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Hipk promotes photoreceptor differentiation through the repression of Twin of eyeless and Eyeless expression

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ABSTRACT

Organogenesis is a complex developmental process, which requires tight regulation of selector gene expression to specify individual organ types. The Pax6 homolog Eyeless (Ey) is an example of such a factor and its expression pattern reveals it is dynamically controlled during development. Ey's paralog Twin of eyeless (Toy) induces its expression during embryogenesis, and the two genes are expressed in nearly identical patterns during the larval stages of development. While Ey must be expressed to initiate retinal specification, it must subsequently be repressed behind the morphogenetic furrow to allow for neuronal differentiation. Thus far, a few factors have been implicated in this repression including the signaling pathways Hedgehog (Hh) and Decapentaplegic (Dpp), and more recently downstream components of the retinal determination gene network (RDGN) Sine oculis (So), Eyes absent (Eya), and Dachshund (Dac). Homeodomain-interacting protein kinase (Hipk), a conserved serine–threonine kinase, regulates numerous factors during tissue patterning and development, including the Hh pathway. Using genetic analyses we identify Hipk as a repressor of both Toy and Ey and show that it may do so, in part, through Hh signaling. We also provide evidence that Ey repression is a critical step in ectopic eye development and that Hipk plays an important role in this process. Because Ey repression within the retinal field is a critical step in eye development, we propose that Hipk is a key link between eye specification and patterning.

Introduction

The developmental events leading to the formation of the adult Drosophila compound eye involve numerous signaling networks and cellular processes that must be tightly coordinated (reviewed in Kumar, 2009). Eye specification begins in the embryo, where a small group of cells expressing the Pax6 homologs Twin of eyeless (Toy) (Czerny et al., 1999) and Eyeless (Ey) (Quiring et al., 1994) are set aside as precursors to the eye-antennal imaginal disc (Garcia-Bellido and Merriam, 1969; Kammermeier et al., 2001). Toy and Ey are positioned atop the retinal determination gene network (RDGN); a group of nuclear proteins responsible for mediating eye development (reviewed in Kumar, 2009, 2011). The core members of this group include Toy, Ey, Eyes absent (Eya) (Bonini et al., 1993), Sine oculis (So) (Cheyette et al., 1994; Serikaku and O'Tousa, 1994), and Dachshund (Dac) (Mardon et al., 1994). In the first and second instars the eye imaginal disc grows and polarizes into anterior–posterior and dorsal–ventral compartments. Then in the third larval instar, patterning initiates in the eye disc (reviewed in Domínguez and Casares, 2005). Patterning is progressive and begins when the morphogenetic furrow, a physical indentation in the tissue, initiates at the posterior margin of the eye disc and begins travelling anteriorly (reviewed in Heberlein and Moses, 1995). As the furrow passes through the tissue, the expression patterns of the RDGN and activities of key signaling networks including Decapentaplegic (Dpp), Hedgehog (Hh), and Wingless (Wg) adjust accordingly, and at any given moment, the morphogenetic furrow divides the eye disc into two distinct regions (Curtiss and Mlodzik, 2000). The anterior region encompasses a pool of undifferentiated cells holding eye progenitor fate and the posterior region contains eye progenitor cells which terminally differentiate to become photoreceptors (R1–R8), cone cells, and pigment cells (reviewed in Baker and Firth, 2011; Roignant and Treisman, 2009).

Although paralogs, Toy and Ey have distinct properties (Czerny et al., 1999; Kammermeier et al., 2001; Punzo et al., 2004; Weasner et al., 2009). Ey holds the dual role of being both a transcriptional activator and repressor, while Toy appears to only act as an activator (Bessa et al., 2002; Punzo et al., 2001; Weasner et al., 2009). The two genes are expressed throughout the larval eye disc until the third larval stage when the furrow begins migrating and at this point they are progressively restricted to the anterior

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(Atkins et al., 2013; Czerny et al., 1999). It is known that the restriction of these factors away from the retinal field is a critical step in eye development, however, little is known about the coordination or mechanism of this developmental switch. Thus far, few factors have been shown to repress Ey within the posterior region of the eye disc (Atkins et al., 2013; Baker and Firth, 2011; Firth and Baker, 2009; Halder et al., 1998). These include Dpp, Hh, So, Eya, and Dac (Atkins et al., 2013; Firth and Baker, 2009). Here we identify Homeodomain-interacting protein kinase (Hipk) as a novel repressor of Toy and Ey.

Hipks are a conserved family of serine-threonine kinases and have been shown to be involved in numerous developmental contexts (Lee et al., 2009; Rinaldo et al., 2008; Swarup and Verheyen, 2011). Hipk regulates the size of the eye in both flies and vertebrates (Inoue et al., 2010; Lee et al., 2009). Additionally, Hipk was shown to phosphorylate Ey and Pax6 although the physiological significance of this event is unknown (Choi et al., 2005; Kim et al., 2006). Previously we showed that Hipk inhibits the repressive effects of the global transcriptional co-repressor Groucho (Gro), and thus promotes Notch-induced eye growth (Lee et al., 2009). Furthermore, Hipk acts to promote the Hh, and thus indirectly, Dpp pathways through its ability to stabilize the Hh signaling effector Cubitus interruptus (Ci). More specifically, Hipk indirectly stabilizes the full length, activator form of Ci (Ci^{ACT}) by inhibiting Slimb, the E3 ubiquitin ligase that targets Ci^{ACT} for proteasomal cleavage into the truncated repressor form of Ci (Ci^{REP}) (Swarup and Verheyen, 2011). Based on our work and these studies, we sought to further investigate the role of Hipk in eye specification and the regulation of Ey and Toy. Our findings reveal that both Pax6 homologs Toy and Ey are repressed by Hipk, and this relationship may be a key step in the developmental switch from specification to differentiation of retinal tissue.

Toy and Ey are termed eye selectors, in part, based on their ability to re-program non-retinal tissue to take on eye fate in ectopic eye induction assays (Czerny et al., 1999; Halder et al., 1995). Given the multitude of feedback loops occurring during eye specification and patterning, researchers have turned to ectopic eye assays to dissect the individual contributions of RD factors in a simplified context (Anderson et al., 2012; Furukubo-Tokunaga et al., 2009; Halder et al., 1995; Pignoni et al., 1997; Shen and Mardon, 1997). Due to its simplicity, the ectopic eye may reveal developmental defects more clearly. In this study we have utilized this technique to aid in our understanding of the relationship between Ey repression and Hipk.

Materials and methods

Drosophila strains and crosses

Flies and crosses were raised on standard media at 25 °C unless stated otherwise. *w^{1118}* was used as wild type. *hs-flp;ubi-GFP,FRT79/TM6B, tub-GAL80^{ts}/CyO,;dpp-lacZ^{M1-1};,;UAS-eGFP;;, hipk^{BG0085}-GAL4/TM6C, w*; P{arm-GFP.P}57*, (Bloomington Stock Centre). *UAS-toy-RNAi/TM3* (VDRC15919),;*UAS-hipk-RNAi;;* (VDRC108254) were obtained from Vienna Drosophila RNAi Center (Dietzl et al., 2007). *hs-flp;;Act > CD2 > UAS-GAL4,UAS-GFP/SM6∼TM6* (gift of Bruce Edgar) and *yw,hsp70-flp tub-GAL4,UAS-GFP; tub-GAL80,FRT40/CyO* (gift of Gary Stuhl). *hipk^{2}/TM6B*, *hipk^{3}/TM6B*, *hipk^{4},FRT79/TM6B* were used as loss-of-function alleles and;*UAS-HA-hipk^{1M};UAS-HA-hipk^{3M}* (Lee et al., 2009).;;*UAS-ey^{UE10};* (Halder et al., 1995),;*UAS-toy^{UO4};;* (Czerny et al., 1999).;*ey-lacZ;* (Halder et al., 1998), *so-lacZ/CyO* (Cheyette et al., 1994) and;;*toy-lacZ* (gifts of Uwe Walldorf). *dpp^{blk}-GAL4/TM6B* (Staehling-Hampton et al., 1994), *GMR-GAL4* (Moses and Rubin, 1991), *Oc2-GAL4/CyO* (gift of Blanco et al. (2009)). *smo^{Q},FRT40/CyO,UAS-GFP, Kr-GAL4* (Biehs et al., 2010), *UAS-ci5m/CyO* (Price and Kalderon, 1999).

Clonal analysis

Somatic clones were generated by crossing *hipk^{4},FRT79/TM6B* to *hs-flp;ubi-GFP,FRT79/TM6B.* MARCM40 clones were generated by crossing *yw,hsp70-flp tub-GAL4,UAS-GFP; tub-GAL80,FRT40/CyO* to corresponding lines. Flip-out clones were generated by crossing *hs-flp;;Act > CD2 > UAS-GAL4,UAS-GFP/SM6∼TM6* to corresponding UAS lines. Hatched progeny were heat shocked at 38 °C, 48 h AEL for either 90 min (somatic and MARCM40 clones) or for 19 min (flip-out clones).

tub-GAL80^{ts} experiment

To temporally control UAS-gene expression, *tub-GAL80^{ts};dpp-GAL4* flies were crossed to *UAS-toy* at 29 °C. At mid-third instar, progeny were shifted to 18 °C until adulthood. Positive and negative controls were crossed at 29 °C/18 °C respectively, and progeny remained there until adulthood.

In situ hybridization

Digoxigenin labelled RNA probes for *hipk* were transcribed from pOT2-Hipk (Drosophila Genomics Resource Center clone ID SD08329) using the Roche DIG RNA Labeling kit. The sense probe was transcribed from XhoI digested plasmid using T7 polymerase and the antisense probe was transcribed from EcoRI digested plasmid using SP6 polymerase. The fluorescent *in situ* hybridization was performed as described in Wilk et al. (2010) with the following specifications: the *hipk*-DIG probe was detected with peroxidase conjugated mouse anti-DIG (Jackson ImmunoResearch) and cyanine-3 tyramide (Perkin Elmer), and picric acid was omitted from the PBTT solution.

Immunostaining and microscopy

Antibody staining was performed as described in Lee et al. (2009) using: rat anti-Ci (1:20), mouse anti-Dac$^{2-3}$ (1:75), rat anti-Elav (1:100), mouse anti-Ey (1:200), mouse anti-Eya (1:200), mouse anti-Pros (1:10) (DSHB), mouse anti-β-galactosidase (1:5000) (Promega), rabbit anti-β-galactosidase (1:1000) (Cappel-MP Biomedicals), rabbit anti-Ey (1:1000) (Halder et al., 1998) and guinea pig anti-Toy (1:1000) (Furukubo-Tokunaga et al., 2009) (gifts of Uwe Walldorf), rabbit anti-Ato (1:800) (gift of Yuh Nung Jan, Jarman et al., 1994), guinea pig anti-Sens (1:1000) (gift of Hugo Bellen, Nolo et al., 2000). Secondary antibodies (Jackson Immuno Research) Dylight-549 (anti-guinea pig, mouse, rabbit), Dy-649 (anti-guinea pig, mouse and rat), and FITC (anti-rat) were used at 1:200.

Imaginal discs were imaged with a Nikon Air laser-scanning confocal microscope. Identical microscopy settings were used to image all discs. Images of all adult flies except in Fig. 1F–I were obtained with a Canon EOS Rebel T1i digital camera mounted to a Leica MZ6 dissecting microscope while submerged in 95% ethanol. For ectopic eye assays, pharate adults unable to eclose were dissected from their pupal cases in 95% ethanol. To image adults in Fig. 1F–I, the fly heads were severed and mounted in Aquatex. The mounted heads were imaged with an Axioplan 2 microscope.