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The HLH protein Extramacrochaetae is required for R7 cell and cone cell fates in the *Drosophila* eye

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ABSTRACT

Notch signaling is one of the most important pathways in development and homeostasis, and is altered in multiple pathologies. Study of *Drosophila* eye development shows that Notch signaling depends on the HLH protein Extramacrochaetae. Null mutant clones show that *extramacrochaetae* is required for multiple aspects of eye development that depend on Notch signaling, including morphogenetic furrow progression, differentiation of R4, R7 and cone cell types, and rotation of ommatidial clusters. Detailed analysis of R7 and cone cell specification reveals that *extramacrochaetae* acts cell autonomously and epistatically to Notch, and is required for normal expression of bHLH genes encoded by the E(spl)-C which are effectors of most Notch signaling. A model is proposed in which Extramacrochaetae acts in parallel to or as a feed-forward regulator of the E(spl)-Complex to promote Notch signaling in particular cellular contexts.

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Introduction

The Notch signaling pathway is one of the cell–cell communication pathways that are most widely used for cell fate specification (Bray, 2006). During *Drosophila* eye development, Notch signaling is important for the growth of the eye imaginal disc (the retinal primordium), for the definition of its dorsal and ventral hemispheres, and for the movement of the wave of differentiation that crosses the eye disc called the morphogenetic furrow. Within the morphogenetic furrow, Notch is essential for the lateral inhibition that specifies an array of single R8 photoreceptor cells through the negative regulation of a proneural bHLH gene, *atonal* (*ato*). Posterior to the morphogenetic furrow, Notch signaling is required for the induction of other retinal cell types including R4 photoreceptor cells, R7 photoreceptor cells, and non-neuronal cone cells, as well as rotation of the developing ommatidial clusters (Nagaraj et al., 2002).

Specification of R7 photoreceptor cells also requires Notch signaling as well as the receptor tyrosine kinase Sevenless (Sev) (Cooper and Bray, 2000; Tomlinson and Struhl, 2001; Doroquez and Rebay, 2006). A group of cells that include the precursors of the R1, R6 and R7 photoreceptor cells, and the cone cells, constitute the “R7 equivalence group”. Contact with the R8 cell induces activation of Sev in the R7 precursor. Contact with the R1 and R6 photoreceptors that express the ligand Delta (Dl) activates Notch in the R7 and cone cell precursors. In this combinatorial system, synergistic activation of Sev and Notch signaling is required for R7 development. Failure to activate receptor tyrosine kinases causes the presumptive R7 photoreceptor to acquire a cone cell fate. Conversely, ectopic Sev activity transforms cone cells into supernumerary R7 cells. In the absence of Notch activity the presumptive R7 photoreceptor acquires R1/R6 photoreceptor fate instead. Conversely, ectopic activation of Notch signaling in the R1/R6 photoreceptor pair directs these photoreceptors to develop as ectopic R7 photoreceptor cells.

The canonical Notch signaling pathway involves ligand-dependent release of the Notch intra-cellular domain, which enters the nucleus and activates transcription by complexing with the DNA-binding protein Suppressor-of-Hairless [Su(H)] and the co-activator Mastermind (Mam) (Bray, 2006). As each Notch molecule can be activated once only, and the cleaved intracellular domain is thought to turn over rapidly, the response to the binding of each ligand molecule may be short-lived (Fryer et al., 2004). Many aspects of Notch function are mediated through the transcription of target genes within the E(spl)-Complex, which includes seven bHLH proteins that act as transcriptional repressors of other genes. The function of Notch was first studied during neurogenesis, where Notch mediates lateral inhibition through E(spl)-mediated repression of proneural bHLH genes. Class II bHLH genes, such as the *ato* gene that is required for R8 photoreceptor specification (Jarman et al., 1994), define proneural regions competent to give rise to neural precursor cells, as heterodimers with the ubiquitously-expressed Class I bHLH gene Daughterless (Da) (Doe and Skeath, 1996; Hassan and Vassin, 1996; Massari and Murre, 2000).

In addition to transcriptional regulation by Notch, proneural bHLH gene function can also be modulated post-translationally by the Extramacrochaetae protein (Campuzano, 2001). The *extramacrochaetae* (*emc*) gene encodes a helix–loop–helix protein without any basic

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DNA-binding domain. Emc antagonizes bHLH proteins' function by forming non-functional heterodimers with them. Emc has mammalian homologs, the Inhibitor of differentiation (Id) proteins, that are implicated in development and cancer (Ruzinova and Benezra, 2003; Iavarone and Lasorella, 2004). In *Drosophila*, the *emc* gene has been thought to provide an initial prepattern that influences the patterning of neurogenesis (Ellis et al., 1990; Garrell and Modolell, 1990; Brown et al., 1995; Campuzano, 2001). This conclusion, however, has been based on the study of weak, hypomorphic mutant alleles. Imaginal disc clones homozygous for null alleles of *emc* do not survive, suggesting that the gene must have additional roles that remain to be elucidated (Garcia Alonso and Garcia-Bellido, 1988; de Celis et al., 1995; Campuzano, 2001). In addition, more recent studies suggest that Emc function may be linked to Notch signaling. Studies of wing and ovary development show that Notch signaling enhances expression of *emc* enhancer traps, and that *emc* is required for aspects of Notch function in those organs (Baonza et al., 2000; Adam and Montell, 2004). By contrast, *emc* was reportedly repressed by Notch signaling during eye development (Baonza and Freeman, 2001).

In the course of investigating *emc* as a possible cell cycle target of Notch signaling, we have discovered that the lethality of *emc* null mutant cells can be delayed very substantially using the Minute technique to provide a growth advantage, and through their study that *emc* is required for many aspects of *Drosophila* eye development. We present an outline of these requirements for *emc*. In addition, we now find that *emc* transcription is not repressed by Notch signaling in eye development as reported previously, but may be enhanced as also reported for the wing and ovary. A detailed analysis of the role of *emc* in R7 and cone cell development shows that Notch requires *emc* to induce R7 and cone cell fates. These findings add to the evidence that *emc* contributes to Notch signaling, perhaps by promoting E(spl)-C expression.

Methods

Mosaic induction

Clones of cells homozygous mutant for genes were obtained by FLP-FRT mediated mitotic recombination technique (Xu and Rubin, 1993; Newsome et al., 2000). For non-Minute genotypes, larvae were subjected to 1 hour heat shock at 37 °C at 60±12 h after egg laying and were dissected 72 h later. For Minute genotypes, heat shock was administered at 84±12 h after egg laying and dissection 72 h later. 'Flip-out' clones were generated by subjecting larvae to heat shock at 37 °C for 30 min at 60±12 h after egg laying and dissection 72 h later.

Flies were maintained at 25 °C unless mentioned otherwise.

All genotypes are described in the figure legends.

Drosophila strains

The following *Drosophila* strains were used: *w;* P{PZ}*emc*^{04322} (Rottgen et al., 1998); P{PZ}*emc*^{04322} (Castrillon et al., 1993); UAS-*Ser* [line #19] (Li and Baker, 2004); UAS-*Dl* (Jönsson and Knust, 1996); UAS-*N*^{intra} (Fuerstenberg and Giniger, 1998); *act*>*CD2*>*GAL4*, UAS-*GFP* (Pignoni and Zipursky, 1997; Neufeld et al., 1998); *mam*^{10} (Lehmann et al., 1983); *Su(H)*$^{\Delta47}$ [*w*$^+$ *l(2)35Bg*$^+$] (Morel and Schweisguth, 2000); *E(spl)gro*$^{b32.2}$*p*[*gro+*] (Heitzler et al., 1996); *emc*^{AP6} (Ellis, 1994); [*UbiGFP*] *M*(3)*67C* FRT80 (Janody et al., 2004); *E(spl)m*δ *0.5-lacZ ry*$^+$ (Cooper and Bray, 1999) and *Cyo* [*w*$^+$*, sev-N*^{act}] (Fortini et al., 1993); UAS-*Da* (Hinz et al., 1994); *sev-Gal4* (Brand and Perrimon, 1993); UAS-*E(spl)-m*δ (de Celis et al., 1996).

Temperature-sensitive studies

N^{ts}/Y larvae were reared at 25 °C (Cagan and Ready, 1989). Larvae were transferred to the restrictive temperature 31 °C for 3 h prior to dissection.

Immunohistochemistry

Labeling of eye discs involving guinea pig anti-Runt 1/1500 (Duffy et al., 1991), mouse anti-Svp 1/1000 (Kanai et al., 2005), mouse anti-Pros 1/25 (MR1A), mouse anti-Cut 1/20 (2B10) (both were obtained from Developmental Studies Hybridoma Bank) and rabbit anti-DPax-2 1/50 (Fu and Noll, 1997) were performed as described (Domingos et al., 2004). Other antibody and DRAQ5 labelings were performed as described (Firth et al., 2006). Images were recorded using BioRad Radiance 2000 Confocal microscope and processed using NIH Image J and Adobe Photoshop 9.0 software. Other primary antibodies used were: mouse anti-βGal 1/100 (mAb40-1a), rat anti-ELAV 1/50 (7E8A10) (both were obtained from DSHB), guinea pig anti-Sens 1/500 (Nolo et al., 2000), rabbit anti-Emc 1/8000 [a gift from Y. N. Jan] (Brown et al., 1995), rabbit anti-Salm 1/50 (Kuhnlein et al., 1994), mouse anti-Hairy 1/50 (Brown et al., 1995), anti-E(spl) (mAb323) 1/1 (Jennings et al., 1994) and anti-GFP 1/500 (Invitrogen).

RNA in situ hybridization

RNA in situ probe design, preparation and detection were performed as described (Firth and Baker, 2007). Hybridization was performed at 55 °C.

Primers used for the first PCR reaction [see Materials and methods (Firth and Baker, 2007)] to amplify transcribed regions of *emc* genomic DNA:

Forward Primer 5′ GGCCGCGGGCATCTCTTCAACGCTCCTT 3′

Reverse Primer 5′ CCCGGGGCTGCTGCTGAGTTGGTTGTTC 3′.

Results

Emc transcriptional reporters coincide with Notch activity

To evaluate the relationship between *emc* and *Notch* signaling, expression of the *emc* gene was visualized during developing third instar *Drosophila* larval eye using enhancer trap lacZ insertion lines P{PZ}*emc*^{04322} and P{PZ}*emc*^{03970} (Figs. 1 and 2 and data not shown). *emc-lacZ* was expressed in all cells in the developing eye, but the level of expression varied. Expression was reduced inside the morphogenetic furrow, just before Senseless expression started, and rebounded posterior to the furrow at around columns 2 to 3, similar to previous observations made with an antibody (Fig. 1A) (Brown et al., 1995).

Anterior to the morphogenetic furrow, *emc-lacZ* expression was higher in the ventral disc compared to the dorsal disc, and especially elevated along the dorso-ventral equator. Posterior to the morphogenetic furrow, *emc-lacZ* levels remained constant in undifferentiated cells that have basal nuclei, but were dynamic in differentiating ommatidial cells (Fig. 1E). As soon as R3, R4 and R8 nuclei were identified by Elav expression, their *emc-lacZ* levels were at a high level similar to that of basal nuclei of undifferentiated cells. In addition, *emc-lacZ* was sometimes even higher in R4 than in R3. R2 and R5 cells always had lower *emc-lacZ* levels. *emc-lacZ* was high in R1/R6 nuclei when first identified around column 6, but decreased from column 8 onwards (Figs. 1B, C). By contrast, *emc-lacZ* was high in nuclei of R7 and cone cell precursors from their appearance in columns 8 and 10, respectively (Figs. 1C, D). *emc-lacZ* remained high in R3/R4 and R7 photoreceptors and in cone cells (Fig. 1D), while dropping in R8 cells (Fig. 1E). In conclusion, *emc* transcription was often elevated where Notch signaling is required, such as at the equator, and in the developing R4, R7 and cone cells.

An Emc transcription reporter is elevated by Notch signaling

The *emc-lacZ* pattern was not what was expected if *emc* transcription is repressed by Notch signaling (Baonza and Freeman, 2001). The relationship between Notch signaling and *emc* expression

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