during stage 1 and 2 expression but migrate apically

during stage 3 and remain near the apical surface of the eye disc throughout stage 4 expression (Frankfort and Mardon, 2002).

An important aspect of Ato proneural function during retinal neurogenesis is the transient and restricted pattern of Ato expression. Ato is expressed only during the crucial time of proneural patterning and R8 founder selection at or near the furrow, and subsequently repressed. This repression of Ato is crucial for maintaining the precisely spaced pattern of ommatidial arrays established posterior to the furrow and therefore needs to be highly regulated during retinal differentiation. A recent study has provided evidence that the repression of *ato* posterior to the furrow is regulated by the *Bar* genes (Lim and Choi, 2003).

Bar class homeodomain proteins are evolutionarily conserved and have been implicated in cell-fate specification and neuronal differentiation in *Drosophila* as well as other species (Higashijima et al., 1992; Jones et al., 1997; Saito et al., 1998; Bulfone et al., 2000; Patterson et al., 2000; Lim and Choi, 2003; Saba et al., 2003; Mo et al., 2004; Poggi et al., 2004). A pair of redundant *Drosophila* *Bar* proteins encoded by two adjacent genes *BarH1* and *BarH2* (hereafter '*Bar*' for both) are specifically expressed in the nuclei of R1/6 photoreceptor neurons and are required for their differentiation (Fig. 1A) (Higashijima et al., 1992), although this requirement appears to be partial (J.L. and K.-W.C., unpublished). Importantly, *Bar* shows a specific expression pattern in the basal undifferentiated retinal precursor cells (hereafter 'basal undifferentiated cells') posterior to the furrow (Fig. 1A). Intriguingly, the anterior boundary of *Bar* expression in the basal undifferentiated cells is juxtaposed to the posterior boundary of Ato expression in the furrow, resulting in a complementary expression pattern across the eye disc along the furrow (Fig. 1B) (Lim and Choi, 2003). Loss of *Bar* in the undifferentiated basal cells results in ectopic *ato* gene expression and ectopic photoreceptor clusters, indicating that *Bar* proteins are essential for transcriptional repression of *ato* and thus for prevention of ectopic retinal neurogenesis posterior to the furrow (Lim and Choi, 2003).

Because *Bar* genes play an essential role in the negative control of *ato* proneural gene, the expression of *Bar* must be precisely regulated in coordination with the initiation and progression of retinal differentiation in the developing eye disc. Hence, the identification of signaling factors that regulate *Bar* expression in the basal undifferentiated cells is important to understand how the complementary expression domains of Ato and *Bar* proteins are established and maintained during eye morphogenesis.

We have addressed this question of *Bar* gene regulation and show that levels of *Bar* protein in the basal undifferentiated cells posterior to the furrow are dynamically regulated by multiple mechanisms during retinal differentiation. First, Hh signaling from the posterior margin induces the initial *Bar* expression at the posterior region of the disc at the early third instar stage. Second, during furrow migration, Ato-mediated EGFR activation in the furrow is required for the induction of *Bar* expression right posterior to the furrow. Finally, *Bar* expression can be positively autoregulated, which may be necessary for maintaining an even level of *Bar* expression from the posterior margin to the region right behind the furrow. Thus, the induction and maintenance of *Bar* expression in the

basal undifferentiated cells are tightly linked to the mechanism of furrow initiation and progression.

Materials and methods

Drosophila stocks

The following mutant and transgenic flies were used in this study: *lz^{r15}* (Daga et al., 1996), *ato¹* (Jarman et al., 1994), *egfr^{CO}* (Lesokhin et al., 1999), *smo³* (Chen and Struhl, 1998) and *UAS-BarH1^{M13}*, *UAS-BarH2^{F11}*, *Bar^{P058}-lacZ* (Sato et al., 1999). Other strains are described at FlyBase (www.flybase.org).

Misexpression and generation of loss-of-function (LOF) mosaic clones

Progeny flies from the cross between *UAS-BarH1^{M13}* (or *UAS-BarH2^{F11}*) females and *Bar^{P058}-lacZ*; *decapentaplegic (dpp)-GAL4/+* males were cultured at 25°C until dissection at the third instar larval stage. LOF clones were generated by the FLP/FRT system (Xu and Rubin, 1993). First instar larvae of the following genotypes were heat-shocked for 1 hour at 37°C and then incubated at 25°C until dissection: (1) *lz* LOF clones were obtained in *lz^{r15} FRT18A/Ubi-GFP FRT18A;hs-FLP3*; (2) *ato* LOF clones were obtained in *ey-FLP; ato¹ FRT82B/Ubi-GFP FRT82B*; (3) *egfr* LOF clones were obtained in *yw; hs-FLP; egfr^{CO}, GMR-P35 FRT42D/arm-lacZ, M(2)561 FRT42D*; and (4) *smo* LOF clones were obtained in *hs-FLP; smo³ FRT40A/arm-lacZ FRT40A*.

Immunocytochemistry

Third instar eye imaginal discs were dissected in phosphate-buffered saline (PBS) on ice, fixed in 2% paraformaldehyde-lysine-periodate fixative and stained as described (Carroll and Whyte, 1989). The following primary antibodies were used in this study: mouse anti-β-gal (1:250; Promega), mouse anti-Elav [1:10; Developmental Studies Hybridoma Bank (DSHB)], mouse anti-GFP (1:200; Upstate biotechnology), mouse anti-Lz (1:500; DSHB), mouse anti-dpERK (1:250; Sigma), rabbit anti-Ato (1:5000) (Jarman et al., 1995), rabbit anti-BarH1 (1:100) (Higashijima et al., 1992), rabbit anti-GFP (1:2000; Molecular Probes), guinea pig anti-Dlg (1:1000; provided by P. Bryant) and guinea pig anti-Ato (1:1000) (Hassan et al., 2000). Secondary antibodies were anti-mouse-CY3, anti-mouse-fluorescein isothiocyanate (FITC), anti-rabbit-CY3, anti-rabbit-FITC and anti-guinea pig-CY5 (Jackson Immunochemicals). Fluorescent images were scanned using Zeiss LSM laser-scanning confocal microscope and processed with Adobe Photoshop.

Results

Lozenge and Glass are required for *Bar* expression in R1/6 photoreceptors but not in the basal undifferentiated cells

To identify activators of *Bar* expression in the basal undifferentiated cells, we first focused on two different transcription factors Lozenge (Lz) and Glass (Gl) as candidates. Both proteins are known to be required for normal *Bar* expression in R1/6 photoreceptor cells (Higashijima et al., 1992; Daga et al., 1996), but it has not been demonstrated whether they are also required for *Bar* expression in the basal undifferentiated cells.

Lz is expressed in R1, 6 and 7 photoreceptor cells and is required for normal level of *Bar* expression in R1/6 cells (Daga et al., 1996; Flores et al., 1998). In the basal undifferentiated cells, Lz is co-expressed with *Bar* in a majority of *Bar*-expressing cells (Fig. 1C, white arrow), except in a group of cells just posterior to the furrow (Fig. 1C, yellow arrow). To

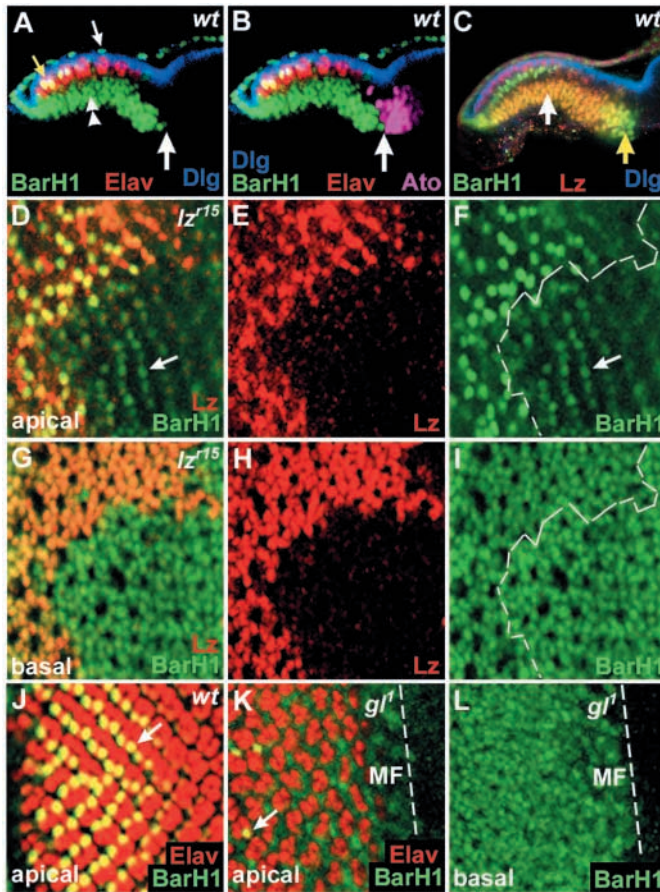


Fig. 1. Bar expression in the basal undifferentiated cells and R1/6 photoreceptor cells is independently regulated. Antibodies used for staining are as indicated in each panel with matched colors in this and all subsequent figures. Posterior is towards the left and dorsal is upwards in all discs unless mentioned otherwise. (A) Two tier nuclei layers in the longitudinally sectioned eye disc. Photoreceptor nuclei stained by Elav (red) localize apically, whereas undifferentiated cell nuclei stained by BarH1 (green) localize in the basal region of the disc. R1/6 photoreceptors (yellow arrow) are labeled by both Elav and BarH1 antibodies. Bar is also expressed in peripodial cells (small white arrow) and basal undifferentiated cells (double arrowheads) behind the furrow (marked by large white arrow).

(B) Complementary expression patterns of Bar and Ato in the basal undifferentiated cells along the furrow (marked by arrow). Two confocal images are combined to illustrate the Bar-Ato pattern. (C) Co-expression of Bar and Lz in a majority of basal undifferentiated cells (marked by white arrow) except in some Bar-expressing cells immediately posterior to the furrow (marked by yellow arrow). An eye disc is longitudinally sectioned. (D-I) Lz is not required for Bar expression in the basal undifferentiated cells. A *lzr¹⁵* LOF clone is identified by the absence of Lz staining (D,E,G,H; red) and marked with broken lines in F,I. Bar expression is downregulated in R1/6 cells (D-F, arrows), but not in the basal undifferentiated cells (G-I) within the *lzr¹⁵* LOF clone. (J) Bar is expressed in R1/6 cells in the apical photoreceptor level. An arrow indicates Bar expression in R1/6 cells of wild-type eye disc. (K,L) *Gl* is not required for Bar expression in the basal undifferentiated cells. A *gl¹* mutant eye disc stained with antibodies for Elav (red) and BarH1 (green) is shown at the apical photoreceptor nuclei level (K) and the basal undifferentiated cell level (L). Bar expression in most R1/6 photoreceptors is strongly downregulated or absent, except a few cells marked by an arrow (K), while its expression in the basal undifferentiated cells is not much changed (L). Broken lines in K,L mark the position of morphogenetic furrow; Bar is expressed only behind the furrow.

test whether Lz is also required for Bar expression in the basal undifferentiated cells, we examined Bar expression in homozygous *lzr¹⁵* mutants (data not shown) and loss-of-function (LOF) clones of *lzr¹⁵*, a null allele of *lz* (Daga et al., 1996) (Fig. 1D-I). We found that the expression level of Bar was strongly decreased but not completely eliminated in R1/6 photoreceptor cells within *lzr¹⁵* mutant clones (Fig. 1D-F), consistent with the previous report (Daga et al., 1996). However, Bar expression in the basal undifferentiated cells was little changed compared with its expression level in adjacent wild-type cells (Fig. 1G-I). These results suggest that Lz is necessary to activate Bar expression in R1/6 cells, but not in the basal undifferentiated cells behind the furrow.

Next, we tested whether *Gl*, a zinc-finger protein expressed in all cells posterior to the furrow (Moses and Rubin, 1991), is required for Bar expression in the basal undifferentiated cells (Fig. 1K,L). *Gl* was not necessary for Bar expression in the basal undifferentiated cells although it was essential for Bar expression in R1/6 photoreceptor cells (Fig. 1K,L) (Higashijima et al., 1992). Taken together, these results suggest that Bar expression requires other activators in the basal undifferentiated cells.

Bar expression in the basal undifferentiated cells depends on nonautonomous signals from the posterior margin

To identify the genes involved in activating Bar expression in the basal undifferentiated cells, we examined where this activator(s) is required in the developing eye disc. This

factor(s) may be expressed in the Bar-expressing cells for cell-autonomous activation of Bar expression in the basal undifferentiated cells. Alternatively, it may be expressed in differentiating photoreceptor cells and secreted to induce nonautonomous Bar expression in the basal undifferentiated cells. To test whether Bar expression in the basal undifferentiated cells depends on differentiating cells, we examined Bar expression in *ato¹* mutant eye disc. Ato protein encoded by *ato¹* is non-functional due to a mutation in the DNA-binding domain and thus fails to induce neural differentiation (Jarman et al., 1994; Jarman et al., 1995).

Morphogenetic furrow can be formed and progresses anteriorly to a certain distance in the *ato¹* mutant eye disc, although retinal differentiation fails to occur (Fig. 2A-C) (Jarman et al., 1994; Jarman et al., 1995). Even in *ato¹* mutant eye disc, we detected Bar expression posterior to the Ato stripe expression (Fig. 2D-F; more than 20 discs observed). This suggests that Bar expression depends on some activator(s) produced from non-neuronal cells as no photoreceptors are generated in *ato¹* mutant eye disc (Jarman et al., 1994; Jarman et al., 1995). Interestingly, Bar expression level in *ato¹* mutant was high near the posterior margin of eye disc but significantly decreased near the furrow (Fig. 2F). This suggests that Bar expression depends on a molecule secreted by non-neuronal cells in the posterior margin, and its concentration becomes limited in the region near the furrow as it progresses anteriorly from the posterior margin (Fig. 2F).

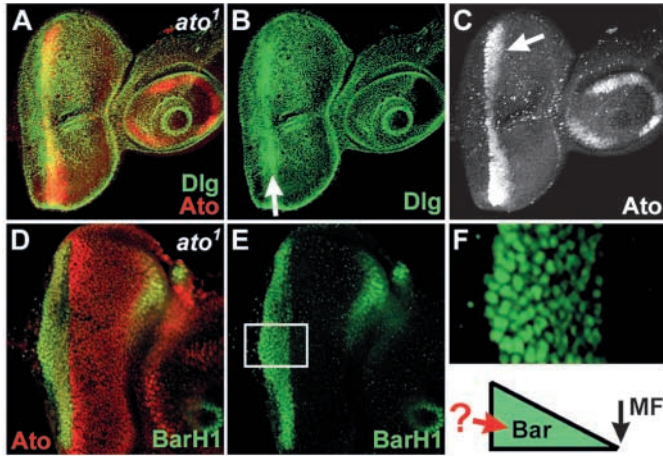


Fig. 2. Bar expression in the basal undifferentiated cells depends on a posterior secreted signal. (A-C) Morphogenetic furrow can be formed and progresses in *ato*¹ mutant eye disc. The furrow is shown by apical membrane constriction intensely stained by Dlg membrane marker (B, arrow) and by Ato expression (C, arrow) in the middle of *ato*¹ mutant eye disc. (D-F) A graded Bar expression in *ato*¹ mutant eye disc. Bar is expressed posterior to the furrow in *ato*¹ mutant eye disc. Bar expression level is high near the posterior margin but decreases significantly near the furrow, suggesting that Bar expression may depend on a secreted factor(s) from the posterior margin (F). An area marked with a rectangle in E is magnified in F. MF, morphogenetic furrow.

Hh signaling is required for initial Bar expression

We reasoned that this posterior activator(s) for Bar expression may be Hh because it is expressed and secreted from the posterior margin at the time of furrow initiation (Heberlein et al., 1993; Ma et al., 1993; Dominguez and Hafen, 1997; Borod and Heberlein, 1998; Curtiss and Mlodzik, 2000). After furrow initiation, photoreceptors generated behind the furrow secrete Hh anteriorly and thus allow the furrow to progress further anteriorly (Heberlein et al., 1993; Ma et al., 1993; Dominguez and Hafen, 1997; Borod and Heberlein, 1998; Greenwood and Struhl, 1999; Curtiss and Mlodzik, 2000). During this process, Hh is also essential for the initial pattern of Ato stripe expression in the furrow regulated by 3'-regulatory element of *ato* (Borod and Heberlein, 1998; Sun et al., 1998; Dominguez, 1999). In *ato*¹ mutant, no Hh expression can be detected in the eye disc proper posterior to the furrow, owing to the lack of photoreceptor differentiation, but Hh expression from the

posterior margin still takes place (Borod and Heberlein, 1998). Therefore, Hh secreted by the posterior margin might be responsible for a graded Bar expression as well as furrow initiation and Ato stripe expression in *ato*¹ mutant eye disc (Fig. 2).

To test whether Hh signaling from the posterior margin plays a role for initial Bar expression in the basal undifferentiated cells, we generated LOF clones of *smoothened* (*smo*), which is an essential component for the transduction of Hh signaling (Alcedo et al., 1996; van den Heuvel and Ingham, 1996; Strutt and Mlodzik, 1997). We examined Bar expression within *smo* LOF clones generated adjacent to the posterior margin of the disc (Fig. 3A-D; more than 20 clones observed). Bar expression was strongly reduced or absent within the relatively large *smo* LOF clone (Fig. 3C, white arrow), although its expression was partially rescued near clone borders (Fig. 3C, yellow arrow). Photoreceptor differentiation failed to occur within *smo* LOF clones when the posterior margin of the disc was included in the clone (data not shown). Taken together, this suggests that Hh from the posterior margin is crucial for initial Bar expression in the basal undifferentiated cells near the posterior margin and that the graded Bar expression in *ato*¹ mutant eye disc (Fig. 2D-F) is probably due to Hh secreted from the posterior margin of the disc.

Next, to test whether Hh signaling is also required for the activation of Bar expression in the middle of eye disc, we examined Bar expression within *smo* LOF clones generated at different positions in the middle region of eye disc (Fig. 3E-H; more than 20 clones observed). Bar expression was almost completely lost within *smo* LOF clones when they were located within and right posterior to the furrow (Fig. 3G, white arrow). However, Bar expression was not eliminated near the posterior border of *smo* LOF clone (Fig. 3G, yellow arrow), suggesting that Bar expression may become independent of Smo as the furrow moves further anteriorly. Interestingly, Ato was ectopically expressed within *smo* LOF clone region whenever Bar expression was lost posterior to the furrow (Fig. 3D,H, red arrows). This is consistent with the role of Bar as a transcriptional repressor of *ato* expression behind the furrow (Lim and Choi, 2003).

Bar expression requires Ato-mediated EGFR signaling from the morphogenetic furrow

Unlike the graded Bar expression pattern in *ato*¹ mutant eye disc (Fig. 2D-F), Bar expression in the basal undifferentiated cells in the wild-type eye disc is relatively even from the posterior

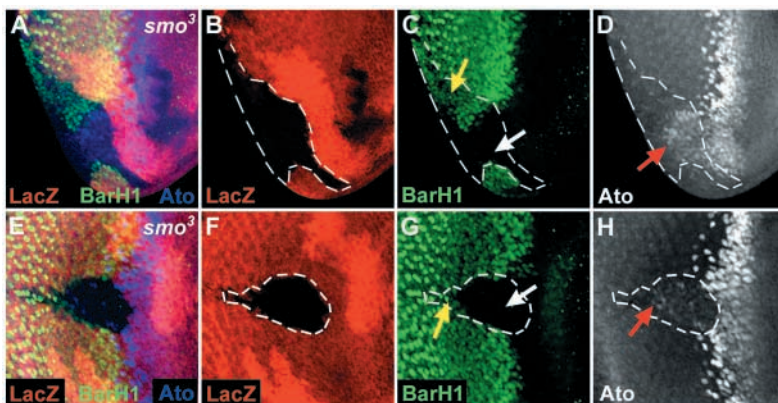


Fig. 3. Hh signaling is required for Bar expression in the basal undifferentiated cells. Eye discs containing *smo*³ LOF clones at different positions, close to the posterior margin (A-D) and near the furrow (E-H), are stained with antibodies for *lacZ* (clone marker; red), BarH1 (green) and Ato (blue). Bar expression is lost in *smo*³ LOF clones located close to the posterior margin (C, white arrow) or near the furrow (G, white arrow). Some Bar expression remains in part within the *smo*³ LOF clone near wild-type cells (C,G; yellow arrows), and ectopic Ato expression is observed in the region of Bar loss within the clones (D,H; red arrows). Apical through basal sections of confocal images are combined in order to see the BarH1 expression in single image.

margin of the disc to posterior the furrow (Fig. 4A,B). In order to establish such even distribution of Bar level, Bar expression may require additional activators derived from the furrow region in addition to the posterior margin Hh signaling (Fig. 4C).

As Bar expression level was low near the furrow in *ato*¹ mutant eye disc (Fig. 2D-F), it is possible that Ato itself may be necessary for Bar expression during furrow progression. To test this possibility, we generated *ato*¹ LOF clones and analyzed Bar expression right posterior to the furrow within *ato*¹ LOF clones (Fig. 4D-F). Indeed, when *ato*¹ LOF clones were generated close to the furrow far away from the posterior margin, Bar expression was absent or strongly downregulated within the *ato*¹ LOF clone compared with Bar expression in the wild-type region (Fig. 4F, white arrow; more than 20 clones scored). However, Bar expression was not significantly affected in the posterior part of the *ato*¹ LOF clones (Fig. 4F, yellow arrow). As expected, Ato expression in *ato*¹ LOF clones was expanded in cells between proneural clusters due to loss of Ato-mediated non autonomous repression of Ato expression (data not shown) (Chen and Chien, 1999). Ectopic Ato expression was also detected in *ato*¹ LOF clones further posterior to the furrow (data not shown) possibly owing to loss of Bar-mediated *ato* repression (Lim and Choi, 2003). Interestingly, in the *ato*¹ LOF clones, the expanded Ato expression towards the regions posterior to the furrow always showed the complementary expression pattern to the loss of Bar expression (data not shown). These data suggest that Ato may be required for the generation of nonautonomous activator signal for Bar expression in the basal undifferentiated cells near the furrow (Fig. 4C, blue arrow).

An interesting question is what is the Ato-mediated nonautonomous signal for Bar expression near the furrow. During early retinal neurogenesis, Ato induces nonautonomous signals through the activation of EGFR signaling within the proneural clusters (Fig. 4G-I) (Chen and Chien, 1999; Lesokhin et al., 1999), which is essential for ommatidial spacing by repressing Ato expression in cells between the proneural clusters (Kumar et al., 1998; Spencer et al., 1998; Chen and Chien, 1999; Lesokhin et al., 1999; Wasserman et al., 2000; Baonza et al., 2001; Yang and Baker, 2001; Frankfort and Mardon, 2002). As EGFR activity is also known to be required for Bar expression in the leg disc (Campbell, 2002), we tested whether EGFR signaling can mediate Ato effects on Bar expression in the eye disc (Fig. 4J-L). Indeed, loss of *egfr* function failed to induce Bar expression immediately posterior to the furrow within the clone (Fig. 4K, white arrow; six clones scored). Interestingly, Bar expression was not affected in the posterior part of the *egfr* LOF clone (Fig. 4K, yellow arrow), as similarly seen in the *ato*¹ LOF clone (Fig. 4F, yellow arrow). In addition, when *egfr* activity was removed at the restrictive temperature in *egfr*^{Δ5} mutant (Kumar et al., 1998), Bar expression was downregulated in the eye and antenna discs (data not shown). These data suggest that EGFR is required for induction of Bar expression right posterior to the furrow, although Bar expression may be induced by other activators in the posterior region of the *egfr* LOF clones. Consistent with the role of EGFR in the activation of Bar expression, loss of *egfr* caused ectopic Ato expression within *egfr* mutant clones behind the furrow due to loss of Bar expression (Fig. 4L, yellow line). Taken together, these data suggest that Ato-mediated activation of EGFR signaling in the furrow induces

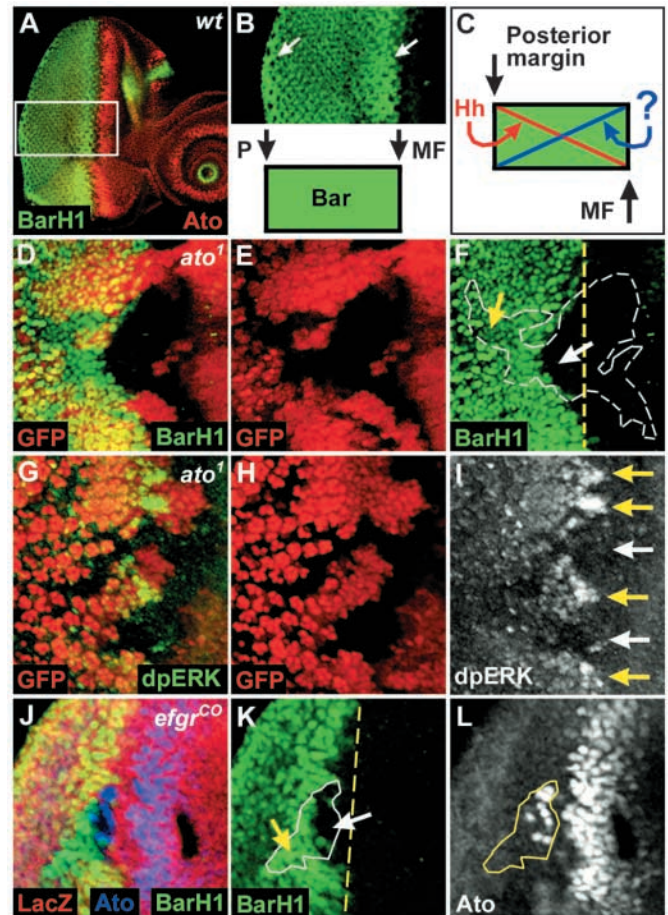
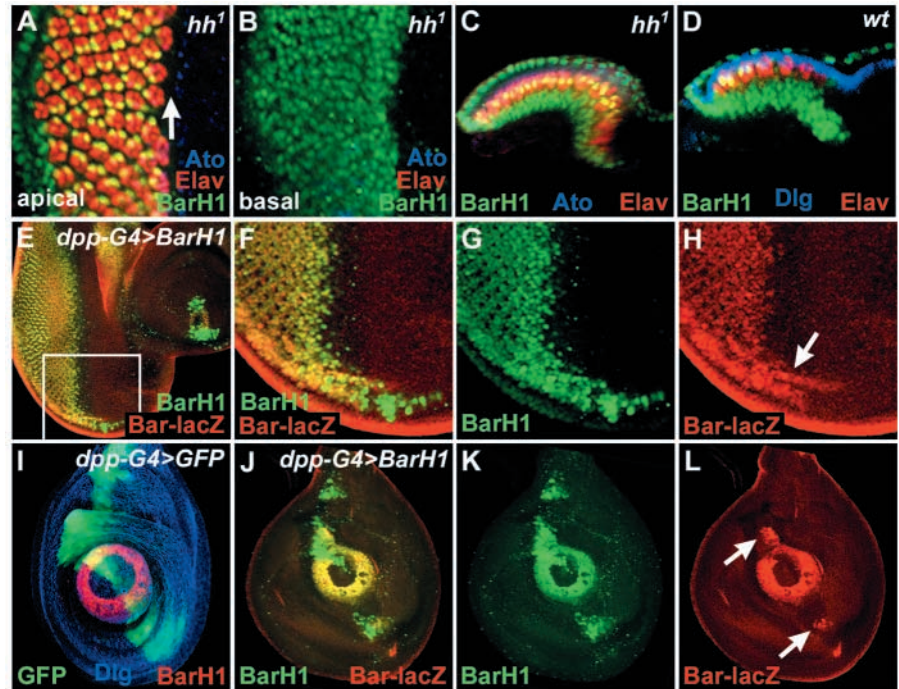


Fig. 4. Ato-EGFR signaling is required for the induction of Bar expression during furrow progression. (A-C) Bar expression level in the basal undifferentiated cells in wild-type eye disc is relatively even, although it appears to have a slightly higher level at the posterior margin and immediately posterior to the furrow (B, arrows). An area marked with a rectangle in A is magnified in B. (C) Predicted two activators for Bar expression in the basal undifferentiated cells: posterior margin signal (Hh) and furrow signal (blue arrow). P, posterior margin; MF, morphogenetic furrow. (D-F) Ato is required for Bar expression. Bar expression is absent (white arrow) immediately posterior to the furrow within *ato*¹ LOF clone, marked by broken white line in F. A broken yellow line marks the normal anterior boundary of Bar expression right posterior to the furrow in wild type. Bar expression is rescued in the posterior part of the *ato*¹ LOF clone (F, yellow arrow). Apical through basal sections of confocal images are combined in order to see Bar expression in single image. (G-I) EGFR is not activated within *ato*¹ LOF clones. Yellow and white arrows mark the dpERK activation within the proneural clusters in the wild-type or *ato*¹ mutant regions, respectively. The dpERK (dual phosphorylated extracellular signal regulated kinase) staining indicates the activation of EGFR signaling pathway. (J-L) *egfr*^{CO} LOF clone shows no Bar expression immediately posterior to the furrow (white arrow in K). In K, the broken yellow line marks the normal anterior boundary of Bar expression right posterior to the furrow in wild type. White (K) and yellow (L) lines, respectively, mark the *egfr* LOF clone boundaries. Ectopic Ato expression (L) is observed in the posterior region of the eye disc where Bar expression is lost (lack of green in K).

Bar expression in the basal undifferentiated cells right posterior to the furrow.

Fig. 5. Bar autoregulates its expression. (A-C) Bar expression in the basal undifferentiated cells is maintained after expression of its two activators, Hh and Ato, has ceased. A *hh¹* mutant eye disc stained with antibodies for Elav (red), BarH1 (green) and Ato (blue) is shown at the apical photoreceptor level (A) and the basal undifferentiated cell level (B). Ato expression is strongly downregulated but Bar expression is quite normal in *hh¹* mutant eye disc. In A, an arrow marks the arrested furrow. (C) A longitudinal section of *hh¹* mutant eye disc. (D) A longitudinal section of wild-type eye disc. (E-L) Bar positively autoregulates its transcription. Misexpression of *BarH1* by *dpp-GAL4* in the background of *BarH2^{P058}-lacZ* ectopically induces *lacZ* expression (H,L; arrows) in the region of BarH1 misexpression shown by ectopic BarH1 protein in the ventral region of eye disc (E-H) and along the anteroposterior (AP) border of leg disc (I-L). The ventral region of eye disc marked by a rectangle in E is magnified in F-H. (I) The wild-type pattern of Bar expression (red) in a ring domain and the *dpp* driver pattern by GFP reporter expression (green) along the AP axis.



Bar autoregulates its expression

Bar expression in the basal undifferentiated cells depends on the function of Hh- and Ato-dependent EGFR signaling from posterior and anterior, respectively. Next, we asked whether Bar expression in the basal undifferentiated cells in the middle of the disc is maintained as the furrow proceeds further anteriorly in late third instar stage. To address this question, we used an eye-specific *hh¹* mutant allele that causes precocious furrow stop during third instar larval stage (Heberlein et al., 1993). In *hh¹* mutant eye disc, Hh is normally produced in photoreceptor cells and functions until mid-third instar stage but is lost after mid-third instar stage of development, resulting in a furrow arrest (Fig. 5A-C). As Ato expression depends on Hh signaling (Borod and Heberlein, 1998; Dominguez, 1999), Ato expression was absent or strongly downregulated in the late third instar *hh¹* mutant eye disc (Fig. 5A-C). In the late third instar *hh¹* mutant eye disc, Bar expression was quite normal posterior to the arrested furrow (Fig. 5A-C). This suggests that Bar expression is maintained in the absence of Hh and Ato once it is initiated by these signals.

One possible mechanism for maintaining Bar expression in the absence of Hh and Ato function in *hh¹* mutant is autoactivation after its initial expression is primed by Hh and Ato. To test whether Bar can positively activate its own expression, we misexpressed *BarH1* or *BarH2* with *dpp-GAL4* driver using GAL4/UAS system (Brand and Perrimon, 1993) in the presence of *Bar^{P058}-lacZ* reporter (Sato et al., 1999). The *lacZ* reporter expression is under the control of endogenous enhancers of *BarH2*. In the eye and leg discs, misexpression of *BarH1* using *dpp-GAL4* activated ectopic *BarH2-lacZ* expression where *dpp* drives *BarH1* expression (Fig. 5E-L). Furthermore, misexpression of *BarH2* using the same driver showed more strongly activated *BarH2-lacZ* expression in the domain of *BarH2* misexpression (data not shown). This suggests that Bar expression is maintained by positive autoregulation.

Discussion

Regulation of Bar expression in the basal undifferentiated cells during retinal neurogenesis

Based on the evidence presented here, we propose a model for the regulation of Bar expression in the basal undifferentiated cells as summarized in Fig. 6. Prior to photoreceptor

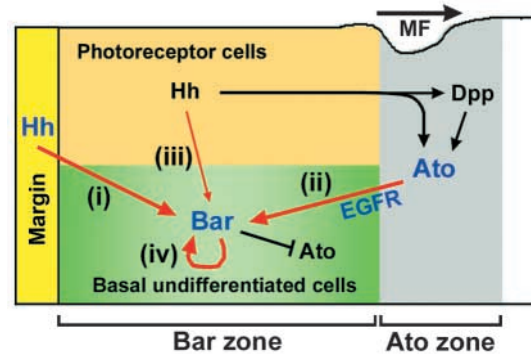


Fig. 6. A model for Bar regulation during retinal neurogenesis. Ato expression (gray region) in the morphogenetic furrow (MF) is activated by Hh from photoreceptor cells (orange region) and initiates the generation of photoreceptor neurons. Bar homeodomain proteins, which are essential for transcriptional repression of *ato* (Lim and Choi, 2003), are expressed in basal undifferentiated cells behind the furrow (green region) by several mechanisms. Positive and inhibitory relationships labeled by arrows may be indirect. (i) Prior to photoreceptor differentiation at the time of furrow initiation, Bar expression in the basal undifferentiated cells is induced initially by a secreted signaling factor, Hh, from the posterior margin (yellow region). (ii) During furrow migration, Bar expression near the furrow is induced by Ato from the furrow. EGFR signaling may partially mediate nonautonomous effects of Ato on Bar expression. (iii) Hh produced in photoreceptor cells induces Dpp expression and may also contribute to Bar expression during furrow migration. Finally (iv), Bar is autoregulated to maintain its expression.

differentiation at the time of furrow initiation, Bar expression in the basal undifferentiated cells near the posterior region of the disc is induced by secreted signaling factors from the posterior margin. Hh signaling from the posterior margin is required for the initial induction of Bar expression. During furrow progression, a narrow region of Bar expression immediately posterior to the furrow depends on Ato from the furrow. EGFR signaling may partially mediate Ato effects on Bar expression. Hh produced by photoreceptor cells generated behind the furrow may also be required in part for Bar expression near the furrow during furrow progression. Finally, Bar is autoregulated to maintain its expression. The properly expressed Bar proteins repress *ato* transcription in the basal undifferentiated cells, thereby preventing ectopic photoreceptor differentiation posterior to the furrow.

Effects of Hh signaling on Bar expression

Hh expression is dynamic, depending on the time and the position in the developing eye disc. In the early third instar eye disc, Hh is expressed in the posterior margin and is required for the furrow initiation (Heberlein et al., 1993; Ma et al., 1993; Dominguez and Hafen, 1997; Borod and Heberlein, 1998; Curtiss and Mlodzik, 2000). During furrow progression, Hh is also produced in the differentiating photoreceptor cells generated posterior to the furrow and secreted anteriorly to promote furrow progression (Heberlein et al., 1993; Ma et al., 1993; Dominguez and Hafen, 1997; Borod and Heberlein, 1998; Greenwood and Struhl, 1999; Curtiss and Mlodzik, 2000). During this process, Bar is specifically expressed in the basal undifferentiated cells posterior to the furrow and inhibits ectopic retinal neurogenesis by repressing proneural gene *ato* expression (Lim and Choi, 2003).

Hh signaling is required for Bar expression in the basal undifferentiated cells during initial eye development because Bar expression was strongly reduced or absent within *smo* LOF clones generated near the furrow or close to the posterior margin of the disc (Fig. 3). Prior to the photoreceptor differentiation, Hh expressed in the posterior margin of the disc is responsible for Bar expression at specific distances from the posterior region of the eye disc proper. A graded expression of Bar near the posterior region in *ato*¹ mutant eye disc (Fig. 2D-F) might be the effects of Hh secreted by the posterior margin.

During furrow progression, Hh signaling is required for Ato expression in the furrow (Borod and Heberlein, 1998; Dominguez, 1999), and Ato-mediated EGFR signaling is required for Bar activation (Fig. 4D-L). Therefore, it is possible that the loss of Bar expression near the furrow in *smo* LOF clones might be caused by indirect effects of reduced Ato expression rather than by direct effects of Hh signaling on Bar expression. Hh may partially contribute to Bar expression by activating normal levels of Ato expression in the furrow. Thus, the Hh-Ato-EGFR cascade activates Bar expression right posterior to the furrow. Alternatively, as Hh signaling may also affect furrow progression (Heberlein et al., 1993; Ma et al., 1993; Dominguez and Hafen, 1997; Strutt and Mlodzik, 1997; Borod and Heberlein, 1998; Greenwood and Struhl, 1999; Curtiss and Mlodzik, 2000), it is possible that the loss of Bar expression near the furrow in *smo* LOF clones might be caused by indirect effects of slow furrow migration rather than by direct effects of Hh signaling on Bar expression.

Effects of Ato-mediated EGFR signaling in Bar expression

Our results suggest that Ato is required nonautonomously for the induction of Bar expression right posterior to the migrating furrow (Fig. 4D-F). Although Ato acts as an activator for Bar expression, expression of these proteins always show a juxtaposed complementary pattern along the furrow (data not shown). This suggests that some mediator(s) is required for transducing Ato effects on Bar expression. EGFR activated by Ato in the furrow is required for Bar expression, suggesting that nonautonomous effects of Ato on Bar expression may be partially mediated by EGFR (Fig. 4D-L). Furthermore, EGFR is required for Bar expression not only in the eye disc but also in the antenna and leg discs in *Drosophila* (data not shown) (Campbell, 2002), suggesting that EGFR signaling may be a common activator for Bar expression in different tissues or even in higher organisms.

Notch (N) signaling is also known to contribute to neuronal differentiation together with Hh and Dpp pathways (Baker and Zitron, 1995; Li and Baker, 2001; Frankfort and Mardon, 2002). Thus, N signaling may play a role for Bar expression in the basal undifferentiated cells during furrow progression. Bar expression was strongly downregulated when N function was removed with a temperature-sensitive mutation (*N^{ts}*) or using the *Enhancer-of-split* [*E(spl)*] mutant clones in the eye disc (data not shown). This suggests that N signaling may be required for Bar expression in the basal undifferentiated cells. However, it is equally possible that loss of Bar expression in the *E(spl)* LOF clones or in the *N^{ts}* eye disc may be an indirect secondary effect of the lack of the basal undifferentiated cells because nearly all cells in the basal region of the eye disc differentiate into photoreceptor cells without N function. Further analysis of Bar regulation at the molecular level will be helpful to identify direct regulators of Bar expression in the undifferentiated cells of the eye disc.

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