Development 137, 273-281 (2010) doi:10.1242/dev.041244

The transcriptional co-factor Chip acts with LIM-homeodomain proteins to set the boundary of the eye field in *Drosophila*

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SUMMARY

Development involves the establishment of boundaries between fields specified to differentiate into distinct tissues. The *Drosophila* larval eye-antennal imaginal disc must be subdivided into regions that differentiate into the adult eye, antenna and head cuticle. We have found that the transcriptional co-factor Chip is required for cells at the ventral eye-antennal disc border to take on a head cuticle fate; clones of *Chip* mutant cells in this region instead form outgrowths that differentiate into ectopic eye tissue. Chip acts independently of the transcription factor Homothorax, which was previously shown to promote head cuticle development in the same region. Chip and its vertebrate CLIM homologues have been shown to form complexes with LIM-homeodomain transcription factors, and the domain of Chip that mediates these interactions is required for its ability to suppress the eye fate. We show that two LIM-homeodomain proteins, Arrowhead and Lim1, are expressed in the region of the eye-antennal disc affected in *Chip* mutants, and that both require *Chip* for their ability to suppress photoreceptor differentiation when misexpressed in the eye field. Loss-of-function studies support the model that Arrowhead and Lim1 act redundantly, using Chip as a co-factor, to prevent retinal differentiation in regions of the eye disc destined to become ventral head tissue.

KEY WORDS: Photoreceptor, Chip, LIM-HD, Arrowhead, Lim1, Homothorax, *Drosophila*

INTRODUCTION

During development, patterning signals progressively restrict cell fates by subdividing large multipotent regions into smaller fields with limited potential. The *Drosophila* head provides a good model system to study such positional fate restrictions. The adult eye, antenna, maxillary palp and head capsule develop from a common epithelial bilayer, the eye-antennal imaginal disc, which is derived from a small group of cells that invaginate from the embryonic ectoderm and proliferate during the larval stages (Haynie and Bryant, 1986; Wolff and Ready, 1993). In the third larval instar, a wave of photoreceptor differentiation led by an indentation called the morphogenetic furrow (MF) initiates at the posterior margin of the eye disc and progresses toward the anterior (Wolff and Ready, 1991). Photoreceptors differentiate in the columnar epithelium posterior to the MF, whereas surrounding regions of the eye disc and the overlying peripodial epithelium instead develop into head cuticle (Dominguez and Casares, 2005). The mechanism by which the eye-head field is subdivided into domains fated to produce distinct adult structures remains poorly understood.

Retinal specification is primarily controlled by two orthologues of the vertebrate *Pax6* gene: *twin of eyeless* (*toy*) and *eyeless* (*ey*) (Czerny et al., 1999; Kronhamn et al., 2002; Quiring et al., 1994). Ectopic expression of *toy* or *ey*, in conjunction with the signaling molecules Hedgehog (Hh) and Decapentaplegic (Dpp), is sufficient to drive retinal development in other imaginal discs (Halder et al., 1995; Kango-Singh et al., 2003; Chen et al., 1999; Czerny et al., 1999). Although the eye-antennal disc is assembled in the embryo from cells that arise from at least three different head segments (Jurgens and Hartenstein, 1993), late in embryogenesis the entire disc expresses *toy* and *ey* and thus seems to have a common identity (Czerny et al., 1999; Daniel et al., 1999). Subdivision of the disc into separate eye and antennal anlagen is first apparent in the early second instar, when the antennal disc downregulates Ey and instead expresses the homeodomain protein Cut, and the eye disc initiates expression of another retinal determination gene, Eyes absent (Eya) (Halder et al., 1998; Kenyon et al., 2003; Dominguez and Casares, 2005). At the same stage, a separate field that gives rise to the maxillary palps and expresses the homeodomain protein Deformed is established in the ventral posterior antennal disc (Lebreton et al., 2008).

Although the mechanism that limits Ey expression to the retinal field is unknown, some factors that restrict the location of photoreceptor differentiation have been identified. One of these is the signaling molecule Wingless (Wg), which is secreted from the anterior lateral regions of the eye disc and from cells surrounding the eye field (Baker, 1988; Tomlinson, 2003), and acts to promote head capsule differentiation and restrict eye development (Heslip et al., 1997; Ma and Moses, 1995; Royet and Finkelstein, 1996; Treisman and Rubin, 1995; Legent and Treisman, 2008). Wg counteracts Ey activity by repressing the expression of the retinal determination genes *eya*, *sine oculis* (*so*) and *dachshund* (*dac*), which are direct targets of Ey (Baonza and Freeman, 2002; Niimi et al., 1999; Pappu et al., 2005; Ostrin et al., 2006). Wg also enhances the expression of two homeodomain transcription factors important for head development: Homothorax (Hth) and Orthodenticle (Otd) [Ocelliless (Oc) – FlyBase] (Pichaud and Casares, 2000; Royet and Finkelstein, 1997; Blanco et al., 2009). These expression patterns are maintained by feedback loops; Eya represses *wg* expression at

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Accepted 17 November 2009

the posterior of the eye disc (Hazelett et al., 1998), whereas Hth maintains ventral *wg* expression (Pichaud and Casares, 2000). Hth has a dual role in eye-antennal disc development; anterior to the MF, it acts together with Ey and the zinc finger protein Teashirt to maintain retinal progenitor cells in a proliferative state (Bessa et al., 2002), but in the ventral head it acts in combination with the co-factor Extradenticle (Exd) to suppress the eye fate (Pai et al., 1998; Pichaud and Casares, 2000; Gonzalez-Crespo and Morata, 1995).

Members of the family of LIM-homeodomain (LIM-HD) transcription factors direct regional specification in the wing and leg imaginal discs (Blair et al., 1994; Cohen et al., 1992; Diaz-Benjumea and Cohen, 1993; de Navascues and Modolell, 2007; Pueyo et al., 2000; Tsuji et al., 2000). In the CNS, LIM-HD proteins define neuronal identity in a combinatorial manner (Dawid and Chitnis, 2001; Lumsden, 1995; Thor et al., 1999). LIM-HD proteins, which contain two LIM protein-protein interaction domains followed by a DNA-binding homeodomain, require the ubiquitous co-factor Chip (known in vertebrates as NLI, Ldb or CLIM) in order to regulate gene expression (Retaux and Bachy, 2002). Chip mediates the formation of multimeric complexes by binding to LIM-HD proteins through its LIM interaction domain (LID) and dimerizing through a separate domain (Jurata et al., 1998; Milan and Cohen, 1999; Rincon-Limas et al., 2000; van Meyel et al., 1999). Loss of *Chip* function leads to pleiotropic defects that mimic the absence of multiple LIM-HD proteins (Morcillo et al., 1997; Pueyo and Couso, 2004; Torigoi et al., 2000; van Meyel et al., 2000). Chip also functionally interacts with other classes of transcription factors during embryonic segmentation and neural development (Ramain et al., 2000; Torigoi et al., 2000; Heitzler et al., 2003).

Here we show that Chip acts independently of Hth to establish the ventral boundary between eye and head tissue. *Chip* mutant clones at the ventral margin of the eye-antennal disc autonomously differentiate as ectopic eyes. We have identified two LIM-HD proteins, Arrowhead (Awh) and Lim1, as likely partner proteins for Chip in the eye disc. These transcription factors restrict eye development by limiting the expression of Ey and downstream genes.

MATERIALS AND METHODS

Drosophila strains

The lethal allele $Chip^{45F}$ was isolated in a mosaic screen for genes required for the normal pattern of photoreceptor differentiation (Janody et al., 2004). The *45F* mutation was mapped by meiotic recombination with $P(w^+)$ elements (Zhai et al., 2003) to a region containing the *Chip* gene; it failed to complement the previously described strong allele $Chip^{e5.5}$ (Morcillo et al., 1997) and had indistinguishable phenotypes. The coding region of *Chip* was amplified by PCR from homozygous $Chip^{45F}$ mutant larvae and found to contain a premature stop codon at position 111 (Q111@). Other alleles used were FRT19A, $Lim1^{7B2}$ (Tsuji et al., 2000), FRT82, hth^{B2} (Pichaud and Casares, 2000), Awh^{11}, Awh^{13} and Awh^{16} (Curtiss and Heilig, 1995). Transgenic flies used were wg^{P} (Kassis et al., 1992), UAS-ChipFL, UAS-ChipΔLID, *UAS*-ChipΔDD (van Meyel et al., 1999), UAS-Hth-GFP (Casares and Mann, 1998), UAS-Lim1 (Tsuji et al., 2000), UAS-Lim3 (Thor et al., 1999), UAS-Awh (Curtiss and Heilig, 1997), UAS-Ap (Milan et al., 1998), UAS-Tup (Thor and Thomas, 1997) $mirr^{cre2}$ (Netter et al., 1998), *fng-lacZ* (Grammont and Irvine, 2001), *eyg-lacZ* (Dominguez et al., 2004) and *E*(*spl*)*m*β-*lacZ* (Cooper et al., 2000).

Mosaic analysis

Stocks used to generate clones of *Chip* mutant cells were *y, w, eyFLP1*; FRT42D, *P*(*Ubi-GFP*)*; y, w, eyFLP1*; FRT42D, *P*(*arm-lacZ*)*; y, w, eyFLP1*; FRT42D, *M*(*2*)*58F*, *P*(*Ubi-GFP*)/CyO, $P(y^+)$*;* and *y, w, eyFLP1*; FRT42D, *M*(*2*)*58F*, *P*(*arm-lacZ*)/CyO, $P(y^+)$. Stocks used to generate clones of *hth* mutant cells were *y, w, eyFLP1*; FRT82B, *P*(*Ubi-GFP*)/TM6B; and *y, w, eyFLP1*; FRT82B, *P*(*Ubi-GFP*)*, M*(*3*)*96C*/TM6B. Rescue and misexpression experiments were conducted using the mosaic analysis with a repressible cell marker (MARCM) system using the following stocks: *hsFLP122*, UAS-GFP; FRT42D, *tub*-GAL80; *tub*-GAL4/TM6B; FRT42D*,* $Chip^{45F}$/CyO, $P(y^+)$; UAS-ChipFL (or UAS-ChipΔLID or UAS-ChipΔDD)/TM6B; FRT42D; UAS-LIM-HD; FRT42D, $Chip^{45F}$/CyO, $P(y^+)$; UAS-LIM-HD; *hsFLP122*, UAS-GFP; *tub*-GAL4; FRT82B, *tub*-GAL80; and UAS-LIM-HD; FRT82, hth^{B2}. Generation of *Lim1* clones in an *Awh* mutant background was done using the stocks FRT19, *Ubi-GFP*, *eyFLP1*; Awh^{11} (or Awh^{13})/TM6B and FRT19, $Lim1^{7B2}$/FM7; FRT80, Awh^{16}/TM6B. When using *hsFLP122*, clones were induced by a 1 hour heat shock at 38.5°C in both the first and second larval instars.

Immunohistochemistry

Staining of eye discs with antibodies or X-gal was performed as described (Lee et al., 2001). Primary antibodies were rat anti-Elav (1:100; Developmental Studies Hybridoma Bank), rabbit anti-Eyeless (1:1000) (Halder et al., 1998), mouse anti-Eya (1:10; Developmental Studies Hybridoma Bank), mouse anti-β-galactosidase (1:200; Promega), rabbit anti-β-galactosidase (1:5000; Cappel), rabbit anti-GFP (1:1000; Molecular Probes), mouse anti-GFP (1:200; Santa Cruz Biotechnology), rabbit anti-Atonal (1:5000) (Jarman et al., 1995), rabbit anti-Lim1 (1:1000; gift of Juan Botas), rabbit anti-Chip (1:500) (Morcillo et al., 1997) and rabbit anti-Homothorax (1:500) (Kurant et al., 1998). Secondary antibodies were from Jackson ImmunoResearch; FITC, TRITC or Cy5 conjugates were used at 1:200 and Alexa fluor 488 conjugates at 1:1000. Images were captured on a Leica TCS NT confocal microscope or on a Zeiss LSM 510 confocal microscope.

In situ hybridization

In situ hybridization to eye-antennal imaginal discs was performed using antisense RNA probes labeled with digoxigenin-UTP (Roche). Sense RNA probes were used as negative controls (data not shown). Riboprobe preparation and subsequent procedures were as detailed in Roignant et al. (Roignant et al., 2006).

Adult head preparation

Adult heads were dissected in PBS, mounted in Hoyer's solution and cleared at 65°C for 2 days.

RESULTS

Chip prevents photoreceptor differentiation at the ventral eye-antennal disc border

We have previously described a mosaic genetic screen for mutations that alter the normal pattern of photoreceptor differentiation in the *Drosophila* eye disc (Janody et al., 2004). Clones homozygous for one mutation isolated in this screen resulted in ectopic eye tissue in the ventral head capsule (Fig. 1A; see Fig. S1E in the supplementary material). Mapping and complementation revealed that this mutation was allelic to *Chip*, which encodes a co-factor for a variety of transcription factors (Pueyo and Couso, 2004; Weihe et al., 2001; Chen et al., 2002; Heitzler et al., 2003; Torigoi et al., 2000; van Meyel et al., 1999; Rincon-Limas et al., 2000; Ramain et al., 2000) that is ubiquitously expressed in the eye-antennal disc and other tissues (Morcillo et al., 1997) (see Fig. S2G-I in the supplementary material). Clones homozygous for the previously described strong allele $Chip^{e5.5}$ also differentiated into ectopic eye tissue (data not shown), confirming that this phenotype is caused by loss of *Chip* function. Our allele, $Chip^{45F}$, has a stop codon predicted to produce an early truncation of the protein (see Fig. 4A), and is therefore likely to be null.

In addition to producing ectopic eye differentiation, *Chip* mutant clones resulted in head cuticle defects, including abnormal clustering of sensory vibrissae on the ventral head (see Fig. S1C,D in the supplementary material), malformations of the

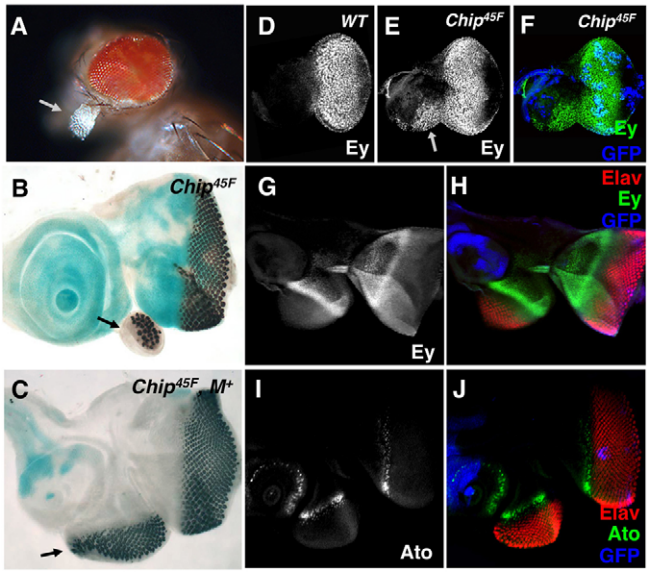

Fig. 1. Chip inhibits retinal differentiation at the ventral eye-antennal disc boundary. (**A**) A *w*⁻ *Chip* mutant clone in the adult head autonomously forms an ectopic ventral eye (arrow). (**B**,**C**) Late third instar eye-antennal discs with *Chip* mutant clones identified by the absence of blue X-gal staining. Photoreceptors are stained with anti-Elav (brown). Anterior is to the left in this and all subsequent figures. Small ventral clones (B) and large clones generated in a *Minute* background (C) differentiate ectopic photoreceptors (arrows). (**D-F**) Early third instar discs stained with anti-Ey (D,E, green in F). (D) Wild type. (E,F) *Chip* mutant clones generated in a *Minute* background marked by the absence of GFP (blue in F). Ey is misexpressed in the antennal disc in the absence of *Chip*. (**G-J**) *Chip* mutant clones generated in a *Minute* background in late third instar eye-antennal discs are marked by the absence of GFP (blue in H,J). Photoreceptors are stained with anti-Elav (red in H,J). Outgrowths from the ventral eye-antennal disc show strong Ey expression (G, green in H) and a stripe of Ato (I, green in J) proximal to the region of Elav expression, indicating that a morphogenetic furrow initiated at the distal tip of the outgrowth.

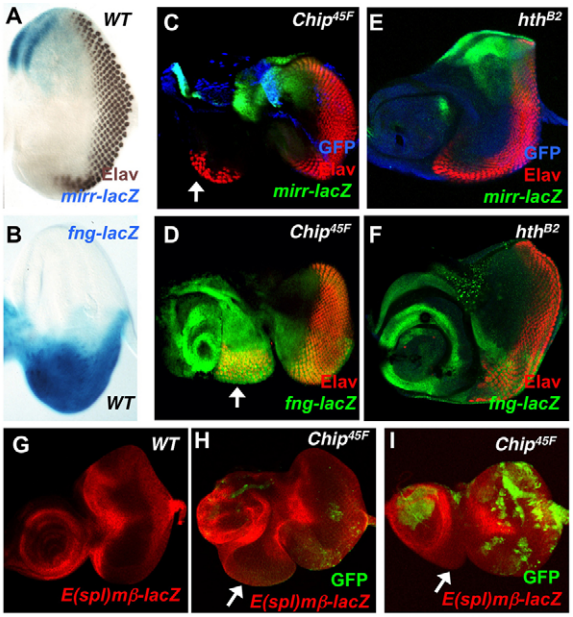

Fig. 2. *Chip* does not regulate dorsoventral polarity. (**A**,**B**) Wild-type eye discs stained with X-gal to reveal *mirr-lacZ* expression (A) or *fng-lacZ* expression (B). (**C-F**) Eye discs with *Chip* mutant clones generated in a *Minute* background (C,D) and eye discs with *hth*^{B2} mutant clones generated in a *Minute* background (E,F), marked by the absence of GFP (blue in C,E). Staining with anti-β-galactosidase (green) shows the expression pattern of *mirr-lacZ* (C,E) or *fng-lacZ* (D,F). Photoreceptors are stained with anti-Elav (brown in A, red in C-F). Ventral outgrowths within *Chip* mutant clones (arrows in C,D) express *fng* in all cells and do not express *mirr*. *hth* mutant clones also show normal *mirr* and *fng* expression, but usually do not produce outgrowths. (**G-I**) Eye discs expressing *E*(*spl*)*m*β-*lacZ*, stained with anti-β-galactosidase (red). (G) Wild type. (H,I) *Chip* mutant clones generated in a *Minute* background, marked by the absence of GFP (green). *E*(*spl*)*m*β-*lacZ* marks the equator in wild-type discs, but is not expressed in *Chip* mutant outgrowths (arrows) in late third instar (H) or early third instar (I).

rostral membrane separating the antennae (see Fig. S1C in the supplementary material) and asymmetric placement of the ocelli on the dorsal head (see Fig. S1D in the supplementary material). In the most extreme cases, the vibrissae, antennae and maxillary palps were absent (see Fig. S1E,F in the supplementary material). Although the size of the endogenous eye was sometimes reduced (see Fig. S1F in the supplementary material), ommatidial differentiation appeared normal. *Chip* is thus required for correct differentiation of the head cuticle, but not the retina.

We further investigated the role of Chip in preventing presumptive head cuticle cells from differentiating into ectopic eyes. Using a *P*(*white*$^{+}$) transgene to mark wild-type tissue, we found that the ectopic ventral eyes were entirely composed of *Chip* mutant tissue, indicating that *Chip* acts autonomously (Fig. 1A). To understand the origin of this phenotype, we examined *Chip*^{45F} mutant clones during the third larval instar at the time of photoreceptor differentiation. We observed ectopic expression of the neuronal nuclear marker Elav only within *Chip* mutant clones arising from the ventral margin of the eye-antennal disc, indicating that Chip has an autonomous and region-specific function. *Chip* mutant clones within the retinal field did not alter the normal pattern of Elav staining (Fig. 1B).

When *Chip* was removed from almost the entire eye-antennal disc by making clones in a *Minute* background (Morata and Ripoll, 1975), abnormal growth was sometimes observed at the dorsal margin, but ectopic photoreceptor differentiation still occurred only in an outgrowth arising from the ventral border between the eye and antennal discs (Fig. 1C). This outgrowth appeared to behave as an independent eye field, with an MF labeled by the basic helix-loop-helix (bHLH) protein Atonal (Ato) (Jarman et al., 1995) that initiated at its distal tip and progressed towards the junction with the endogenous eye-antennal disc (Fig. 1I,J). The eye selector gene *ey* was expressed in the outgrowth and downregulated in the region of photoreceptor differentiation (Fig. 1G,H), as in the normal eye disc. Ey misexpression in the antennal disc was already observed in *Chip* mutant clones at the early third instar, before any overgrowth or ectopic photoreceptor differentiation (Fig. 1D-F), suggesting that it might have a causative role in ectopic eye formation.

Loss of *Chip* had no effect on dorsoventral patterning; the entire outgrowth expressed *fringe* (*fng*), a marker of the ventral half of the eye disc, and failed to express *mirror* (*mirr*), a marker of the dorsal half of the eye disc (Cho and Choi, 1998; McNeill et al., 1997) (Fig. 2A-D). Growth of the wild-type eye disc depends on Notch activation at the dorsoventral boundary triggered by asymmetric *fng*

expression (Dominguez and de Celis, 1998; Cho and Choi, 1998; Papayannopoulos et al., 1998). Similarly, dorsal outgrowths seen in clones lacking the dorsally expressed GATA transcription factor Pannier arise from the juxtaposition of mutant cells with ventral identity and wild-type cells with dorsal identity (Maurel-Zaffran and Treisman, 2000). By contrast, ectopic *Chip* mutant eye fields arise in a field of uniform *fng* expression, suggesting that their growth is Notch independent. Consistent with this interpretation, the Notch target genes *Enhancer of split mβ* [*E*(*spl*)*mβ*] and *eyegone* (*eyg*), which mark the dorsoventral boundary of the eye disc (Chao et al., 2004; Cooper et al., 2000; Dominguez et al., 2004) (Fig. 2G), were not expressed in the central region of *Chip* mutant outgrowths (Fig. 2H,I and data not shown).

Chip inhibits eye differentiation independently of Hth

Similar ectopic eyes have been reported to arise from the ventral margin of the eye-antennal disc in clones mutant for *hth* or its co-factor *exd*, which encode interacting homeodomain transcription factors (Pichaud and Casares, 2000; Pai et al., 1998; Gonzalez-Crespo and Morata, 1995; Ryoo et al., 1999). Removal of *hth* also does not alter dorsoventral identity as assessed by *mirr* and *fng* expression (Fig. 2E,F). We therefore investigated whether Chip might repress eye differentiation by promoting the expression or function of Hth. Hth is expressed in the most anterior domain of the eye disc (Bessa et al., 2002) as well as in undifferentiated cells near the posterior. In *Chip* mutant clones differentiating into ectopic eyes, Hth was expressed normally in the original eye disc, and additional Hth expression was induced posterior to the MF in the ectopic eye disc (Fig. 3D,E). Hth was unaffected in early third instar eye discs with large *Chip* mutant clones (Fig. 3A-C). *Chip* is thus not required to initiate or maintain Hth expression. Likewise, *hth* mutant clones had no effect on Chip expression (see Fig. S2J-L in the supplementary material).

As *Chip* encodes a transcriptional co-factor capable of interacting with homeodomain-containing proteins (Torigoi et al., 2000), it is possible that it might be a co-factor for Hth. Hth represses ectopic ventral eye development in part by maintaining the expression of *wingless* (*wg*) (Pichaud and Casares, 2000). By contrast, *wg* was still expressed in the ventral anterior eye disc, and ectopically expressed at the lateral edges of the outgrowth, in *Chip* mutant clones (Fig. 3F,G). As *Chip* does not affect the expression of the Hth target gene *wg*, Chip is likely to act in parallel to or downstream of Hth to prevent eye differentiation. To investigate whether Chip acts downstream of Hth, we used the MARCM approach (Lee and Luo, 1999) to ectopically express Hth specifically within *Chip* mutant clones. Misexpression of Hth in clones of cells in the retina prevents normal photoreceptor differentiation (Pichaud and Casares, 2000; Pai et al., 1998) (Fig. 3H,I). We found that ectopic Hth expression was still able to inhibit photoreceptor differentiation in *Chip* mutant cells, indicating that *Chip* is not required downstream of Hth (Fig. 3J,K). In addition, large *hth* mutant clones generated in a *Minute* background resulted in ectopic ventral photoreceptor differentiation, but did not cause outgrowths like those seen in large *Chip* mutant clones (Fig. 2E,F). Taken together, these results suggest that Chip and Hth use distinct mechanisms to prevent ectopic eye differentiation at the ventral boundary of the eye-antennal disc.

Chip requires its LIM interaction domain to prevent eye differentiation

Chip is known to interact with LIM-homeodomain proteins such as Apterous (Ap) through its LID (Milan and Cohen, 1999; van Meyel et al., 1999), as well as with the GATA factor Pannier through its N-terminal proline-rich domain (P-rich) (Ramain et al., 2000) and with Bicoid, Fushi tarazu and other homeodomain proteins through its other interaction domain (OID) (Torigoi et al., 2000) (Fig. 4A). To identify potential interaction partners for Chip in the eye disc, we first examined whether the LID was required for limiting eye differentiation. We found that expression of a full-length UAS-Chip transgene completely prevented ectopic photoreceptor differentiation within *Chip* mutant clones (Fig. 4C), although it had no effect when expressed in wild-type eye discs (see Fig. S3F in the supplementary material). By contrast, *Chip* mutant clones expressing UAS-ChipΔLID, a transgene with the LID deleted (van Meyel et al., 1999), still differentiated ectopic photoreceptors in the ventral anterior eye disc (Fig. 4B,C). UAS-Chip and UAS-ChipΔLID show equivalent activity when misexpressed in the wing and leg discs (van Meyel et al., 1999; Pueyo and Couso, 2004). The failure of ChipΔLID to rescue *Chip* mutant clones therefore indicates that the LID is required for Chip to limit eye development, and suggests that Chip acts with one or more LIM protein(s) in the ventral head primordium. Chip forms multimeric complexes with Ap by dimerizing through its dimerization domain (DD, Fig. 4A) (van Meyel et al., 1999; Rincon-Limas et al., 2000). A form of Chip lacking this domain (UAS-ChipΔDD) (van Meyel et al., 1999) also failed to prevent ectopic photoreceptor differentiation when expressed in *Chip* mutant clones (Fig. 4C), suggesting that dimerization is important for the function of Chip in the eye-antennal disc.

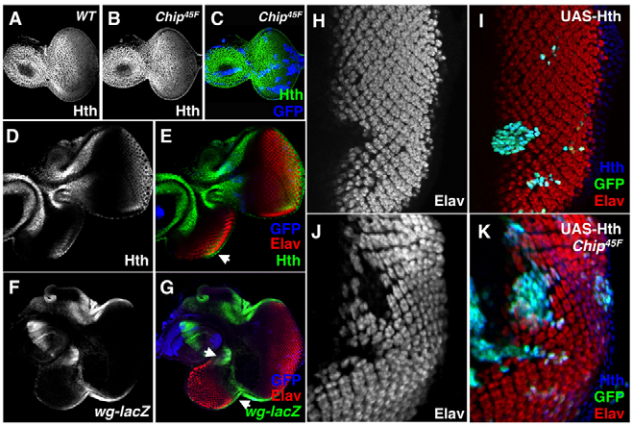

Fig. 3. Hth represses photoreceptor differentiation independently of *Chip*. (**A-C**) Early third instar discs stained with anti-Hth (A,B, green in C). (A) Wild type. (B,C) *Chip* mutant clones generated in a *Minute* background marked by the absence of GFP (blue in C). Loss of *Chip* does not affect Hth expression. (**D-G**) *Chip* mutant clones generated in a *Minute* background in late third instar discs are marked by the absence of GFP (blue in E,G). Photoreceptors are stained with anti-Elav (red in E,G). Hth (D, green in E) and *wg-lacZ* (F, green in G) are still expressed in the anterior eye disc and show new expression domains within the outgrowth in *Chip* mutant clones (arrows). Hth is thus not downstream of Chip and does not require Chip as a co-factor to control *wg* expression. (**H-K**) Eye discs misexpressing Hth (blue in I,K) in wild-type or *Chip* mutant cells marked by co-expression of GFP (green in I,K). Both types of clones failed to express Elav (H, J, red in I,K) posterior to the morphogenetic furrow. Hth does not require Chip to prevent photoreceptor differentiation.

Arrowhead and Lim1 inhibit photoreceptor differentiation only in the presence of Chip

The *Drosophila* genome encodes seven LIM-HD proteins (Fig. 5A) (Hobert and Westphal, 2000). To identify potential LIM-HD partners that might act with Chip to limit the eye field, we examined

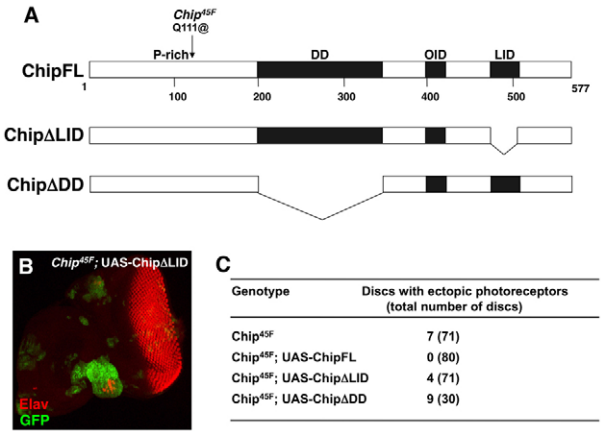

Fig. 4. Chip requires its LIM interaction domain to repress photoreceptor differentiation. (**A**) Full-length Chip construct (ChipFL) and the Chip constructs with the LID deleted (ChipΔLID) or the dimerization domain deleted (ChipΔDD). The proline-rich (P-rich), dimerization (DD), other interaction (OID) and LIM interaction (LID) domains are indicated. The *Chip*^{45F} allele has a stop codon at position 111. (**B**) An eye-antennal disc with *Chip*^{45F} mutant clones expressing ChipΔLID marked by GFP co-expression (green). Photoreceptors are stained with anti-Elav (red). (**C**) The number of eye discs containing *Chip* mutant clones with no rescue construct, or with UAS-ChipFL, UAS-ChipΔLID or UAS-ChipΔDD, that show ectopic photoreceptor differentiation. All discs containing *Chip* mutant clones were counted, regardless of the position of the clones. The LID and DD domains are required for Chip to suppress eye differentiation.

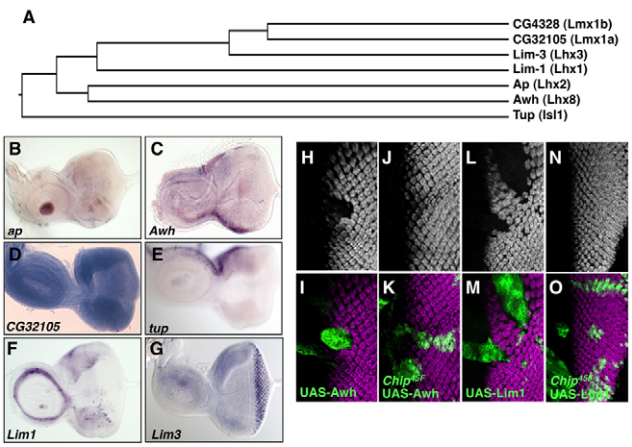

Fig. 5. Arrowhead (Awh) and Lim1 inhibit photoreceptor differentiation in a *Chip*-dependent manner. (**A**) Seven LIM-HD proteins are encoded in the *Drosophila* genome. The closest human homologue for each is given in parentheses. (**B-G**) Whole-mount in situ hybridization to eye-antennal discs with *LIM-HD* probes. (B) *ap*; (C) *Awh*; (D) *CG32105*; (E) *tup*; (F) *Lim1*; (G) *Lim3*. Only *Awh*, *Lim1* and *CG32105* are expressed in the ventral eye-antennal boundary region. (**H-K**) Eye discs that ectopically express Awh in clones of wild-type cells (H,I) or in *Chip* mutant clones (J,K) marked by co-expression of GFP (green in I,K). (**L-O**) Eye discs that ectopically express Lim1 in clones of wild-type cells (L,M) or in *Chip* mutant clones (N,O), marked by co-expression of GFP (green in M,O). Photoreceptors are stained with anti-Elav (H,J,L,N, magenta in I,K,M,O). Both Awh and Lim1 inhibit photoreceptor differentiation in wild-type, but not *Chip* mutant, cells.

their expression patterns in the eye-antennal disc by in situ hybridization. As shown previously (Cohen et al., 1992), *ap* transcription was confined to the presumptive arista in the antennal disc (Fig. 5B), making *ap* unlikely to control the development of the ventral eye-antennal boundary region. We could exclude three additional LIM-HD genes not expressed in this region: *CG4328* showed no detectable expression in the eye-antennal disc (data not shown), *Lim3* was specifically expressed in differentiating photoreceptor cells (Fig. 5G), and *tailup* (*tup*) was only expressed at the dorsal margin of the eye-antennal disc (Fig. 5E). By contrast, *Arrowhead* (*Awh*) expression was specific to the ventral margin (Fig. 5C) (Curtiss and Heilig, 1997). *Lim1* was expressed in the most distal domain of the antennal disc and also in a proximal ring (Fig. 5F) (Lilly et al., 1999; Tsuji et al., 2000) adjacent to or overlapping with the *Awh* domain at the ventral boundary between the eye and antennal discs. Earlier in development, Lim1 was present throughout the antennal disc (see Fig. S2D in the supplementary material), and *Awh* expression appeared to extend more dorsally along the eye-antennal boundary (see Fig. S2A,B in the supplementary material). Finally, *CG32105* was ubiquitously expressed (Fig. 5D).

We next tested whether these LIM-HD proteins could prevent photoreceptor differentiation when ectopically expressed in the eye field. We found that misexpression of Ap or Lim3 had no effect on photoreceptor differentiation (see Fig. S3A,E in the supplementary material). By contrast, misexpression of Awh, Lim1 or Tup with *ey*-GAL4 reduced or abolished the eye (see Fig. S3B-D in the supplementary material), and clones of cells misexpressing these LIM-HD proteins failed to differentiate as photoreceptors (Fig. 5H,I,L,M and see Fig. S3G,H in the supplementary material), consistent with previous studies of Awh (Curtiss and Heilig, 1995; Curtiss and Heilig, 1997). Interestingly, Awh, Lim1 and Tup were unable to block photoreceptor differentiation when misexpressed in *Chip* mutant cells (Fig. 5J,K,N,O and see Fig. S3I,J in the supplementary material). Because *tup* is not expressed at the ventral margin of the disc, these results identify Awh and Lim1 as the most likely proteins to act with Chip to prevent eye differentiation at the ventral eye-antennal boundary. We tested their activity in this region of the disc by asking whether their overexpression could rescue the ectopic photoreceptor differentiation seen in *hth* mutant clones at the ventral disc margin (see Fig. S4A-C in the supplementary material). We found that Awh expression fully rescued this phenotype of *hth* mutant clones, and Lim1 expression provided significant but not complete rescue (see Fig. S4D-J in the supplementary material). The ability of these proteins to block eye development is thus largely independent of the presence of Hth.

Lim1 is required to limit Ey expression

One of the earliest indications of boundary formation between the eye and antennal discs is restriction of Ey expression to the eye disc (Halder et al., 1998; Kenyon et al., 2003). We found that misexpression of either Lim1 or Awh in the anterior eye disc inhibited Ey expression (Fig. 6A,B,E,F). This repression was largely Chip dependent, as expression of Lim1 or Awh in *Chip* mutant cells had a much weaker effect on Ey (Fig. 6C,D,G,H). Surprisingly, ectopic expression of these LIM-HD proteins posterior to the normal Ey domain led to ectopic Ey expression (Fig. 6A,B), perhaps as a secondary consequence of the failure of these cells to differentiate (Fig. 5I,M). Consistent with this gain-of-function experiment, we found that Ey expression expanded into *Lim1* mutant clones in the

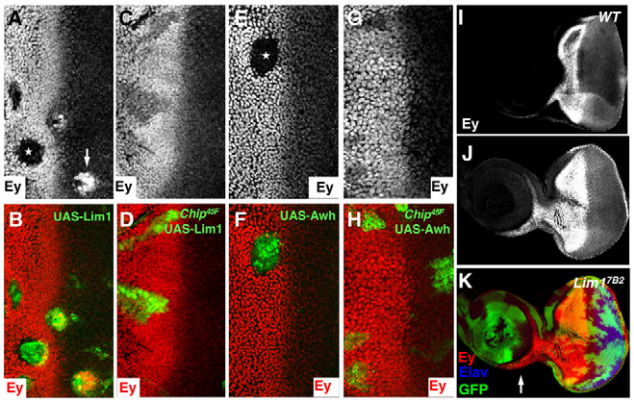

Fig. 6. Lim1 represses Ey in the antennal disc. (**A-H**) Eye discs that ectopically express Lim1 (A-D) or Awh (E-H) in clones of wild-type cells (A,B,E,F) or in *Chip* mutant clones (C,D,G,H), marked by co-expression of GFP (green in B,D,F,H), stained with anti-Ey (A,C,E,G, red in B,D,F,H). Lim1 and Awh can repress Ey expression in its normal anterior domain (stars), but inappropriately maintain a high level of Ey expression in posterior cells (arrow in A). *Chip* is required for full repression of Ey. (**I**) A wild-type eye-antennal disc stained with anti-Ey. (**J**,**K**) An eye-antennal disc with *Lim1* mutant clones marked by the absence of GFP (green in K), stained with anti-Ey (J, red in K) and anti-Elav (blue in K). Ey expression expands into the ventral antennal disc in *Lim1* mutant regions (arrow in K).

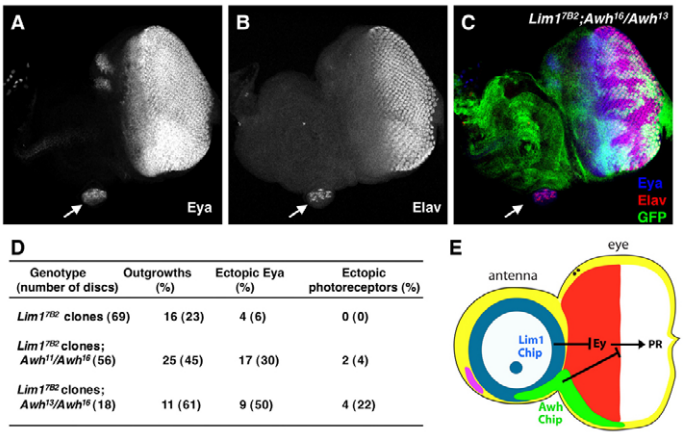

Fig. 7. *Lim1* and *Awh* redundantly contribute to limiting the eye field. (**A-C**) *Lim1* mutant clones marked by the absence of GFP (green in C) in an *Awh* mutant eye-antennal disc, stained with anti-Eya (A, blue in C) and anti-Elav (B, red in C). A clone in the ventral antennal disc (arrow) misexpresses Eya and differentiates photoreceptors. (**D**) The numbers of discs with *Lim1* clones in wild type and *Awh* mutant backgrounds that misexpress Eya and Elav and that form outgrowths. Removing *Awh* increases the frequency of all these phenotypes. (**E**) Schematic of an eye-antennal disc illustrating a model for the functions of Chip, Lim1 and Awh. Chip acts with Lim1 to prevent Ey expression in the anterior eye disc from spreading into regions fated to form the ventral head, and with Awh to regulate additional genes that inhibit photoreceptor differentiation. Yellow regions give rise to adult head cuticle, and the pink region to the maxillary palp.

ventral antennal disc (Fig. 6I-K). By contrast, Ey was unaffected in *Awh* mutant clones (data not shown), showing that Awh is sufficient but not necessary to repress *ey* expression.

If either Awh or Lim1 were the sole partner of Chip in the ventral eye-antennal disc, *Awh* or *Lim1* mutant clones should differentiate as ectopic eyes. However, no ectopic Elav staining was observed in clones lacking either LIM-HD protein (Fig. 6K, see Fig. S5A-F in the supplementary material, and data not shown) (Curtiss and Heilig, 1995). In addition, the misexpression of *ey* in *Lim1* mutant clones was usually not accompanied by expression of the downstream retinal determination genes *eya* or *dac* (see Fig. S5A-F in the supplementary material, Fig. 7D), indicating that transformation of these cells to the eye fate is incomplete. By contrast, *Chip* mutant clones misexpress Eya in the antennal disc even before the onset of ectopic photoreceptor differentiation (see Fig. S5G-I in the supplementary material). To test whether Awh and Lim1 might act redundantly, we generated *Lim1* mutant clones in *Awh* mutant eye-antennal discs. Both ectopic Eya expression and ectopic photoreceptor differentiation were observed in some clones located in the ventral eye-antennal disc (Fig. 7A-D). This indicates that Lim1 and Awh both contribute to preventing photoreceptor differentiation in this region. The weaker phenotype in comparison to *Chip* mutants could reflect either residual function of Awh in these allelic combinations, or an additional contribution from other partners for Chip. Together, these data support a combinatorial model in which LIM-HD proteins act on distinct targets to keep the ventral head cuticle devoid of retinal differentiation.

DISCUSSION

We have shown that the co-factor Chip promotes head development and prevents inappropriate retinal differentiation at the ventral eye-antennal disc boundary. This function requires a domain of Chip known to interact with LIM-HD proteins. Two such proteins, Awh and Lim1, are likely partners for Chip based on their expression in the ventral eye-antennal disc, their ability to inhibit photoreceptor differentiation only in the presence of Chip, and the ectopic eye differentiation observed in *Awh Lim1* double mutant cells. Finally, we found that Chip and Lim1 repress the expression of the selector gene *ey*. These findings implicate LIM-HD/Chip complexes in establishing the boundary between the eye and head fields.

Chip and LIM-HD proteins restrict the eye fate by repressing *ey* and other targets

Regionalization of the eye-antennal disc is a progressive process in which selector genes and signaling pathways specify the fates of different head structures. Clones of eye-antennal disc cells induced during the second larval instar can contribute to multiple organs (Morata and Lawrence, 1979), indicating that these cells retain developmental plasticity at this stage. The anteroposterior boundary of the wing disc is established much earlier; expression of the selector gene *engrailed* (*en*) specifically in the posterior cells during embryogenesis generates an affinity border that keeps the two compartments clonally separated (Zecca et al., 1995; Tabata et al., 1995). By contrast, the eye selector gene *ey* is uniformly expressed throughout the early eye-antennal disc, and only retracts to the eye field in the second instar (Kenyon et al., 2003). It was initially proposed that localized Notch signaling controls this retraction, as expression of dominant-negative forms of Notch in the eye disc abolishes *ey* expression and leads to antennal duplications (Kurata et al., 2000; Kumar and Moses, 2001). However, a later study demonstrated that loss of Notch function does not affect *ey* expression directly, but reduces cell proliferation in the retinal field, preventing the initiation of *eya* expression (Kenyon et al., 2003). We

show here that Chip and Lim1 are both necessary to repress *ey* expression in the anterior of the antennal disc (Fig. 7E). Additional factors probably help to restrict *ey* expression to the eye disc, because *ey* expression does not extend throughout the normal Lim1 expression domain in *Lim1* or *Chip* mutant clones in the antennal disc.

As *Lim1* mutant clones always misexpress Ey, but rarely misexpress Eya and never differentiate ectopic photoreceptors, additional proteins must interact with Chip to repress retinal differentiation. Awh is a good candidate because it is expressed at the ventral margin of the eye-antennal disc, its misexpression in the retina represses photoreceptor differentiation in a Chip-dependent manner, and loss of both *Lim1* and *Awh* leads to ectopic photoreceptor differentiation in the ventral eye-antennal disc. As ectopic photoreceptors differentiate only in the absence of both *Lim1* and *Awh*, whereas Ey expansion is observed in *Lim1* single mutants, Awh must control the expression of target genes other than *ey* (Fig. 7E). It may negatively regulate other genes involved in retinal determination, such as *eya*, or positively regulate genes important for head capsule development, such as *Deformed* and *odd-paired* (Lee et al., 2007; Lebreton et al., 2008).

Chip and Hth repress photoreceptor differentiation by independent mechanisms

Like Chip, Hth is required to prevent retinal differentiation at the ventral eye-antennal disc boundary (Pai et al., 1998; Pichaud and Casares, 2000). Our investigation of the relationship between Chip and Hth indicates that Chip is not required for Hth expression or activity. The ability of Hth to repress photoreceptor differentiation in *Chip* mutant clones rules out the possibility that Chip acts as a co-factor for Hth or an essential downstream mediator of its effects. The normal expression of Hth and its target gene *wg* in *Chip* mutant clones also make it unlikely that Chip controls the expression of Hth or its co-factor Exd. However, the possibility that Hth and Chip act in parallel poses the paradox that misexpressed Hth is sufficient to repress photoreceptor development in the eye field in the absence of Chip, but endogenous Hth is insufficient to do so in the head field. It is possible that Hth expression levels in the head field early in development are too low to repress the eye fate in the absence of Chip. Consistent with this hypothesis, we have found that overexpression of Hth in *Chip* mutant cells prevents ectopic photoreceptor differentiation (data not shown). Similarly, overexpression of Awh or Lim1 prevents ectopic photoreceptor differentiation in *hth* mutant cells, suggesting that endogenous levels of these LIM-HD proteins are not sufficient to compensate for the absence of Hth. The two classes of transcription factors may normally act on different sets of target genes, but show some cross-regulatory ability when overexpressed.

Distinct mechanisms control dorsal and ventral head differentiation

The boundary between the eye and the dorsal head appears to be established differently from the boundary in the ventral region. The LIM-HD gene *tup* is expressed at the dorsal eye-antennal disc boundary, in a pattern resembling the mirror image of the *Awh* pattern, and is capable of repressing photoreceptor development in a *Chip*-dependent manner. However, loss of *Chip* in this region does not lead to ectopic eye formation, although it can cause overgrowth and mispatterning of the head. In the absence of Chip, the GATA transcription factor Pannier (Pnr) and its target gene *wg* may be sufficient to maintain dorsal head fate (Maurel-Zaffran and Treisman, 2000). The ventral margin of the eye-antennal disc may be particularly susceptible to ectopic photoreceptor differentiation because of the high level of Dpp signaling there. A 5′ enhancer element has been shown to direct *dpp* expression specifically in the ventral marginal peripodial epithelium of the eye-antennal disc (Stultz et al., 2006). The ability of Dpp and Ey to synergize to drive retinal differentiation (Chen et al., 1999) therefore makes it critical to repress Ey in this region, which is fated to form head capsule.

In addition, this domain of Dpp overlaps with Wg present at the anterior lateral margin of the eye disc; the combination of these two growth factors induces proximodistal growth of the leg (Lecuit and Cohen, 1997). One function of Chip and its partner proteins might thus be to repress the outgrowth that would otherwise be triggered by the combination of Dpp and Wg. Unlike growth of the wild-type eye disc (Chao et al., 2004; Reynolds-Kenneally and Mlodzik, 2005), growth of *Chip* mutant regions appears to be Notch-independent, as they do not contain a *fng* expression boundary and do not show activation of the Notch target genes *E*(*spl*)*m*β or *eyg*. Notch has been thought to trigger growth by inducing the expression of the JAK/STAT ligand Unpaired (Upd) (Chao et al., 2004; Reynolds-Kenneally and Mlodzik, 2005); however, a recent report describes an earlier function for Upd upstream of Notch (Gutierrez-Avino et al., 2009), raising the possibility that *upd* expression is activated independently of Notch in *Chip* mutant clones. As *hth* mutant clones, or clones lacking the Odd skipped family member Bowl (Bras-Pereira and Casares, 2008), frequently show ectopic ventral photoreceptor differentiation but rarely induce outgrowths like those seen in *Chip* mutants, the functions of Chip in growth and differentiation are likely to be separable.

LIM-HD proteins establish developmental territories

LIM-HD proteins also set developmental boundaries in other imaginal discs, acting in concert with other classes of transcription factors. In the wing disc, Tup specifies the notum in collaboration with homeodomain transcription factors of the Iroquois complex (de Navascues and Modolell, 2007), and Ap specifies the dorsal compartment (Diaz-Benjumea and Cohen, 1993). Ap interacts with the homeodomain protein Bar and Lim1 with Aristaless to establish specific tarsal segments within the leg disc (Pueyo and Couso, 2004). LIM-HD proteins have also been implicated in vertebrate eye development, although those that have been studied appear to play positive roles. The Ap homologue Lhx2 is expressed within the mouse retinal field at the neural plate stage, and contributes to the expression of *Pax6*, *Six3* and *Rx* (Tetreault et al., 2009; Porter et al., 1997). *Lmx1b*, the homologue of *CG32105*, is required for the development of anterior eye structures such as the cornea and iris (Pressman et al., 2000), and is mutated in human patients with nail-patella syndrome, often characterized by glaucoma (Bongers et al., 2002). Within the retina, loss of Lim1 results in mispositioning of horizontal cells within the amacrine cell layer (Poche et al., 2007). *Drosophila Lim3* shows photoreceptor-specific expression, and might therefore have a positive function in eye development.

In the central nervous system, LIM-HD proteins act combinatorially to specify different neuronal cell fates. In both *Drosophila* and vertebrates, combinations of Islet and Lhx3/4/Lim3 proteins regulate motoneuron specification and pathfinding (Thor et al., 1999; Thaler et al., 2002). The ability of Chip to interact with LIM-HD proteins and other transcription factors as well as to dimerize enables it to form heteromeric transcription factor complexes (Torigoi et al., 2000; Ramain et al., 2000). In the wing disc, the active complex is a tetramer containing two subunits each of Chip and Ap (Milan and Cohen, 1999; Rincon-Limas et al., 2000), whereas in motoneuron development the Chip homologue

NLI can form either a tetramer with Lhx3 or a hexamer containing both Isl1 and Lhx3 (Thaler et al., 2002). Our finding that Lim1 and Awh act redundantly to prevent eye development in the ventral head primordium, whereas Chip is absolutely required, seems most consistent with regulation of distinct subsets of target genes by independent Chip-Awh and Chip-Lim1 complexes; however, we cannot rule out a contribution from a complex containing all three proteins, or even additional transcription factors. The role of the Chip co-factor may be to coordinate multiple transcriptional regulatory complexes to restrict developmental fates within the eye-antennal imaginal disc, allowing it to give rise to the head cuticle as well as distinct external sensory structures.

Acknowledgements
We thank Fred Bernard, Juan Botas, Fernando Casares, Steve Cohen, Dale Dorsett, Tetsuya Kujima, Richard Mann, Marco Milan, Stefan Thor, Don Van Meyel, the Developmental Studies Hybridoma Bank and the Bloomington *Drosophila* stock center for fly stocks and reagents. The manuscript was improved by the critical comments of Sergio Astigarraga, Erika Bach, Inés Carrera, Kerstin Hofmeyer, Justine Marsolier, Kara Nygaard, Sylvie Ozon Rickman and Josie Steinhauer. This work was supported by the National Institutes of Health (grant EY13777 to J.E.T.). Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.041244/-/DC1

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