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Segregation of eye and antenna fates maintained by mutual antagonism in *Drosophila*

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SUMMARY

A general question in development is how do adjacent primordia adopt different developmental fates and stably maintain their distinct fates? In *Drosophila melanogaster*, the adult eye and antenna originate from the embryonic eye-antenna primordium. These cells proliferate in the larval stage to form the eye-antenna disc. The eye or antenna differs at mid second instar with the restricted expression of Cut (Ct), a homeodomain transcriptional repressor, in the antenna disc and Eyeless (Ey), a Pax6 transcriptional activator, in the eye disc. In this study, we show that *ey* transcription in the antenna disc is repressed by two homeodomain proteins, Ct and Homothorax (Hth). Loss of Ct and Hth in the antenna disc resulted in ectopic eye development in the antenna. Conversely, the Ct and Hth expression in the eye disc was suppressed by the homeodomain transcription factor Sine oculis (So), a direct target of Ey. Loss of So in the eye disc caused ectopic antenna development in the eye. Therefore, the segregation of eye and antenna fates is stably maintained by mutual repression of the other pathway.

KEY WORDS: *Drosophila*, Eye-antenna, Mutual antagonism, Transcriptional repression

INTRODUCTION

During development, adjacent primordia become specified into different developmental fates and the segregation of these fates has to be stably maintained. Most of the adult head structures of *Drosophila* originate in the embryo head region as two bilaterally symmetric groups of cells called the eye-antenna disc primordium. Cells in the primordium proliferate to form the larval eye-antenna disc. The developmental potential of the eye-antenna disc can be tested by transplanting fragments of the disc into host larva and allowing them to metamorphose. Experiments on eye-antenna disc from late third instar (L3) larva show that it develops into adult head structures, including the compound eye and ocelli from the eye disc, the antenna and maxillary palp from the antenna disc, and the head capsule that surrounds these organs (Haynie and Bryant, 1986). For the sake of simplicity, we will use eye and antenna to represent the two large regions of the eye-antenna disc.

The timing of segregation of the eye and antenna fates can be assessed using several approaches. Based on histological analysis, the eye and antenna primordium of the eye-antenna disc are different in mid-first instar (m-L1) larva in terms of the timing of onset of cell divisions and cell morphology (Madhavan and Schneiderman, 1977). Mitotic clonal analysis suggested that the eye and antenna fates were not clonally restricted 27 hours after egg laying (AEL) but become segregated before 36 hours AEL (Morata and Lawrence, 1979; Postlethwait and Schneiderman, 1971). Using disc transplantion experiments, it was found that the posterior part of late second instar (l-L2) eye-antenna disc produced ommatidia and the anterior part produced antenna structures, while the central part produced both (Bryant, 1974; Vogt, 1946), suggesting that the fate segregation occurred before l-L2. By temperature shift at different time points, the phenocritical period for the eye-to-antenna transfromation due to block of Notch signaling was determined to be at the second half of L2 (Kumar and Moses, 2001). Based on the spatiotemporal gene expression patterns, it was also proposed that L2, which is when the eye and antenna determining genes become expressed specifically in their respective disc (see below), is the time of specification for the two primordia (Kumar and Moses, 2001). Kenyon et al. (Kenyon et al., 2003) proposed that the fate segregation occurred in e-L2 (e-L2), when there are clear differences in gene expression in the two discs. Each of the above observations has its premises and is not conclusive regarding the timing of fate segregation. In this study, we examine the spatiotemporal expression of eye- and antenna-determining genes, and try to correlate the segregation of expression with the segregation of developmental fates.

The early eye-antenna disc expresses several genes that are important for eye development (see Fig. 1). *eyeless* (*ey*) encodes a Pax6 transcription factor (Quiring et al., 1994) required for eye development. It is expressed uniformly in the eye-antenna disc in L1 and becomes restricted to the eye disc in e-L2 (Kenyon et al., 2003; Kumar and Moses, 2001; Singh et al., 2002). *sine oculis* (*so*) is a direct target of *ey* in the developing eye (Niimi et al., 1999). So is a homeodomain protein and is required for eye development (Cheyette et al., 1994). It is uniformly expressed in the L1 eye-antenna disc (supplementary material Fig. S4). In early-L2, *ey* and *so* expression becomes restricted to the eye disc (Kenyon et al., 2003). The timing of the restriction of expression to eye disc roughly correlates with the timing of segregation of the eye and antenna fates.

Similarly, the early eye-antenna disc uniformly expresses *homothorax* (*hth*), which is important for antenna development. Hth is a TALE type homeodomain protein and interacts physically with another homeodomain protein, Extradenticle (Exd), to promote its nuclear localization (Kurant et al., 1998; Pai et al., 1998; Rieckhof et al., 1997). Hth expression in the L1 eye-antenna disc is uniform, but is retracted from the posterior region of the eye disc in e-L2 (Bessa et al., 2002; Cavodeassi et al., 2000; Lebreton et al., 2008; Singh et al., 2002). In L3 eye-antenna disc, it is expressed in the proximal region of the antenna disc and in the

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anterior region of the eye disc (Pai et al., 1998). *hth* or *exd* mutations caused antenna-to-leg transformation (Casares and Mann, 1998; Pai et al., 1998). In fact, the classical antenna-to-leg transformation was due to the suppression of *hth* by the ectopic expressed Hox genes (Yao et al., 1999). Ectopic expression of Hth and another homeodomain protein, Distal-less, can induce ectopic antenna development (Dong et al., 2000). Hth also affects eye development. In the region anterior to the progressing morphogenetic furrow (MF) in the eye disc, Hth expression can be divided into two regions (Bessa et al., 2002). In the anterior margin of eye disc where Ey and Tsh are not expressed, Hth blocks retinal development (Pai et al., 1998). In the region slightly posterior, Hth is co-expressed with Ey and Tsh, and together they maintain the cells in a proliferative and undifferentiated state (Bessa et al., 2002; Lopes and Casares, 2010) through interaction with Yorkie (Peng et al., 2009), a positive component of the Hippo pathway. Cut (Ct), another homeodomain protein, becomes expressed in the antenna disc in e-L2 and is the earliest marker for the antenna disc (Duong et al., 2008; Kenyon et al., 2003; Lebreton et al., 2008). Although Ct is expressed in L2 in the entire antenna disc, the phenotype caused by *ct* mutant clones affected only very restricted domains (Ebacher et al., 2007). In this study, we showed that Ct and Hth function redundantly to repress the retinal determination pathway.

As the timing of fate segregation roughly correlated with the timing of disc-specific expression of genes important for the development of eye and antenna, we examined the mechanism regulating the disc-specific gene expression. We found that the antenna genes *hth* and *ct* repress *ey* transcription, and the eye gene *so* represses Ct and Hth expression. Furthermore, the loss of Hth and Ct in the antenna disc resulted in ectopic eye development in the antenna, and loss of *so* in the eye disc resulted in ectopic antenna development in the eye. This means that the eye and antenna pathways mutually antagonize each other, thereby segregating the two primordia and the two developmental fates. The segregation of the eye and antenna primordia are thus maintained by the mutual repression between the eye and antenna pathways genes.

MATERIALS AND METHODS

Fly stocks

Fly culture and crosses were performed according to standard procedure at 25°C unless otherwise noted. The sources of *hsFLP^{22}; Act5C>y+>GAL4, UAS-GFPS65T* (Ito et al., 1997), *dpp-GAL4, UAS-ey*, *UAS-toy, so^{10}-lacZ, ey-GFP* have been described previously (Yao and Sun, 2005). *UAS-hth* has been described previously (Yao et al., 1999). *UAS-ct, ct^{c145} FRT19A* (Blochlinger et al., 1991) was from Yuh Nung Jan (UCSF, San Francisco, CA, USA). *so^{3} FRT42D* (Salzer and Kumar, 2009) was from Justin Kumar (Indiana University, Bloomington, IN, USA). *eyD02-lacZ* was from Walter Gehring (University of Basel, Switzerland) (Hauck et al., 1999). *dE-GAL4* (Morrison and Halder, 2010) was from Georg Halder (BCM, Houston, TX, USA). *hth-GAL4* (Noro et al., 2006) was from Richard Mann (Columbia University, New York, USA). *UAS-hth-RNAi* (transformant ID 12764) was from the VDRC stock center. Other fly stocks were from Bloomington Drosophila Stock Center or Kyoto stock center.

Clonal induction

Positively labeled flp-out expression clones were generated by crossing *UAS*-lines to *hs-FLP^{22}; Act5C>y+>GAL4 UAS-GFPS65T* (Ito et al., 1997). The females were allowed to lay eggs for 12 hours at 25°C. The eggs were cultured for further 24 hours until 70% of them hatched (24~36 hours AEL), then heat-shocked at 37°C for 45 minutes. The larvae were cultured in 25°C until dissection at different stages. The staging was based on mouth hook morphology and disc size. Mutant clones were induced by the FLP-FRT method (Xu and Rubin, 1993). For *so^{3}* clones, *so^{3} FRT42D* males were crossed to *ey-FLP22;* 2x*P[ubi-nls-GFP]FRT42D* virgins.

miRNA constructs

Target sequences of 22 nucleotides were selected from the coding region of *ct*, *ey* and *toy*, respectively, and the UAS-miRNA constructs were as previously described (Chen et al., 2007; Yao et al., 2008). The miRNA target sites were chosen in the coding region because there is no suitable target site in the 5′UTR and 3′UTR. Therefore, we could not use rescue to rule out the possible off-target effects. We designed two miRNAs to target different sites for each gene. The two constructs for each gene gave similar effects (not shown). Similarly, the *mi-hth* and *hth-RNAi*, with different target sites, also gave similar results. These results argue against off-target site effects. The sequence and cloning procedures are available upon request. The *UAS-mi-(toy+ey)* construct is a tandem fusion of *ey* and *toy* miRNAs under the same UAS control. Germline transformants of each construct were generated as described previously (Jang et al., 2003).

Immunohistochemistry

Antibody staining for imaginal discs was as previously described (Pai et al., 1998). Primary antibodies were mouse anti-Ct (1:500), rat anti-Elav (1:500), mouse anti-Eya (1:200) (DSHB, University of Iowa), goat anti-Hth (dG-20, Santa Cruz Biotechnologies) and rabbit anti-β-galactosidase (1:1500, Cappel). Rabbit anti-Ey and guinea-pig anti-Toy (Furukubo-Tokunaga et al., 2009; Halder et al., 1998) were from Uwe Walldorf (Saarland University, Saarbrücken, Germany). Rat anti-Dll (Vachon et al., 1992) was from Stephan Cohen (IMCB, Proteos, Singapore). Rabbit anti-Lim1 (Lilly et al., 1999) was from Juan Botas (BCM, Houston, TX, USA). 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.

ChIP assay

Eye-antenna discs connected with mouth hooks were dissected from L2 larvae (48~72 hours AEL), collected in PBS on ice and fixed with freshly prepared 1.8% formaldehyde. Dissection time was minimized to process about 100 animals in under 1 hour. Chromatin preparation and immunoprecipitation were performed using Magna ChIP kit (Millipore). Anti-Hth (dG-20) and mouse anti-Ct (1:100) were used for immunoprecipitation as described previously (Peng et al., 2009). Specificity was tested by normal mouse IgG (Millipore). Three sets of PCR primers (ChIP-1, ChIP-2 and ChIP-3) located on the *ey3.6* enhancer (see Fig. 5) were used to check the immunoprecipitated chromatin.

Generation of mutant *ey3.6* enhancer transgenic flies

The *ey3.6* enhancer construct (Chotard et al., 2005) was from Iris Salecker (MRC-NIMR, London, UK). Cut- and Hth-binding sites on *ey3.6* enhancer were individually mutated into *Bgl*II and *Xba*I sites (supplementary material Fig. S7) using the QuikChange Multi Site-Directed Mutagenesis kit (Stratagene). The original and mutated *ey3.6* fragments were cloned into the pH-Stinger enhancer tester vector (Barolo et al., 2000) to drive the GFP reporter. The GFP construct is flanked by gypsy insulators to avoid positional effects on expression. Germline transformants of each construct were generated as described previously (Jang et al., 2003). A minimum of three independent transgenic lines were tested for each construct.

GAL4/GAL80^{ts} temperature shift experiment

tub-GAL80^{ts}; hth-GAL4 males were crossed to *UAS-mi-hth; UAS-mi-ct* virgins. The embryos were collected at 17°C for 24 hours and then kept at 17°C until shifted to 30°C at L2 stage for 24 hours. After temperature shift, the larvae were cultured in 17°C. Progeny without temperature shift were used as control.

Scanning electron microscopy

Adult or pharate flies were fixed in Bouin's solution, dehydrated through an ethanol series before transferring to 100% acetone overnight, followed by crucial point drying with liquid CO_2 and sputter coating with gold. Samples were examined using an Environmental Scanning Electron Microscope (FEI Quanta 200).

RESULTS

Gene expression in the early eye-antenna disc

We first searched for transcription or nuclear factors that showed restricted expression in the early eye or antenna disc. These and the references are summarized in Fig. 1. Expression data from this study are presented in supplementary material Fig. S1-S4. In L1 eye-antenna disc, *ey*, *twin of eyeless* (*toy*), *so*, *eye gone* (*eyg*), *hth* and *teashirt* (*tsh*) are expressed uniformly. There is no *engrailed* (*en*) expression at this stage. In e-L2, *orthodenticle* (*otd*; *oc* – FlyBase) becomes expressed throughout the eye-antenna disc. The segregation of the eye and antenna primordia is apparent with the restriction of *ey*, *toy*, *so*, *eyg* and *tsh* expression to the eye disc, the onset of *eyes absent* (*eya*), *dachshund* (*dac*) and *caupolican* (*caup*) expression in the eye disc, the restriction of *hth* to the antenna disc and the onset of *ct* and *Lim1* expression in the antenna disc. At this time, the eye disc showed dorsoventral segregation, with *caup* expressed only in the dorsal region. Both eye and antenna primordia also showed some degree of anteroposterior regionalization, with the restricted expression of *en*/*invected* (*inv*), *dac* and *eya*. At mid to late L2, *Distal-less* (*Dll*) expression appeared in the center of the antenna disc, where *ct* and *Lim1* expression become excluded.

Ectopic Ct or Hth repressed *ey* transcription

As Ct expression extends throughout the antenna disc at e-L2 (Fig. 1) and is one of the earliest marker for antenna disc (Kenyon et al., 2003), we examined the regulation and function of Ct. Ectopic *ct* expression in the eye disc inhibited Ey expression cell-autonomously (Duong et al., 2008) (Fig. 2A,A′; 100%, *n*=36 clones). We first tested whether *ct* can inhibit *ey*. We used the *eyD02-lacZ* (Hauck et al., 1999) as a reporter for *ey* transcriptional regulation. Clonal induction of *ct* inhibited *eyD02-lacZ* cell-autonomously in L2 eye disc (Fig. 2B,B′; 100%, *n*=25 clones). The *so-lacZ* and Toy expression were also inhibited cell-autonomously in ectopic *ct*-expression clones (Fig. 2C-C″; 73%, *n*=37 for *so-lacZ*, 30%, *n*=30 for Toy). We note that the repression occurred only when Ct is expressed at a higher level, based on the intensity of co-expressed GFP. The repression was weaker when the clones were in the anterior domain of the eye disc (*so-lacZ* repressed in 5/14 anterior clones. (Toy repressed in 2/18 anterior clones.) Clone size did not correlate with the repression. These results indicate that Ct could shut off eye fate by repressing the Toy-Ey-So cascade.

hth is also expressed in L2 antenna disc, but has a broader expression than Ct (Fig. 1). Similar to *ct*, clonal induction of *hth* can inhibit *ey* transcription cell-autonomously in L2 eye disc (Fig. 2D,D′; 64%, *n*=40). Together, these results suggested that both *ct* and *hth* may maintain the antenna identity by repressing the Toy-Ey-SO cascade in the L2 antenna disc.

Ectopic expression of *ey* driven by *dpp-Gal4* (*dpp>ey*) can induce ectopic eyes in adult appendages (Halder et al., 1995) (Fig. 3B; 100%, *n*=15 flies). When *ct* or *hth* was co-expressed with *ey*, ectopic eyes formation was nearly completely suppressed in *dpp>ct+ey* (Fig. 3C; 91%, *n*=23 flies) and *dpp>hth+ey* flies (Fig. 3D; 83%, *n*=18 flies). All of these developed to pharate. Ectopic photoreceptor formation also was suppressed in wing discs (Fig. 3C′,D′). These results suggested that *ct* and *hth* also repressed the eye-inducing effect of the Ey protein.

Hth and Ct are required to repress eye fate

We then tested whether endogenous *ct* and *hth* are required to repress *ey* in L2 antenna disc. We examined clones of cells carrying the ct^{c145}-null mutation (Blochlinger et al., 1991). When ct^{c145} clones occurred in L2 antenna disc, Ey protein was not induced in these clones (not shown). ct^{c145} clones caused distal antenna to distal leg transformation in adults, consistent with a previous report (Ebacher et al., 2007). The absence of Ey induction in the *ct* clone suggested that another gene may activate *ct* to repress *ey*. Hth is an obvious candidate. We made microRNAs that target *hth* and *ct* mRNA individually, and used a *UAS-hth-RNAi* line. The expression of *hth* and *ct* miRNAs was driven by *hth*-GAL4 in L2 antenna disc. Ct or Hth protein level was reduced dramatically in *hth>mi-ct* and *hth>mi-hth* antenna disc, respectively (supplementary material Fig. S5). The reduction of Ct or Hth did not reciprocally affect their expression (supplementary material Fig. S5). This is in contrast to the previous finding that Ct expression in the l-L3 antenna disc is activated by Hth and Exd (Dong et al., 2002; Ebacher et al., 2007), and indicated that their regulatory relationships change during development. Single Ct or Hth knockdown did not induce Ey in the antenna disc (Fig. 4A,B). But in the double knockdown *hth>mi-hth+mi-ct* L2 disc, Ey protein clearly expanded into the entire antenna disc at a level comparable with the level in the eye disc

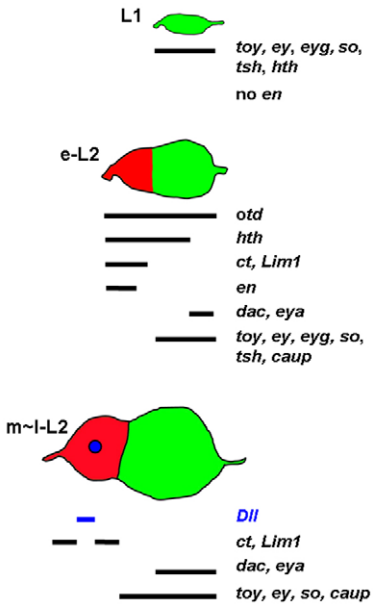

Fig. 1. Gene expression in the early eye-antenna disc. The expression of nuclear factors in the early eye-antenna disc from the literature and from this study is summarized. The names of these genes are listed in italics. The horizontal line indicates the extent of expression. The eye disc is in green and the antenna disc is in red. The line separating the two halves of the eye-antenna disc does not indicate lineage or clonal restriction. The central spot in the antenna disc of Dll expression is marked in blue. As this information come from multiple sources, the relative extent of expression of some of these genes were not based on co-staining in the same sample. The references that describe the expression patterns are listed below. Those based on protein level are labeled with the first character capitalized. Those based on in situ hybridization or reporter expression are labeled as italics. *ey*, *toy*, *so*, Eya, Dac, Notch (Kumar and Moses, 2001); Ey, *hth*, Hth, *tsh* (Singh et al., 2002); *ey*, Ey, Cut, Eya, Dac, Dll (Kenyon et al., 2003); *eyg* (Wang et al., 2008); Hth, Cut, En/Inv (Lebreton et al., 2008); Ey, Cut (Duong et al., 2008); So, Eya, Dac (Halder et al., 1998); Otd (Royet and Finkelstein, 1997); Ey, Caup (Cavodeassi et al., 1999); Hth, Ey, Tsh (Bessa et al., 2002); *so*, Ey, Eya, Dac (Curtiss and Mlodzik, 2000); Toy, *ey*, Ey, *en*, Cut, *so*, Eya, Dac, *tsh*, Hth, Dll, Lim-1 (data from this study; supplementary material Figs S1-S4).

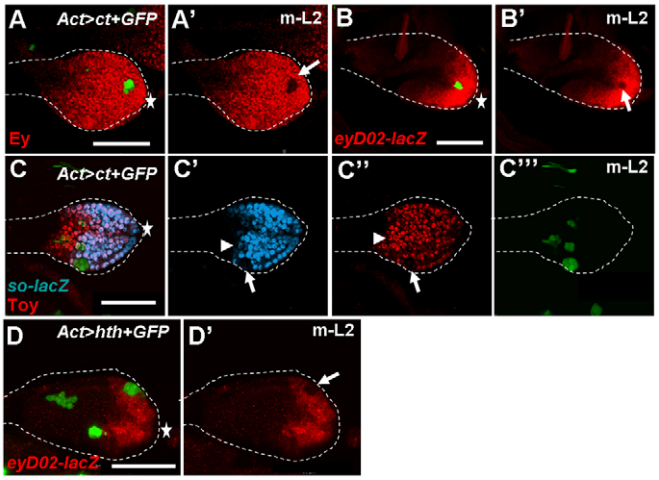

Fig. 2. Ectopic Hth or Ct inhibited transcription of *ey*, *so* and Toy. (**A-C‴**) Flp-out clones of *ct* expression (marked by GFP, green) cell-autonomously inhibited the expression of Ey (A,A′; anti-Ey, red), *eyD02-lacZ* (B,B′; anti-β-galactosidase, red), *so-lacZ* (anti-β-galactosidase, cyan in C,C′) and Toy (anti-Toy, red, C,C″) in L2 eye disc. (**D**,**D′**) Flp-out clones of *hth* expression (marked by GFP, green) cell-autonomously inhibited *eyD02-lacZ* (anti-β-galactosidase, red). Arrows mark the anterior clone that did not repress *so-lacZ* or Toy. Stars indicate the position of the optic stalk. Scale bars: 50 μm.

(Fig. 4C). In *hth>mi-ct* and *hth>mi-hth*, Eya expression remained restricted to the eye disc (Fig. 4D,E). In the double knockdown *hth>mi-hth+mi-ct*, Eya expression expanded into the antenna disc (Fig. 4F). *so-lacZ* showed similar expansion (Fig. 4G). As *hth>mi-hth+mi-ct* flies have high lethality, we had to raise the flies at a lower temperature (17°C) to obtain pharates that could be examined. Knockdown of either *ct* (3/53 died as pharate, the rest eclosed to adult) or *hth* (25/46 died as pharate, the rest eclosed to adult) caused distal antenna-to-leg transformation with complete penetrance (Fig. 4H, *n*=53 for *ct* and *n*=46 for *hth*), similar to the phenotype resulting from *ct* or *hth* mutant clones (Ebacher et al., 2007; Pai et al., 1998). In *hth>mi-hth+mi-ct* flies cultured at 17°C, about 80% died before pharate so cannot be examined. The other 20% died as pharate and small ectopic eyes occasionally appeared in the basal region of antennae or in the transformed A2 segment (Fig. 4I-K; 15%, *n*=20 flies). This is often accompanied by the loss of distal antenna segments or distal antenna-to-leg transformation, loss of maxillary palp, clypeus, labrum, labellum and medial ocellus, and an indentation at the anterior midline commissure (supplementary material Fig. S6). These clearly represented the weakest phenotype. In the discs of *hth>hth-RNAi+mi-ct*, a small ommatidia-like cluster of cells expressing the neuronal marker Elav can be identified in L3 antenna (Fig. 4L,M; 69%, *n*=39 discs) and leg discs (not shown). The location correlated with the *dpp* expression domain (Fig. 4L,M), suggesting that a high level of Dpp is required. This is similar to the requirement for Dpp in Ey-induced ectopic eye development (Chen et al., 1999; Kango-Singh et al., 2003). Stronger ectopic eye phenotype was not observed, perhaps because the lower culture temperature to avoid lethality also reduced the knockdown efficiency. These results suggested that *ct* and *hth* function redundantly to repress Ey, *so* and Eya expression in L2 antenna cells and repressed eye fate.

Ct and Hth repress *ey* transcription directly

As ectopic *ct* or *hth* can inhibit *eyD02-lacZ* expression cell-autonomously (Fig. 2B,D), we tested whether the transcriptional repression is direct. There are several putative Ct- and Hth-binding sites (Andrés et al., 1994; Aufiero et al., 1994; Ebner et al., 2005; Ryoo et al., 1999) in the *ey3.6* enhancer (Fig. 5A, supplementary material Fig. S6). Using chromatin immunoprecipitation (ChIP), we checked whether Hth and Ct can bind to the *ey* locus in L2 eye-antenna disc. Three pairs of PCR primers were used (Fig. 5A). Our results showed ChIP-1 and ChIP-2 regions can be immunoprecipitated by either Ct or Hth (Fig. 5B). We noticed that the signal from Hth is always stronger than from Ct.

We then mutated these binding sites in *ey3.6* and tested the effects in transgenic flies. *ey3.6-GFP* expression was restricted to the L2 eye disc (Fig. 5C). When the three Ct binding sites were mutated, the resulting $ey3.6^{mcutx3}$*-GFP* expression extended partially into the antenna disc (Fig. 5D). Similarly, when the two Hth-binding sites were mutated, $ey3.6^{mhthx2}$*-GFP* expression extended partially into the antenna disc (Fig. 5E). When all binding sites for Hth and Ct were mutated, $ey3.6^{mcutx3+mhthx2}$*-GFP* expression extended into most of the antenna disc (Fig. 5F). These results suggest that both Hth and Ct repress *ey* transcription through direct binding to their respective target sites.

Ct and Hth are repressed in eye disc by So

We next asked whether the antenna program is suppressed in the eye disc by the retinal determination network genes. We first tested the effect of *ey* and *toy* on Ct and Hth expression. Clonal *ey* induction in the antenna disc induced *so* transcription as expected, but did not affect Ct expression (Fig. 6A). We made a single miRNA construct that targets both *ey* and *toy* mRNA, thereby reducing Ey and Toy protein levels at the same time. *dE-GAL4* drives expression in the dorsal part of the eye-antenna disc (Fig. 6G) (Morrison and Halder, 2010). *dE>mi-(toy+ey)+GFP* caused a reduction of eye disc size and Ey expression in the dorsal eye disc (Fig. 6B; 37%, *n*=46 discs). The DV patterning of the eye disc was not lost, as indicated by the dorsal GFP expression (Fig. 6B). Neuronal differentiation and disc size were significantly repressed in L3 eye disc (Fig. 6C; 31%, *n*=22

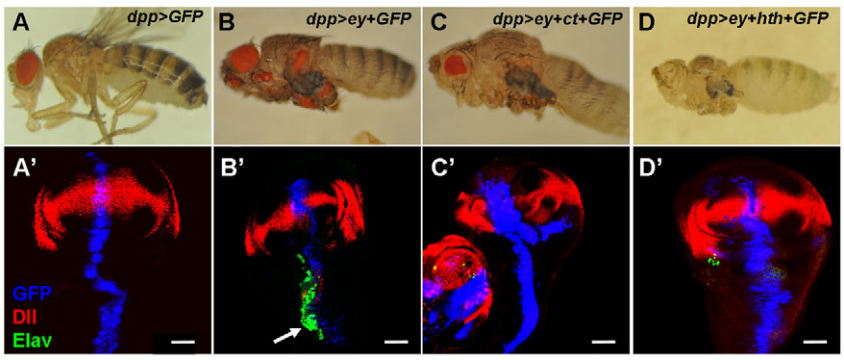

Fig. 3. Ectopic Hth or Ct inhibits activity of Ey. (**A**,**A′**) *dpp>GFP* fly has normal morphology and L3 wing disc. (**B**,**B′**) *dpp>ey+GFP* induced ectopic eyes in wing, antenna and leg. Photoreceptor induction in wing disc is indicated by Elav (arrow, green). (**C**,**C′**) Ectopic eye formation was suppressed by co-expressing *ct* (*dpp>ey+ct*). (**D**,**D′**) Ectopic eye formation was suppressed by co-expressing *hth* (*dpp>ey+hth*). In all L3 wing discs, the *dpp* expression domain was visualized by *dpp>GFP* (blue). Scale bars: 50 μm.

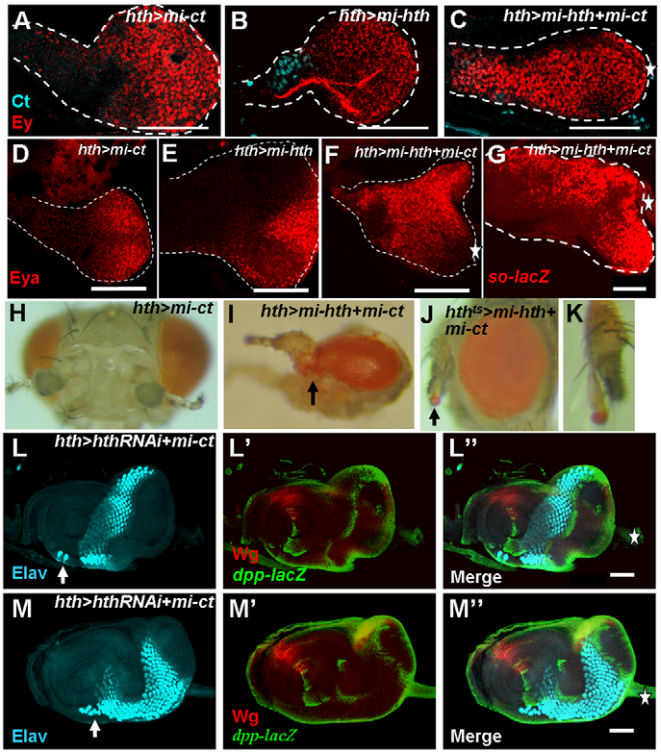

Fig. 4. Hth and Ct function redundantly in repressing *ey*. (A-G) Late L2 eye-antenna discs. (**A**,**B**) Single knockdown of Ct (*hth>mi-ct*; A) or Hth (*hth>mi-hth*; B) did not cause Ey expression in antenna disc (Ey, red; Ct, cyan). (**C**) Double knockdown of Ct and Hth (*hth>mi-hth+mi-ct*) caused Ey (red) de-repressed in antenna disc. Star indicates the position of the optic stalk. (**D**,**E**) Single knockdown of Ct (*hth>mi-ct*; D) or Hth (*hth>mi-hth*; E) did not affect the Eya (red) expression, which remained restricted to the eye disc. (**F**,**G**) In the double knockdown *hth>mi-hth+mi-ct*, Eya (red) expression expanded into the antenna disc (F) and *so-lacZ* (red) showed similar expansion (G). Stars indicate the position of the optic stalk. (**H**) Single knockdown of Ct (*hth>mi-ct*; H) or Hth (*hth>mi-hth*; not shown) caused partial antenna-to-leg transformation. (**I-K**) Double knockdown of Ct and Hth (*hth>mi-hth+mi-ct*) caused the antenna-to-leg transformation and ectopic eye formation in antenna. Arrows indicate the ectopic ommatidia formation in *hth>mi-hth+mi-ct* heads. (J,K) The double knockdown was combined with Gal80^{ts} (denoted as *hth^{ts}>mi-hth+mi-ct*) and cultured at 17°C to prevent the early effect of Hth and Ct knockdown. The larvae were shifted to 30°C at L2 stage for 24 hours, and then returned to 17°C until observation. (**L-M″**) In *hth>hth-RNAi+mi-ct* eye-antenna disc, ectopic cluster of Elav^{+} cells can be found in the mid-L3 antenna disc. These can appear as an isolated cluster (L) or as an anterior extension of or fusion with the endogenous ommatidia development (M-M″). The location of these correlated with the *dpp-lacZ* expression domain (51% discs have Elav clusters located in *dpp-lacZ* domain; 18% discs have Elav clusters located between Wg and *dpp-lacZ* domain; *n*=39.) *dpp-lacZ* is in green; Wg is in red; Elav is in cyan. The stars indicate the location of optic stalk. Arrows indicate the ectopic ommatidia formation. Scale bars: 50 μm.

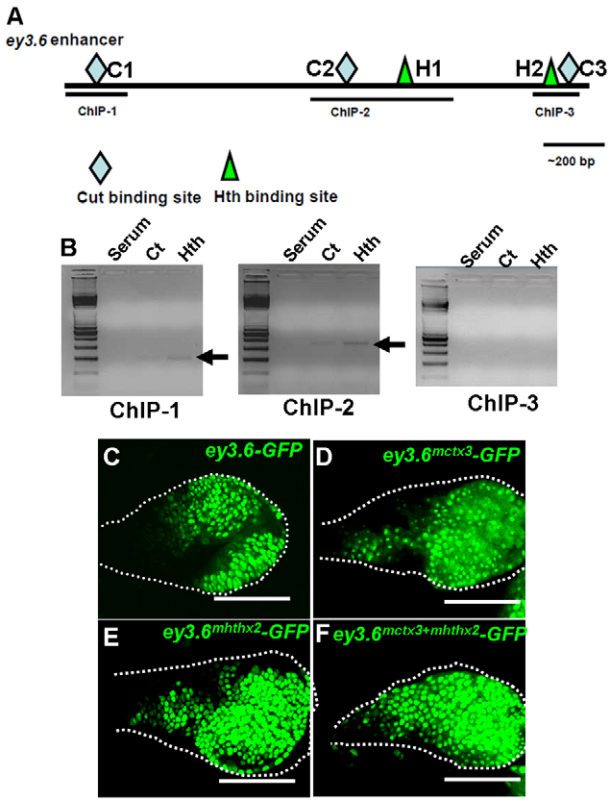

Fig. 5. Ct and Hth directly repress *ey* transcription. (**A**) Three fragments (ChIP-1, ChIP-2 and ChIP-3) from the *ey3.6* enhancer-containing binding sites for Ct and/or Hth were used for the ChIP assay. (**B**) Both Ct and Hth were immunoprecipitated with ChIP-1 (Ct-binding site C1), ChIP-2 (Ct-binding site C2 and Hth-binding site H1), but not with ChIP-3 (Hth-binding site H2 and an incomplete Ct-binding site C3). Serum was used as a negative control in each assay. Arrows indicate the proper band of PCR fragments. (**C-F**) The expression pattern of *ey3.6-GFP* (C), *ey3.6 ^{mcutx3}-GFP* (D), *ey3.6 ^{mhthx2}-GFP* (E) and *ey3.6$^{mcutx2+mhthx2}$-GFP* (F) transgenes (all in the pH-Stinger vector) in mid L2 eye-antenna disc (GFP, green). Scale bars: 50 μm.

discs). However, Ct was not induced in the dorsal eye disc in L2 and L3 disc (Fig. 6B,C). *dE>mi-(toy+ey)+GFP* raised at 25°C all died in the pharate stage with the loss of most head structures, with the labellum, which is derived from the labial disc, remaining (Fig. 6D; 100%, *n*=21 flies; SEM figures not shown). This phenotype is similar to that reported for *ey*-, *toy*- and *eyg*-null mutants (Jang et al., 2003; Jiao et al., 2001; Kammermeier et al., 2001; Kronhamn et al., 2002; Yao and Sun, 2005), and has been interpreted as the loss of the eye-antenna disc (Jang et al., 2003). When raised at 17°C, the adults have small eyes but normal antenna and palp (Fig. 6E; 26%, *n*=34 adult eyes; scanning electron microscope figures not shown). The phenotype is consistent with loss of the corresponding tissue, rather than fate change into head capsule. Clonal induction of *mi-(toy+ey)* in L2 eye cells efficiently inhibited Toy expression but did not affect Ct (Fig. 6F,F′; 100%, *n*=43 clones) or Hth (not shown). These findings suggest that Ct and Hth may not be repressed by Ey and Toy in the L2 eye disc (see Discussion).

We next tested *so* and *eya* for the effect on Ct and Hth expression. Clonal induction of *so* in L2 antenna disc inhibited Ct expression cell-autonomously (Fig. 6H,H′; 100%, *n*=25). Clonally expressing *eya,* the binding partner of So in developing eye, did not affect Ct expression (not shown). These results suggest that So can inhibit Ct in L2 antenna cells. In the hypomorph mutant *so^{1}*, Ct expression expanded into the equatorial region in L2 eye disc (Fig. 6I; 33%, *n*=24 discs). At this stage, the eye disc size is not reduced. Ectopic Ct also was induced in *so^{3}*-null clones in L2 (Fig. 6J; 73%, *n*=15 discs) and L3 (6K; 100%, *n*=22 discs) eye disc. Ectopic Hth was also induced in *so^{3}* clones in L2 eye disc (not shown). Some of the Ct

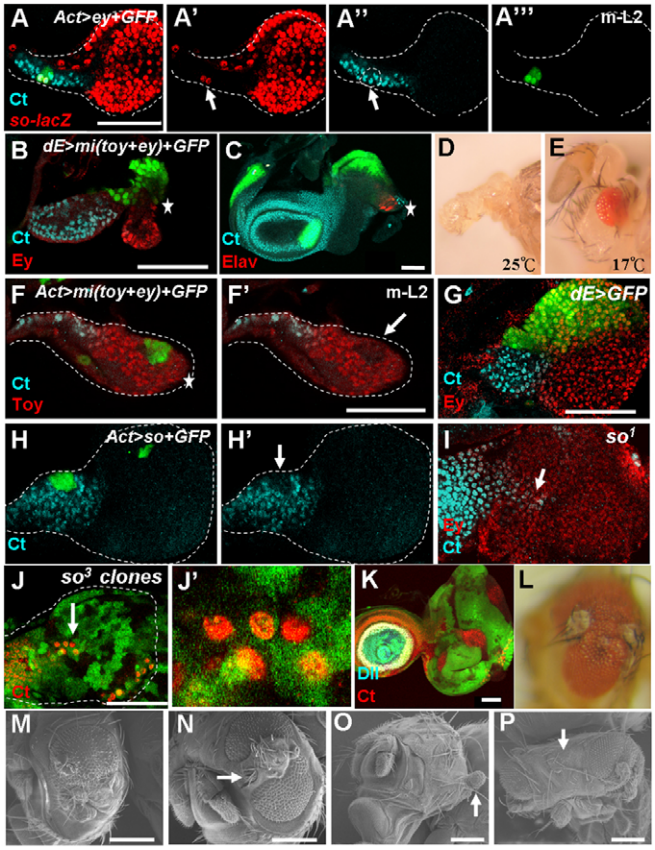

Fig. 6. Ct and antenna cell fate are repressed in eye disc by So. (**A-A‴**) Flp-out clones expressing *ey* (*Act>ey+GFP*, marked by GFP, green) induced *so-lacZ* (A′; anti-β-galactosidase, red) but did not affect Ct expression (cyan; clone is marked by broken white line in A″) in m-L2 disc. Arrows indicate the location of the GFP$^+$ clone. (**B**) *dE>mi(toy+ey)* caused a reduction in eye disc size, elimination of Ey (red), but did not induce Ct (cyan) in the dorsal half of eye disc in l-L2. Star indicates the position of the optic stalk. (**C**) In *dE>mi(toy+ey)* m-L3 eye disc, neuronal differentiation (marked by Elav, red) was inhibited. Star indicates the position of the optic stalk. (**D**) *dE>mi(toy+ey)* adult has no eye and antenna development when cultured at 25°C. (**E**) *dE>mi(toy+ey)* adult has small eye size, but has no ectopic antenna development in the eye disc when cultured at 17°C. (**F**,**F′**) Flp-out clones of *mi(toy+ey)* [*Act>mi(toy+ey)+GFP*, marked by GFP, green] reduced Toy (red) but did not induce Ct (cyan) in l-L2 eye disc. Arrow indicates the location of the GFP$^+$ clone. Star indicates the position of the optic stalk. (**G**) *dE-Gal4* expression pattern in l-L2 eye-antenna disc was marked by GFP (green). Ct (cyan) and Ey (red) marked the antenna and eye disc, respectively. (**H**,**H′**) Flp-out clonal expression of *so* (*Act>so+GFP*, marked by GFP, green) inhibited Ct (cyan) cell-autonomously in l-L2 antenna disc. Arrow indicates the location of the GFP$^+$ clone. (I-K) Reducing So expression caused Ct induction in L2 and L3 eye disc. (**I**) Ct (cyan) expression was expanded into so^1 m-L2 eye disc. Arrow indicates the expansion of Cut. (**J**,**K**) Ct (red) was induced in so^3-null clones (marked by the absence of GFP, green) in m-L2 and l-L3 eye disc. J′ is an enlargement of J. Arrow indicates the region magnified in J′. (K) Dll was not induced in the clones. (**L**) Partial antenna tissue developed from the *ey-flp*-induced so^3 mutant adult eye. (**M-P**) Scanning electron microscopy observations of *ey-flp*-induced so^3 mutant adult heads. These showed ectopic antenna tissue in the eye (M, arrow), head cuticle with antenna-like structures extending into the eye (N, arrow), a small eye become club-like (O, arrow) and loss of ocellus (P, arrow). Scale bars: 200 μm in M-P; 50 μm in A-L.

expression appeared in the wild-type cells adjacent to the so^3 clone (Fig. 6J′), suggesting a non-autonomous mechanism. Not all so^3 mutant cells expressed Ct (Fig. 6J,K). In 76 so^3 clones, Ct was induced in similar frequency in anterior (5), posterior (4), margin (7) and equator (5), suggesting no regional bias. The proportion of so^3 cells with Ct induction increased significantly from L2 to L3 (Fig. 6J,K). This suggested that the expression of Ct in the eye disc requires a positive factor that may be progressively expressed or activated in eye disc, or a negative factor that is progressively lost or inactivated. About 80% of flies with *ey-Flp* induced so^3 clones can eclose as adult, and have small or no eye, but with normal antennae and palp. Ectopic antenna tissue can be found in these adult eye (Fig. 6L; 80%, n=52 adult eyes; Fig. 6M), consistent with previous report (Salzer and Kumar, 2009). Head cuticle with antenna-like structures can invade the eye (Fig. 6N). Some of the small eye become club-like (Fig. 6O), suggesting a partial transformation to antenna, as reported in the misexpression of Dip3 (Duong et al., 2008). There is also occasional loss of ocellus (Fig. 6P), consistent with the role of *so* in ocelli development (Cheyette et al., 1994). These results suggest that Ct and Hth expression are repressed by So in the L2 eye disc. Importantly, the repression served to block the antenna fate.

DISCUSSION

Maintenance of the eye and antenna fate segregation by mutual antagonism

The L1 eye-antenna disc has uniform expression of several transcription factors (Fig. 1), and the eye and antenna fates are not segregated. During e-L2, the uniform disc becomes segregated into two primordia with distinct developmental fates. In the antenna disc, Ct and Hth repressed the Toy/Ey/So eye pathway. In the eye disc, Ct and Hth are repressed by So. Thus, the segregation of the antenna and eye primordia can be stably maintained by their mutual antagonism. These results demonstrated that organ development requires not only the choice of the specific developmental pathway, but also requires the active repression of the opposing developmental pathway. Our results provided the molecular mechanism for such mutual antagonism.

In l-L3 eye-antenna disc, although the expression domain of Ct/Hth and Ey/So are juxtaposed, so^3 clones showed derepression of Ct and Hth only in the most posterior region (zone 4) but not in the more anterior regions behind MF (zones 2 and 3) (Salzer and Kumar, 2009). Thus, there may be an additional mechanism to repress Ct and Hth expression. For Hth, the repression is by Dpp and Hh signaling in L3 eye disc (Lopes and Casares, 2010). It has not been tested whether Ct is also repressed by Dpp and Hh.

For individual cells in the eye-antenna disc, the mutual repression provided a mechanism for a choizce of bistable states, either eye or antenna fate. A bistable state can often be maintained by positive-feedback loop, in addition to mutual repression. Such a positive-feedback loop is known for the eye pathway (Pauli et al., 2005; Pignoni et al., 1997), but has not been reported for the antenna pathway.

The mutual transcriptional repression mechanism is expected to work at the level of individual cells. Therefore, a salt-and-pepper mosaic pattern would be predicted unless there is additional patterning influence. The patterning gene *dpp* is expressed in the posterior margin of e-L2 eye disc, and is required for Eya expression at this stage (Kenyon et al., 2003). However, *dpp* is not required for the restricted expression of Ct and Ey (Kenyon et al., 2003). We propose that there is another patterning gene that biases the antenna disc to express Ct. Thus, the difference between eye and antenna primordia may be predetermined before the onset of Ct and Eya at e-L2.

Subdivision of developmental primordium

The subdivision of a developmental primordium into subprimordia with specific fates is a common requirement in development. For example, the mammalian ventral foregut endoderm differentiates into the adjacent liver and pancreas (Zaret et al., 2008), and a bipotential population of foregut endoderm cells give rise to both liver and pancreas (Deutsch et al., 2001). The maintenance of such division by mutual antagonism has been reported before. For example, the division between the presumptive thalamus and prethalamus in *Xenopus* is due to the mutual repression by the Irx homeodomain proteins and the Fezf zinc-finger proteins (Rodríguez-Seguel et al., 2009). The boundary between optic cup and optic vesicle is maintained by mutual transcriptional repression between Pax6 and Pax2 (Schwarz et al., 2000). Our findings provide a new example, with clear correlation, both temporal and causal, of gene expression changes and developmental fate specification.

The maxillary palp and ocelli are derived from specific regions in the eye-antenna disc (Haynie and Bryant, 1986). The maxillary palp fate does not become segregated from the rest of eye-antenna disc as late as late L3 (Morata and Lawrence, 1978). The timing of ocelli fate decision is not clear. *otd* is required for ocelli development, and is the first marker for the ocellar region: it is ubiquitously expressed in the early L2 eye-antenna disc, and becomes restricted to the ocellar region in the eye disc in early L3 (Finkelstein et al., 1990; Royet and Finkelstein, 1997; Wang et al., 2010; Wieschaus et al., 1992). Thus, the palp and ocelli may be determined as subfields of the antenna disc and eye disc, respectively. This is consistent with our finding that *hth>mi-ct+mi-hth* resulted in the loss of palp (supplementary material Fig. S6), whereas *so* affected ocelli but not palp (Fig. 6).

Repression of Ct and Hth by So

Our results showed that Ct and Hth are repressed by So. Salzer and Kumar (Salzer and Kumar, 2009) also found induction of Ct and Hth expression in so^3 clones in a region far posterior to the MF in l-L3 eye disc. The fact that So represses Ct and Hth in two spatially and temporally distinct situations suggest that this is a conserved function of So.

Whether the repression of Ct and Hth by So is direct transcriptional repression is not clear. Ectopic So expression caused cell-autonomous repression of Ct and Hth (Fig. 6), suggesting that the repression could be direct. Recently it was shown that So acts as a transcriptional repressor to repress *ct* transcription (Anderson et al., 2012). So may interact with a repressor and Groucho (Gro) is a likely candidate. So can bind to Gro (Kenyon et al., 2005; Silver et al., 2003) and the So-Gro complex was postulated to repress Dac transcription in eye disc (Salzer and Kumar, 2009). The zebrafish So homologue Six3 interacts with Groucho and functions as a transcriptional repressor (Kobayashi et al., 2001). The transcriptional co-repressor CtBP has been shown to functionally and physically interact with Ey, Dac and Dan (Hoang et al., 2010). Whether the protein complex also involves So has not been determined. Overexpression of CtBP caused eye and antenna defect, but the phenotype was not affected by reducing *so* dose (Hoang et al., 2010). Therefore, CtBP is probably not the co-repressor for So.

We found that so^3 clones caused non-autonomous induction of Ct in its surrounding wild-type cells (Fig. 6). Salzer and Kumar (Salzer and Kumar, 2009) also reported similar non-autonomous induction of Dac in L3 disc. They observed elevated Delta within the mutant clone and elevated activated N at the border of mutant clone, thus suggesting that the non-autonomous induction is due to N signaling to surrounding cells. Whether a similar mechanism operates in the L2 disc remains to be tested.

The finding that *ey* and *toy* do not repress Ct and Hth, in both gain-of-function and loss-of-function experiments, was initially perplexing. Clonal *ey* expression in the antenna disc did not repress Ct and Hth. In these clones, *so-lacZ* was induced, but not in all ey^+ cells and at a level lower than the endogenous level in most cells in the eye disc (Fig. 6A′). When *ey* was clonally induced at 29°C, Ct level was reduced (supplementary material Fig. S8). These results suggested that the ectopic *ey* and *toy* at 25°C induced *so* at a level not sufficient to repress Ct. The strength of Ey has been shown to be crucial for its ability to induce ectopic eye development (Weasner et al., 2009). In the double knockdown of *ey* and *toy* in the eye disc, Ct and Hth were not induced. Judging from the eye disc phenotype and residual neuronal differentiation, the knockdown was not complete and may account for the failure to detect Ct and Hth derepression. Alternatively, additional factors, independent of *ey* and *toy*, may also repress Ct and Hth expression. This would be consistent with the weak effect of so^3 clones in inducing Ct and Hth expression.

Hth expression is initially uniform in the eye-antenna disc but becomes restricted to the antenna disc in e-L2. In L3 eye disc, Hth expression is downregulated by Dpp and Hh, produced from the progressing MF and developing photoreceptors, respectively (Lopes and Casares, 2010). However, Hth expression retracted from the posterior part of the eye disc in e-L2, even before the initiation of MF and photoreceptor differentiation. At e-L2, *dpp* and *hh* are expressed in the posterior region of the eye disc (Borod and Heberlein, 1998; Cavodeassi et al., 1999; Chanut and Heberlein, 1997; Cho et al., 2000; Kenyon et al., 2003; Royet and Finkelstein, 1997). It is possible that the early Hh and Dpp contributed to the repression of Hth from the eye disc, in addition to the repression by So.

Transcriptional repression of *ey* by Ct and Hth

Our results showed that Ct and Hth represses *ey* transcription. The binding sites for both Hth and Ct in *ey3.6* are required for its repression in the antenna disc, suggesting that both Hth and Ct bind to the *ey3.6* enhancer directly. The ChIP assay results showed that both Hth and Ct can bind to the ChIP-1 fragment, which contains the binding site for Ct but not for Hth. This suggests that the Hth may bind through a Hth-Ct complex. However, as ectopic expression of either Hth or Ct is sufficient to repress *ey* transcription, the repression does not require the formation of the Hth-Ct complex.

In the RNAi experiments, knocking down Ct or Hth individually did not cause de-repression of the eye pathway genes. However, when the Ct- or Hth-binding site in *ey3.6* was separately mutated, the repression of *ey3.6* in the antenna disc was partially lost. One possible explanation for the discrepancy is that the RNAi knockdown was not complete. When the binding sites for both Ct and Hth were mutated, the de-repression of *ey3.6* in the antenna disc was strongly enhanced. It is possible that both Ct and Hth contributed to the repression of *ey* transcription, and a threshold net amount of these repressors is required.

Hth physically interacts with Exd through the MH domain of Hth and the PBC-A domain of Exd to promote Exd nuclear localization (Abu-Shaar et al., 1999; Jaw et al., 2000). Hth generally acts as a transcriptional activator (Inbal et al., 2001), but Hth and Exd can interact with En or UbxIa to repress transcription (Gebelein et al., 2002). Thus, Hth would need to interact with a repressor to repress *ey*. Ct can serve such a role. Ct can act as a transcriptional repressor by direct binding to a target gene (Valentine et al., 1998). The human and mouse Ct homologues generally function as transcriptional repressor (Nepveu, 2001; Sansregret and Nepveu, 2008). However, as ectopic expression of

Hth alone in the eye disc, in the absence of Ct, is sufficient to repress *ey*, Hth must be able to interact with an additional repressor.

We found that Ct can also block the function of Ey when co-expressed with Ey (Fig. 3). It is possible that the block resulted from the repression of *toy* transcription, which may reduce the strength of the feedback regulation of the retinal determination gene network.

Role of Ct and Hth in antenna development

Although Ct is expressed in L2 in the entire antenna disc, the phenotype caused by *ct* clones affected only restricted domains, perhaps owing to its later restricted expression (Dong et al., 2002; Ebacher et al., 2007). In this study, we report a novel function of Ct in antenna development. Ct and Hth function redundantly to repress the retinal determination pathway. Because of this functional redundancy, this Ct function was not revealed in *ct* clones.

hth or *exd* mutations caused antenna-to-leg transformation (Casares and Mann, 1998; Pai et al., 1998). Hth has a role in blocking eye development at the anterior margin of the eye disc (Pai et al., 1998), where Ct is not expressed. In the antenna disc, this function is masked because of the functional redundancy with Ct revealed in this study.

Even when both *ct* and *hth* were knocked down in their endogenous expression domain (*hth>mi-hth+mi-ct*), we did not observe significant transformation of the antenna to eye in adult. One possible reason is that the *hth>mi-hth+mi-ct* caused lethality and the flies have to be raised at a lower temperature, thereby excluding a stronger phenotype. Another possibility is that the *Dll* expression in the antenna disc served to block eye development. *Dll* and *hth* are required in parallel for normal antenna development. Co-expression of *Dll* and *hth* can induce the formation of antenna structures in many ectopic sites (Cohen and Jürgens, 1989; Dong et al., 2000). It may be the presence of Dll that blocked eye development and provided a leg identity to cause the distal antenna-to-leg transformation found in *hth>mi-hth+mi-ct* flies.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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