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# Temporal switching of regulation and function of *eye gone* (*eyg*) in *Drosophila* eye development

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ABSTRACT

The Pax gene *eyg* is important for *Drosophila* eye development. *eyg* expression in the visual system changes dynamically during development. In this study, we found that the transcriptional regulation of *eyg* can be separated into four distinct temporal phases (E, L1, L2, and L3) and each is regulated by distinct *cis*-regulatory elements. Utilizing these enhancers for temporal and spatially specific manipulations, we addressed the regulation and function of *eyg* at different developmental stages. We found that Notch signaling is required and sufficient for *eyg* expression and this activity is restricted only to the L2 stage. We further showed that the function of *eyg* in eye development is required only at the second instar larval stage, while its function for head and antenna development can be provided at any time during embryo and larval development. Thus there is a temporal switch of the regulatory mechanism and function of *eyg*. We propose that *eyg* expression at L2 is induced and maintained by N signaling, and is turned off at L3 by a negative feedback loop involving the morphogenetic furrow.

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## Introduction

The *Drosophila* adult compound eyes originate in the embryo as two bilaterally symmetric groups of cells formed the eye-antenna disc primordium (EADP) in the head region. These cells proliferate during the larval stages to form the eye-antennal imaginal disc. At second instar larval stage (L2), the eye and antennal fields become separately marked by *ey* and *cut* expression, respectively (Kenyon et al., 2003). At mid third instar larval stage (L3), a wave of signaling events initiate from the center of the posterior margin of the eye disc and progressively move toward the anterior side. The moving boundary is characterized by an apical–basal shortening of the cells, thereby forming an indentation, named the morphogenetic furrow (MF). As the MF sweeps forward, cells posterior to MF begin to differentiate into the diverse retinal cell types. This series of proliferation, fate specification and differentiation events are precisely coordinated temporally and spatially. These cells undergo different phases of gene expression. To understand the mechanism for the temporal transition from one phase to the next is of great importance.

*eye gone* (*eyg*) encodes a Pax6-like which is important for eye development (Chao et al., 2004; Dominguez et al., 2004; Jang et al., 2003; Yao and Sun, 2005). During visual system development, *eyg* shows a dynamic expression pattern. Based on in situ hybridization, it is first expressed uniformly in all cells in the EADP at stage 15 (Jones et al., 1998; Jun et al., 1998). As these cells proliferate to form the eye-antennal disc in the larval stages, *eyg* expression becomes restricted to the dorsoventral (DV) midline during L2 (Jang et al., 2003). At L3, *eyg* expression at the DV midline fades, and begins to express in the anterior region of the eye disc (Jang et al., 2003). The expression pattern in the L3 eye disc is confirmed with an anti-Eyg antibody (Dominguez et al., 2004). In some enhancer trap lines, the DV midline (equatorial) expression does not fade (Dominguez et al., 2004; Jang et al., 2003; Sun et al., 1995). In this study, we try to identify the mechanism regulating the spatial and temporal switch of expression patterns of *eyg*. Through the analysis of the *cis*-acting regulatory elements in the *eyg-toe* locus, we found that the regulation of *eyg* transcription can be divided into at least four distinct temporal phases, representing distinct regulatory mechanisms.

Previous studies have shown that Notch (N) signaling regulates *eyg* expression in the equator of eye disc (Chao et al., 2004; Dominguez et al., 2004). Downregulating N signal (in $N^{ts}$, *ey*>$N^{ECD}$, *N* mutant clones, or *Su(H)* mutant clones) resulted in reduction of *eyg* expression, while activation of N signal (clonal expression of $N^{act}$ or the ligand Ser or Dl) resulted in the induction of *eyg* expression (Chao et al., 2004; Dominguez et al., 2004). In this study, we demonstrate that the regulation of *eyg* expression at the DV midline by N occurs only at L2. We further provided evidence that the L2 expression is turned off at L3 by the progressing MF.

Although Eyg is a Pax protein structurally similar to Ey which specifies eye fate, its function is distinct from Ey (Yao and Sun, 2005) and does not affect eye fate specification (Jang et al., 2003; Dominguez et al., 2004). *eyg* has two known functions in eye development:

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promoting cell proliferation and promoting MF initiation (Chao et al., 2004; Tsai et al., 2007; Tsai and Sun, 2004). The first function is primarily mediated through activating the expression of *upd*, which serves as a ligand to activate the Dome receptor and Jak/STAT signaling to induce cell proliferation. The induction of *upd* is restricted to the point of intersection of the equatorially expressed Eyg and the posterior margin of the eye disc (Chao et al., 2004). This point has been called the posterior center (PC) and the firing center, because it is the site of MF initiation (Ma and Moses, 1995; Tsai et al., 2007). Upd is able to distribute over a long range to promote cell proliferation in the region anterior of the MF (Tsai and Sun, 2004). In addition to acting through *upd*, ectopic *eyg* can promote cell proliferation in a cell-autonomous manner without inducing *upd* (Chao et al., 2004), so some of Eyg function may be independent of *upd*. The second function of *eyg* in promoting MF initiation is mediated by repressing the expression of *wg* (Hazelett et al., 1998; Jang et al., 2003; Yao and Sun, 2005), which blocks MF initiation (Blackman et al., 1991; Heslip et al., 1997; Treisman and Rubin, 1995). Since Eyg functions as a transcriptional repressor (Yao and Sun, 2005), it is possible that Eyg directly represses *wg* expression. The repression on *wg* also occurs indirectly through the induction of *upd*, which then acts through the Jak/STAT pathway to repress *wg* transcription (Ekas et al., 2006; Tsai et al., 2007). Wg has a broad expression in early L2 eye disc, and becomes restricted to the lateral margins in late L2 eye disc (Baker, 1988; Cavodeassi et al., 1999; Cho et al., 2000). Upd expressed at the PC caused the retraction of *wg* from the posterior margin, thereby allowing MF initiation (Tsai et al., 2007). Since *upd* RNA is detected at the PC at L2 and early L3 (Halder et al., 1995; Pignoni and Zipursky, 1997; Tsai and Sun, 2004; Zeidler et al., 1999) and this expression is lost in *eyg* mutant (Chao et al., 2004), the regulation by *eyg* must occur at L2 and early L3. The retraction of *wg* to the lateral margins also occurred at L2. Therefore, the two major functions of *eyg*, inducing *upd* and repressing *wg*, probably both occur at L2. Whether the early and late expression of *eyg* has any function in eye development is unknown. In this study, we demonstrated that the role of *eyg* in eye development is required only at L2. We also demonstrated that *eyg* has a role in head and antenna development, and this function can be provided by *eyg* expression at any time during the embryo and larval stages.

## Materials and methods

### *Fly stocks*

Fly culture and crosses were performed according to standard procedure at 25 °C unless otherwise noted. The source of *hsFLP*$^{22}$; *Act5C>y+>GAL4*, *UAS-lacZ* (Ito et al., 1997), *UAS-eyg* (Jang et al., 2003), *UAS-N*$^{act}$ (Go et al., 1998), *Su(H)*$^{SF8}$*FRT40A/Cyo* (Schweisguth and Posakony, 1992), *N*$^{ts}$ (Shellenbarger and Mohler, 1978), *eyg*$^{M3\text{-}12}$ (Jang et al., 2003) and *upd-lacZ* (Sun et al., 1995) was described in Chao et al., 2004. *E(spl)-mβ-lacZ* (Nellesen et al., 1999) was from M. Milán. *UAS-FLP*; *Act5C>Draf>lacZ* was from U. Bhadra (CCMB, India). *tubP-GAL80 [ts]* (McGuire et al., 2003) was from the Bloomington stock center. *UAS-eyg*-VP16 was described in (Yao and Sun, 2005).

### *Clonal induction*

Positively labeled flp-out expression clones were generated by crossing *UAS*-lines to *hs-FLP*$^{22}$; *Act5C>y+>GAL4 UAS-lacZ* (Ito et al., 1997). Heat-shock induction of *hs-FLP*$^{22}$ was at 37 °C for 30 min at 24–48 h after egg laying (AEL). Mutant clones were induced by the FLP-FRT method (Xu and Rubin, 1993). For *Su(H)*$^{SF8}$ mutant clones, *Su(H)*$^{SF8}$ *FRT40A* males were crossed to *hs-FLP*$^{22}$; 2x*P[ubi-nls-GFP] FRT40A* virgins. Heat-shock induction of *hs-FLP* was at 37 °C for 90 min at 24–48 h AEL.

### *Temperature shift experiments*

*N*$^{ts}$ eggs were laid at 18 °C for 24 h and maintained at 18 °C, except for a 24 h shift to 29 °C at the indicated time. After culture at 29 °C for 24 h, larvae were dissected for antibody staining. For GAL4/GAL80$^{ts}$ experiments, the embryos were collected at 22 °C for 24 h and then kept at 18 °C until shift to 29 °C at distinct developmental stages, and then returned to 18 °C. Progeny without temperature shift was used as control. Pharate adults and newly eclosed adults were observed.

### *Immunohistochemistry*

Antibody staining for imaginal discs was as previously described (Pai et al., 1998). Primary antibodies were rat anti-Elav (1:500), mouse anti-Dac (1:200), mouse anti-Eya (1:200), mouse anti-N (intra cellular domain) (1:200) (Developmental Studies Hybridoma Bank, University of Iowa) and rabbit anti-β-galactosidase (1:1500, Cappel). Guinea-pig anti-Eyg was kindly provided by Natalia Azpiazu (Aldaz et al., 2003). Secondary antibodies (Jackson ImmunoResearch) were Cy3 anti-rabbit, Cy5 anti-rabbit, Cy3 anti-rat, Cy5 anti-rat, Cy3 anti-mouse and Cy5 anti-mouse. Fluorescent images were obtained using a Zeiss LSM 510 confocal microscope.

### *In situ hybridization*

*eyg* antisense probe and hybridization procedure are as described previously (Yao et al., 2008). The pcDNA3-eGFP was similarly transcribed for the *gfp* antisense RNA probe and followed the same hybridization procedure.

### *eyg enhancer dissection*

EcoRI and BamHI fragments of BAC R49A02 (including the *eyg-toe* genomic region) were cloned first into pBluescript KS(+) vector, then into *pH-Stinger* and *pH-Pelican* (Barolo et al., 2000). The reporters in *pH-Stinger* and *pH-Pelican* are nuclear GFP and β-galactosidase, respectively. Subfragments derived from B8 and all subsequent derivatives were obtained by PCR amplification using primers with artificial Bgl II–Xho I restriction site extensions. The series of E2 constructs were cloned using available restriction sites. The relative position and length of each construct were shown in Figs. 1, 2 and Supplementary Fig. S2.

Constructs carrying mutated Su(H)-binding sites of CD fragment were generated by PCR mutagenesis by the QuikChange® Site-Directed Mutagenesis kit (Stratagene). Germline transformants of each construct were generated as described previously (Jang et al., 2003). A minimum of three independent transgenic lines was tested for reporter activity for each construct.

Detailed construction history of the GAL4 lines generated in this study is available on request.

## Results

### *eyg expression can be divided into four distinct phases*

The *eyg-toe* locus contains the gene pair *eyg* and *toe*, separated by about 30 kb (Fig. 1A). The two Pax genes share high homology in two conserved DNA-binding domains, the paired domain and the homeodomain, and have very similar functions (Aldaz et al., 2003; Dominguez et al., 2004; Jang et al., 2003; Yao and Sun, 2005, Yao et al., 2008). In situ hybridization showed that *eyg* and *toe* are expressed in almost identical pattern throughout development (Jang et al., 2003; Yao et al., 2008). In the visual system, their expression begins in the embryonic EADP and continues to the larval eye disc. *eyg* first attracted our attention because of its expression at the equator in the eye (Sun et al., 1995). This DV midline expression was shown to be

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