Published in final edited form as:

Dev Dyn. 2010 September ; 239(9): 2367–2385. doi:10.1002/dvdy.22380.

***Drosophila* CtBP regulates proliferation and differentiation of eye precursors and complexes with Eyeless, Dachshund, Dan and Danr during eye and antennal development**

Chinh Q. Hoang^{*}, Micheal E. Burnett^{*}, and Jennifer Curtiss[†]

Department of Biology, New Mexico State University, Las Cruces, NM 88003

Abstract

Specification factors regulate cell fate in part by interacting with transcriptional co-regulators like CtBP to regulate gene expression. Here, we demonstrate that CtBP forms a complex or complexes with the *Drosophila melanogaster* Pax6 homolog Eyeless (Ey), and with Distal antenna (Dan), Distal antenna related (Danr) and Dachshund to promote eye and antennal specification. Phenotypic analysis together with molecular data indicates that CtBP interacts with Ey to prevent overproliferation of eye precursors. In contrast *CtBP,dan,danr* triple mutant adult eyes have significantly fewer ommatidia than *CtBP* single or *dan,danr* double mutants, suggesting that the CtBP/Dan/Danr complex functions to recruit ommatidia from the eye precursor pool. Further, *CtBP* single and to a greater extent *CtBP,dan,danr* triple mutants affect the establishment and maintenance of the R8 precursor, which is the founding ommatidial cells. Thus, CtBP interacts with different eye specification factors to regulate gene expression appropriate for proliferative versus differentiative stages of eye development.

Keywords

Drosophila melanogaster; CtBP; Eyeless; Distal antenna; Distal antenna related; Dachshund; eye; antenna

INTRODUCTION

Selector genes are required for the specification of cells, tissues and organs. More strikingly, misexpression of selector genes can convert one cell type into another. Since all selector genes encode transcription factors, they are presumed to lie at (or near) the top of a hierarchy that regulates transcription of tissue-specific genes (Mann, 2000).

One classic example of a selector gene is the *eyeless* (*ey*) gene of *Drosophila melanogaster*. In the absence of *ey* function the eye fails to develop (Quiring et al., 1994). Conversely, misexpression of *ey* in cells fated to form antenna, wing or leg can convert them to an eye fate. Remarkably, the ectopic eye that forms as a result contains all of the cell types of an endogenous eye, and these cells develop specialized organelles and assort in the same patterns as in the endogenous eye (Halder et al., 1995). The *ey* gene encodes a *Drosophila* Pax6 (Paired Homeodomain-6) homolog, and is thus part of class of genes thought to have roles in eye specification throughout Metazoa (Gehring and Ikeo, 1999; Gehring, 2005).

[†]Corresponding author: curtij01@nmsu.edu phone: (575) 646-5611 Fax: (575) 646-5665.

^{*}Authors contributed equally to this work

Other than Ey, a small network of transcription factors that includes Twin of Eyeless (Toy), Eyes absent (Eya), Sine oculis (So) and Dachshund (Dac) cooperate to specify eye fate. All of these genes are required for eye development, and all can trigger ectopic eye formation, albeit to greater or lesser degrees. In terms of expression initiation, the factors in the eye regulatory network form a (mostly) linear hierarchy. For example, Ey is required for Eya expression, but Eya is not required for Ey expression (Halder et al., 1998). That they form an interconnected network once expressed is indicated by the fact that they can regulate each other's expression through feedback loops (e.g. Eya can activate Ey expression ectopically), and that they are capable of direct physical interaction (reviewed in (Silver and Rebay, 2005; Kumar, 2009)).

The probable transcription factors Distal antenna (Dan) and Distal antenna related (Danr) have recently been shown to participate in the eye regulatory network (Curtiss et al., 2007). As with other network members, Dan and Danr are required for normal eye development and are capable of converting antennal precursors to an eye fate. With respect to initiation of expression, Dan and Danr lie at the bottom of the eye specification factor hierarchy, and thus appear to act at or near the transition from specification and differentiation. However, Dan and Danr participate in expression feedback loops characteristic of the eye regulatory network. For instance, misexpression of either results in ectopic Ey expression in the antenna. Furthermore, both are capable of direct physical interactions with Ey and Dac.

In spite of decades of work, very little is known about the mechanisms by which the eye specification transcription factor network regulates gene expression during eye development, or of the exact functions of the individual eye specification factors during the different stages of *Drosophila* eye development. During early larval stages, the cells of the eye-antennal disc proliferate asynchronously. About halfway through the larval period, a structure called the morphogenetic furrow forms, marking the beginning of eye differentiation. The furrow propagates across the eye disc from posterior to anterior, leaving differentiating ommatidia in its wake. Cells ahead of the furrow continue to proliferate asynchronously, and arrest in G1 slightly anterior to the furrow (reviewed in (Wolff and Ready, 1993)).

Ey is known to be expressed at early larval stages and later ahead of the furrow in proliferating eye precursors. Recent work suggests that Ey promotes proliferation of eye precursors (Bessa et al., 2002; Peng et al., 2009; Lopes and Casares, 2010). In addition, Ey is directly involved in activating expression of *so* and the proneural gene *atonal* (*ato*) in the pre-proneural zone immediately ahead of the furrow (Niimi et al., 1999; Zhang et al., 2006). However, Ey is subsequently downregulated at the furrow, and it is Eya, So, Dac, Dan and Danr that participate in the transition of cells from proliferation to differentiation (Pignoni et al., 1997; Curtiss et al., 2007). The genes that are directly regulated by the eye specification factors and that participate directly in these developmental events are largely unknown, as are the mechanisms by which their transcription is regulated.

Besides the eye, Dan and Danr also participate in specification of another organ, the distal antenna. In loss-of-function *dan* or *danr* mutants the distal part of the antenna is transformed into a leg. Conversely, expression of Dan or Danr in distal leg precursors converts them to distal antennal structures. These phenotypes are similar to the phenotypes of mutants for the antennal selector genes, *Distal-less* and *Homothorax* (Casares and Mann, 1998; Dong et al., 2000), as well as genes functioning downstream of these two factors (Duncan et al., 1998; Johnston et al., 1998; Dong et al., 2002). Evidence suggests that Dan and Danr lie at the bottom of the antennal specification hierarchy (Emerald et al., 2003; Suzanne et al., 2003), as they do in eye specification, again suggesting that they act at or near the transition from specification and differentiation.

The Dan and Danr protein sequences contain motifs that suggest a link to epigenetic mechanisms of regulating gene expression. Dan and Danr are closely related proteins that contain only two recognizable motifs, a Pipsqueak domain and a PxDLS sequence. Pipsqueak domains have a helix-turn-helix structure that is similar to a homeodomain, and have been shown to bind DNA in a sequence-specific manner when present in other proteins (e.g. Iwahara et al., 1998; Wang et al., 1999; Huang et al., 2002; Lours et al., 2003). Pipsqueak domains are often found in proteins that have been linked to chromatin modification (Siegmond and Lehmann, 2002). The PEDLS sequence present in Dan and Danr is related to motifs known to bind to the transcriptional co-regulator C-terminal Binding Protein (CtBP) (reviewed in (Chinnadurai, 2007). Accordingly, Danr has been shown to bind *Drosophila* CtBP (dCtBP) in a genome-wide two-hybrid screen (Giot et al., 2003).

CtBP is a highly conserved transcriptional co-regulator first identified for its ability to bind to the C-terminus of the adenovirus E1A oncoprotein (Turner and Crossley, 2001; Chinnadurai, 2007). Both vertebrate and *Drosophila* CtBPs have been characterized as co-repressors for both short and long-range repressors, including Hairy, Snail, Krüppel, and mammalian Basic Krüppel-like Factor/Krüppel-like Factor 3 (Nibu et al., 1998a; Nibu et al., 1998b; Poortinga et al., 1998; Turner and Crossley, 1998). Whereas vertebrates have two CtBP genes, invertebrates including *Drosophila* have a single CtBP gene (dCtBP) that encodes two major protein isoforms, one long (~50kD), and one short (~42kD) (Nibu et al., 1998a; Nibu et al., 1998b; Poortinga et al., 1998; Sutrias-Grau and Arnosti, 2004; Mani-Telang and Arnosti, 2007). Based on current knowledge, the major function of vertebrate CtBP homologs is to link DNA-binding transcription factors to the chromatin modification machinery (reviewed in (Turner and Crossley, 2001; Chinnadurai, 2007).

Here, we use both molecular and genetic evidence to show that Dan and Danr bind directly to *Drosophila* CtBP, and that they form a complex or complexes with CtBP in eye-antennal discs that play functional roles in both eye and antennal development. Additional molecular and genetic experiments show that CtBP also complexes with Ey and Dac during eye development. *CtBP* loss-of-function mutant eyes have more ommatidia than wild-type eyes, and *CtBP* mutant clones overproliferate anterior to the morphogenetic furrow. Based on this result and the fact that *ey* has previously been shown to promote proliferation anterior to the furrow (Bessa et al., 2002; Peng et al., 2009; Lopes and Casares, 2010), we speculate that the presence of CtBP in a complex containing Ey ahead of the furrow modulates the effects of Ey on proliferation, limiting the pool of ommatidial precursors. In contrast, *CtBP,dan,danr* triple mutant adult eyes have significantly fewer ommatidia than either *CtBP* single or *dan,danr* double mutant eyes, suggesting that CtBP/Dan/Danr complex(es) promote differentiation of eye precursors into ommatidia. Recruitment and maintenance of the founding R8 precursor is affected in *CtBP* single mutants and to a much greater extent in *CtBP,dan,danr* triple mutants, suggesting that a function of the CtBP/Dan/Danr complex during differentiation is to firmly establish R8 cell fate. Taken together, our results suggest that CtBP is part of multiple complexes with different eye specification factors to participate in the transition of eye precursors from proliferation to differentiation.

RESULTS

Dan and Danr interact with CtBP *in vitro* and *in vivo*

Dan and Danr have been shown to physically interact with Ey, Dac and with each other in GST-pulldown and in yeast two-hybrid assays (Curtiss et al., 2007). A genome-wide yeast two-hybrid screen (Giot et al., 2003) also predicted a direct physical interaction between Danr and CtBP. CtBP has been shown to interact with many transcription factors and co-

factors via motifs related to the PxDSL CTBP-binding motif originally identified in the adenovirus E1A protein (Schaeper et al., 1995) (reviewed in Chinnadurai 2007).

Although functional PxDSL motifs can exhibit considerable sequence variability – Giot et al., 2003 identified 13 functional sequence variants – the PEDLS motifs in both Dan and Danr are perfectly conserved among *Drosophila* species (Figure 1A). For *D. melanogaster* Dan, the PEDLS sequence lies 191 amino acids from the end of the protein; *D. melanogaster* Danr's PEDLS motif is 17 amino acids from the C-terminus. Dan and Danr are likely to have arisen from a gene duplication event, since they are only ~45 kb apart in the genome and their sequences are more closely related to each other than to other known related proteins (Emerald et al., 2003). The duplication event appears to have occurred after the divergence between Nematocera and Brachycera suborders in Diptera, because the mosquito *Aedes aegypti* has only a single Dan/Danr ortholog. The predicted mosquito protein (included in the Dan alignment) also has a PEDLS sequence located 175 amino acids from the end of the protein. The presence of the PEDLS sequence among these species suggests that it has an important and conserved function.

The data above prompted us to test whether Dan or Danr binds to CtBP *in vitro*, using GST pull-down assays. We incubated labeled *in vitro* translated Dan or Danr with GST, GST-Six HD (a truncated So protein containing only the Six domain and the Homeodomain), GST-CtBP, GST-Danr and GST-Dan (Figure 1B), the latter two serving as positive control (Curtiss et al., 2007). Labeled *in vitro* translated Dan or Danr were both pulled down by GST-CtBP, GST-Dan and GST-Danr, but not by GST-Six HD or by GST alone. Thus, CtBP binds directly with both Dan and Danr *in vitro*.

Next, we investigated whether CtBP, Dan and/or Danr can complex with each other *in vivo*. We performed co-immunoprecipitation (co-IP) experiments on lysates derived from eye-antennal disc/brain complexes, using either anti-Dan or anti-Tubulin antibody (Fig 1C). On Western blots, a ~50 kDa band was detected with anti-CtBP in immunoprecipitates obtained with anti-Dan but not anti-Tubulin. This corresponds to the size of the long CtBP isoform (Mani-Telang and Arnosti, 2007). In addition, a ~45 kDa band was detected with anti-Danr, which is the predicted size for Danr.

We also carried out the converse experiment, in which we performed co-IPs with anti-CtBP and probed Western blots with anti-Dan. The anti-Dan antibody recognizes a ~90 kD band on Western blots of wild-type eye-antennal disc lysates, and this band is missing from eye-antennal disc lysates from *dan,danr^{ex56}* homozygotes (Fig 1D; we have previously shown that Dan protein is undetectable by immunofluorescence in *dan,danr^{ex56}* homozygous clones; (Curtiss et al., 2007). A ~90 kD protein was detected by anti-Dan antibody only in immunoprecipitates obtained with anti-CtBP, but not in immunoprecipitates obtained with anti-Tubulin (Fig 1E). These results suggest that Dan forms a complex with both Danr and CtBP. Due to lack of sufficient anti-Danr anti-sera, we were unable to perform the same experiment with Danr.

CtBP interacts genetically with *dan* and *danr* during eye development

Overexpression of genes involved in eye specification interferes with eye development, resulting in smaller, rougher eyes than wild type (Curtiss and Mlodzik, 2000; Curtiss et al., 2007). To test whether overexpression of CtBP interferes with eye development, we used the Gal4/UAS system (Brand and Perrimon, 1993). *P[GSV]^{A396}* and *P[GSV]^{A132}* are bidirectional EP elements located in the first intron of *CtBP* that have previously been used to study CtBP function (Toba et al., 1999; Fang et al., 2006).

Similar to the effects of overexpressing *ey*, *dac*, *dan* or *danr*, overexpression of CtBP in eye precursors under *ey-Gal4* control disrupts eye development. The two CtBP EP lines gave similar effects with *ey-Gal4*, although *P[GSV]^{A396}* gave the strongest effects and was used in the experiments described below. 100% of adult eyes from *ey-Gal4/+; P[GSV]^{A396}/+* (hereafter *ey>CtBP*) individuals raised at 30°C were smaller and rougher than wild-type eyes (Fig 2A,B; 4 separate experiments, 169 flies total). Eyes ranged from 40%-90% of wild type size, with most (68%) greater than 50% wild-type size. In addition, the eyes were moderately rough and showed duplications of interommatidial bristles (Fig 2B inset, arrows). Stern et al, (Stern et al., 2007) also observed duplicated interommatidial bristles when they expressed CtBP under *GMR-Gal4* control, corroborating our findings. These results provide evidence that CtBP functions in eye development.

Besides the eye defects, *ey-CtBP* flies had patterning defects in various regions of the head. These defects included: (1) loss of vibrissae and replacement by bristles resembling orbital or vertical bristles (not shown); (2) occasional splitting of the eye field, sometimes accompanied by formation of antennal-like structures in the cuticular field between eye domains (Fig 2B, black arrow); (3) loss of distal antennal structures (not shown, ~1% of flies); (4) apparent clefts in the vertex region of the head with loss of ocelli and mispatterning of bristles (31.7% of flies, *N*=101) (Fig 2D, cleft denoted by black arrow; extra orbital bristles surrounded by a patch of orbital-like cuticle denoted by white arrow).

To explore the functional relationships among *dan*, *danr* and *CtBP*, we tested the effects of overexpressing CtBP in individuals heterozygous for *dan^{ems3}*, *danr^{ex35}* and *dan danr^{ex56}* mutations (Fig 3 and Table 1). Importantly, *dan^{ems3}*, *danr^{ex35}* and *dan danr^{ex56}* heterozygotes all have normal-sized eyes with no roughness. We crossed *ey>CtBP* flies to either control *w¹¹¹⁸* flies or to flies containing *dan/danr* mutations. This experiment was performed several times, with similar results. In one particular experiment carried out at 25°C, 31% (*N*=35) of *ey>CtBP* individuals had eyes that were smaller and rougher than wild-type eyes (Fig 3A,B). In contrast, in the same experiment only 16% (*N*=19) of *ey>CtBP*, *dan^{ems3}/+* (Fig 3C), and only 13% (*N*=30) of *ey>CtBP/+; danr^{ex35}/+* (Fig 3D) individuals had smaller than wild-type eyes, and the eye roughness and extra interommatidial bristle phenotypes were also considerably suppressed. These data suggest that CtBP interacts in the same developmental process as Dan and Danr, and that *dan* and *danr* are required for CtBP function.

We also performed converse experiments in which we compared *ey-Gal4*, *UAS-dan/+* (*ey>dan*) and *ey-Gal4*, *UAS-danr/+* (*ey>danr*) to *ey>dan*, *CtBP^{87De-10}/+* and *ey>danr*, *CtBP^{87De-10}/+* (*CtBP^{87De-10}* heterozygotes have normal-sized eyes with no roughness). As described previously (Curtiss et al., 2007), misexpression of either *dan* or *danr* in eye precursors results in small, rough eyes in a significant percentage of the population. Removing a copy of *CtBP* in this background suppresses these defects in terms of both eye size and roughness (Table 1; compare Fig 3E,F). Similarly, removing one copy of *CtBP* suppresses the small rough eye phenotype of *ey>danr* (Table 1; compare Fig 3G,H). These data suggest that CtBP is required for the functions of *dan* and *danr*.

CtBP functions in complexes with Eyeless and Dachshund during eye development

It has previously been shown that Dan physically interacts with Ey and Dac *in vitro* and that *dan* mutations interact genetically with *ey* and *dac* mutations (Curtiss et al., 2007). However, it is not known whether Dan can complex with the Ey and Dac proteins *in vivo*. To answer this question, we performed co-IP experiments with anti-Dan as above, but probed the Western blots with antibodies specific for Ey and Dac (Fig 4A). A ~115 kD protein and a ~130 kD protein were detected by anti-Ey and anti-Dac antibodies, respectively, only in immunoprecipitates obtained with anti-Dan, but not in immunoprecipitates obtained with

anti-Tubulin. These apparent molecular weights have previously been reported for Ey (Callaerts et al., 2001; Punzo et al., 2001) and for Dac (Chen et al., 1997).

Conversely, we were able to pull down Dan from eye-antennal discs expressing a FLAG-tagged version of Ey (kind gift of Rui Chen), using sepharose beads conjugated to anti-FLAG; sepharose beads alone did not pull down Dan (Fig 4B). Thus, results from GST-pulldowns (Curtiss et al., 2007) and co-IP assays indicate that Dan can directly bind Ey and Dac, and suggest that these factors form a complex or complexes *in vivo*.

Since Dan associates with both CtBP and Ey *in vivo* (Fig 1C,D; Fig 4A), we wondered whether CtBP and Ey might also associate *in vivo*. We therefore used anti-CtBP to probe Western blots containing co-IPs from eye-antennal discs expressing FLAG-tagged Ey. CtBP was detected in co-IPs obtained with anti-FLAG conjugated sepharose (Fig 4B), suggesting that CtBP and Ey associate in eye-antennal disc tissue during larval stages. The top-most band runs at an apparent molecular weight of ~50 kD, which corresponds to the long CtBP isoform. It is unclear whether the lower bands are degradation products, or if Ey is also capable of associating with the short ~42 kD CtBP isoform (Mani-Telang and Arnosti, 2007). Finally, we were able to pull down Ey from eye-antennal discs using anti-CtBP (Fig 4C). We have not reliably detected Dac in co-IPs obtained with anti-CtBP. These results suggest that CtBP interacts with both Dan and Ey during eye development.

We next tested whether *CtBP* also interacts functionally with *ey* or *dac* during eye development. Removing a copy of *ey* or *dac* in an *ey>CtBP* background using the *ey*², *ey*^R, *dac*⁴, *dac*³ or *dac*^P alleles reproducibly suppresses the *ey>CtBP* phenotype (Table 1; Fig 4D-F; not shown). Although the percentages of *ey>CtBP,ey*²/+; *ey>CtBP,ey*^R/+; *ey>CtBP,dac*^P/+ and *ey>CtBP,dac*⁴/+ with smaller than wild-type eyes is not suppressed compared to *ey>CtBP* alone, the eyes in these genotypes tend to be larger and much less rough. Eyes of *ey*², *ey*^R, *dac*^P and *dac*⁴ heterozygotes are normal in size and show no roughness. These data suggest that, like Dan and Danr, Ey and Dac are required for CtBP function.

Not all eye specification factors have the same relationship to CtBP as Dan, Danr, Ey and Dac. For instance, *ey>CtBP,eya*²⁰¹⁵/+ flies have slightly smaller and rougher eyes than *ey>CtBP* flies (compare Fig 4G,H), suggesting that CtBP and Eya have antagonistic functions. Likewise, removing a copy of *so* (*so*³) in an *ey>CtBP* background has no discernible effect on eye size or roughness (compare Fig 4G,I).

Finally, we showed that reducing the function of *CtBP* suppresses the effects of expressing *ey* under *ey-Gal4* control. We have previously demonstrated that *ey>ey* flies have smaller, rougher eyes than wild type (Curtiss and Mlodzik, 2000; Curtiss et al., 2007). In one particular experiment, 100% (*N*=63) of *ey>ey* flies had smaller eyes than wild type. In contrast, only 54% (*N*=26) of *ey>ey, CtBP*^{87De-10}/+ flies had smaller eyes than wild type (Table 1; compare Fig 4J-L), suggesting that CtBP is required for Ey function. Taken together, these data suggest that Ey and Dac function in a complex or complexes with CtBP.

CtBP is required for development of eye-antennal disc-derived tissues

Ey, Dac, Dan and Danr have known roles in eye and antennal specification. CtBP is a well-characterized co-regulator that participates in many developmental contexts including oncogenesis and apoptosis (Chinnadurai, 2006), but no published studies have looked for a role in either eye or antennal specification in *Drosophila*. Because CtBP interacts with Ey, Dac, Dan and Danr *in vitro* and in eye-antennal discs, we asked whether CtBP is necessary for eye or antennal development.

We used two independent *CtBP* mutant alleles (*CtBP*^{87De-10} and *CtBP*⁰³⁴⁶³ – see Materials and Methods for details) for our analysis. Although Poortinga et al. (Poortinga et al., 1998) describe *CtBP*⁰³⁴⁶³ as homozygous lethal at the pharate adult stage, in our hands both *CtBP*^{87De-10} and *CtBP*⁰³⁴⁶³ are lethal prior to late larval stages. We generated flies transheterozygous for *CtBP*^{87De-10} and *CtBP*⁰³⁴⁶³, and flies containing these mutations *in trans* with a deletion encompassing the *CtBP* locus [*Df(3R)ry615*]. In addition, we generated *CtBP*^{87De-10} homozygous loss-of-function clones using the FLP/FRT system (Xu et al., 1993) together with an *ey-FLP* transgene (Newsome et al., 2000), which induces FRT-mediated recombination in both eye and antennal portions of the eye-antennal discs. We also combined this with the *Minute* technique (Newsome et al., 2000) to generate individuals in which wild type or *CtBP*^{87De-10} clones comprised most of the adult head (+/M, *CtBP*^{87De-10}/M).

Homozygous mutant or wild type tissue was marked in the larval eye-antennal disc by the absence of GFP, and examination of +/M or *CtBP*^{87De-10}/M discs using fluorescence microscopy confirmed that the majority (>95%) of the eye-antennal disc cells were mutant (not shown). Clones were marked in the adult eye by the absence of pigment, but were not marked in other regions of the adult head. Wild-type clones produced via *ey-FLP/FRT* with or without the *Minute* technique resulted in no discernible defects (Fig. 5A and not shown). In all cases, a substantial number of *CtBP* transheterozygotes survived to the pharate adult stage, but none was ever observed to emerge from its pupal case; pharate adults were thus dissected from pupal cases for analysis. Many *CtBP*^{87De-10}/M, and almost all *CtBP*^{87De-10} mosaic individuals survived to adulthood. Mutant phenotypes in the thorax of all of the transheterozygous combinations resembled those seen in other studies (Poortinga et al., 1998; Barolo et al., 2002; Biryukova and Heitzler, 2008; Stern et al., 2009). *CtBP*^{87De-10}/M individuals and *CtBP* transheterozygotes all had similar phenotypes in the eye, the bristles of the head, and the antennae (described in more detail below).

CtBP is required for development of head bristles—Head bristle phenotypes of the *CtBP* mutant combinations tested here were similar to those described for the thorax (Barolo et al., 2002; Stern et al., 2009), with some bristles duplicated, others missing, and some normal. Duplicated bristles were of two varieties: extra bristles (additional bristle and socket) or double bristles (two bristles in the same socket). In stronger mutant combinations (e.g. *CtBP*^{87De-10}/M – Fig 5B) extra or double bristles were always observed for one or more vertical (ver) bristles (arrows); most post-vertical (pv) bristles were missing; orbital (orb) bristles were mostly present, but occasionally missing or duplicated (not shown); all interocellar bristles, and most ocellar, frontal and postorbital bristles were missing. Weaker combinations (e.g. *CtBP*^{87De-10}/*CtBP*⁰³⁴⁶³ – Fig 5C) had similar but milder head bristle phenotypes, with occasional duplicated vertical and orbital bristles (arrow), occasional double and/or misoriented orbital (arrow) and post-vertical bristles (not shown), and variable loss of interocellar and frontal bristles. Barolo et al., (Barolo et al., 2002) previously mentioned similar head bristle phenotypes at a much-reduced frequency in *CtBP*^{87De-10}/+ heterozygotes, and at a higher frequency in *CtBP*^{87De-10}/*CtBP*⁰³⁴⁶³ pharate adults.

In addition to the bristle phenotypes described above, the ocelli and surrounding ocellar cuticle appeared somewhat larger in *CtBP*- mutants compared to wild-type (compare Fig 5A to Fig 5B,C), although the distance across the dorsal head between eyes appeared to be approximately the same as in wild-type heads. The larger size of the ocellar region does not appear to be due to transformation of other regions of the head: although bristles are often missing or altered, distinctive regions of cuticle (e.g. the ridged frons cuticle, the orbital cuticle, etc) all appeared to be present. Rather, the relative sizes of the various regions may be changed in *CtBP*- mutants. These results suggest a minor role for CtBP in development of the morphology and bristles of the head.

CtBP is required for antennal bristle development and for development of the aristae—We also noted defects in antennae in the *CtBP* loss-of-function mutants. Wild-type antennae comprise 5 segments plus the arista (Fig 5D; a4 is not readily visible in this preparation). 2nd and 3rd antennal segments (a1, a2 and a3) appeared somewhat larger in *CtBP* loss-of-function mutant combinations (compare Fig 5D,E). In addition, the arista appeared to be slightly shorter, with the main shaft tapering more prominently along the proximal-distal axis (arrow in Fig 5E). Branches from the main shaft were curved. Unlike *dan* and *danr* single or double mutants, we saw no bracted bristles or other obvious evidence of antenna-to-leg transformation. Finally, the number of bristles on a2 was reduced; the loss of bristles appeared more severe in *CtBP*^{87De10}/*Df(3R)ry615* individuals than in other mutant combinations. Johnston's organs in a2 appeared normal (not shown). These data suggest a minor role for *CtBP* in development of the size and morphology as well as the bristles of the antennae.

CtBP is required to limit eye size, and for interommatidial bristle formation—*CtBP* mutant combinations have larger than wild-type eyes (compare Fig 5F,G) with an increase in the number of ommatidia. In our hands wild-type (Oregon R) eyes (*N*=5) have a mean of 740 ommatidia, similar to what has been found by others (~750; (Wolff and Ready, 1993). In contrast, *CtBP*⁰³⁴⁶³/*Df(3R)ry615* (*N*=2), *CtBP*^{87De10}/*Df(3R)ry615* (*N*=3) and *CtBP*^{87De10}/*CtBP*⁰³⁴⁶³ (*N*=3) have means of 938, 818 and 906 ommatidia, respectively (Fig 5H). A significant overall difference among the mean number of ommatidia of the 4 genotypes was detected (one-way ANOVA, *P*<0.0001). A Tukey-Kramer post-hoc test revealed multiple significant differences among individual pairs of genotypes (Fig 5H). In particular, the test demonstrates that *CtBP*⁰³⁴⁶³/*Df(3R)ry615*, *CtBP*^{87De10}/*CtBP*⁰³⁴⁶³ and *CtBP*^{87De10}/*Df(3R)ry615* eyes have statistically significantly more ommatidia than wild-type eyes. These results suggest that *CtBP* functions to prevent eyes from growing too large.

Besides the effects on eye size, the *CtBP* mutant eyes were mildly rough and showed a loss of interommatidial bristles (Fig 5F,G insets). The latter effect is opposite to the extra interommatidial bristles that result from overexpression of *CtBP* (Stern et al., 2007), and suggests that *CtBP* is required for interommatidial bristle formation.

CtBP regulates proliferation ahead of the morphogenetic furrow during eye development—Loss of *CtBP* could result in an increased number of ommatidia for several reasons. It is possible that the entire head is larger in *CtBP*- mutants. However, based on our observations (e.g. Fig 5), the *CtBP*- eyes appear to be proportionally larger than other structures that make up the head capsule. Although at present we have no definitive evidence, as noted above *CtBP* may also affect growth of other discrete regions of the eye-antennal disc, including the ocelli (compare Fig 5A with Fig 5B,C) and parts of the antennae (compare Fig 5D with Fig 5E). In addition, we have not noticed major patterning defects in *CtBP* mutant heads. For instance, as described above the extra ommatidia in *CtBP*- eyes do not appear to replace particular regions of head cuticle. Nor have we observed obvious ectopic dorsal/ventral margins, which can serve as organizers of ectopic growth in the eye-antennal disc (c.f. Cavodeassi et al., 1999; Gutierrez-Avino et al., 2009), as assessed by staining *CtBP* transheterozygous eye-antennal discs for markers of the D/V boundary.

To address the reason for overgrowth, we asked whether *CtBP* mutants affect the number of cells generated during larval eye-antennal disc development, increasing the pool of cells from which ommatidia form. Any additional cells that may be present in *CtBP*- mutants would likely be generated ahead of the furrow: because the number of ommatidia is determined in the furrow, increased proliferation behind the furrow would not be expected to lead to additional ommatidia, but instead to additional cells available for recruitment into existing ommatidia. In cases where extra cells are recruited into ommatidia or are otherwise

allowed to survive (e.g. if apoptosis fails during pupal stages), the resulting adult eyes are very rough (c.f. Wolff and Ready, 1991). *CtBP*-eyes exhibit little or no roughness.

Consistent with the idea of additional prefurrow cells in *CtBP*- mutants, we noticed that *CtBP*^{87De-10} prefurrow clones appeared larger than wild-type clones in third instar larval eye-antennal discs, often tapering off in size close to and behind the furrow (Fig 6A,B). We also tested *CtBP*^{87De-10},*dan,danr*^{ex56} triple clones (described in more detail below), and these also appeared larger compared to either wild-type clones or *dan,danr*^{ex56} double mutant clones (Fig6C,D).

We measured the areas of wild-type, *CtBP*^{87De-10}, *CtBP*^{87De-10},*dan,danr*^{ex56} and *dan,danr*^{ex56} prefurrow clones, and divided the total prefurrow clone area by the total prefurrow area to obtain a ratio. Mean ratios for *CtBP*^{87De-10} (0.45 ± 0.05), and *CtBP*^{87De-10},*dan,danr*^{ex56} (0.48 ± 0.05) were larger than for wild-type (0.31 ± 0.04) or *dan,danr*^{ex56} (0.32 ± 0.04) (Fig 6E), and we detected a significant overall difference among the means of the ratios (one-way ANOVA, $P < 0.02$). A Tukey-Kramer post-hoc test found a significant difference between the wild-type and *CtBP*^{87De-10},*dan,danr*^{ex56} ratios.

To determine whether the difference in clone size might be caused by an increase in proliferation in *CtBP*^{87De-10} mutant tissue, we stained eye-antennal discs containing wild type or *CtBP*^{87De-10} clones with anti-Phospho-Histone H3 (anti-PH3) to mark cells in mitosis. We then counted the number of anti-PH3 positive cells in all clones ahead of the furrow, and divided this number by the total prefurrow clone area in each disc. We obtained a mean ratio of 0.0024 ± 0.0003 for homozygous *CtBP*^{87De-10} clones, versus 0.0015 ± 0.0003 for wild-type clones, a difference that is statistically significant (t test; $P < 0.05$) (Fig 6F). These results suggest that CtBP controls the number of cells anterior to the furrow by regulating their proliferation.

This result is particularly interesting in light of the fact that Ey has been implicated in promoting proliferation in eye precursors anterior to the furrow (Bessa et al., 2002; Peng et al., 2009; Lopes and Casares, 2010), in the same region where we have shown that CtBP is limiting proliferation. In addition, we have demonstrated that CtBP and Ey can associate in eye-antennal disc extracts (Fig 4). Based on this information, we propose that the purpose of the interaction between CtBP and Ey ahead of the furrow in the larval eye disc is to restrain the effects of Ey on eye precursors, allowing their proliferation to be tightly controlled.

To summarize our results, *CtBP*^{87De-10} and *CtBP*^{87De-10},*dan,danr*^{ex56} triple mutant clones ahead of the furrow in the larval eye disc are larger than wild-type clones. Further, *CtBP*^{87De-10} single mutant clones contain more mitotic figures anterior to the morphogenetic furrow, where cells are proliferating but have not yet begun to differentiate, suggesting that more cells are being produced in these clones. In the case of the *CtBP* single mutant clones, this is consistent with the fact that *CtBP* transheterozygous or *CtBP*^{87De-10}/*M* mosaic mutant adult eyes contain increased numbers of ommatidia. Thus, in *CtBP* single mutants increased numbers of eye precursors are produced, and are able to differentiate into increased numbers of ommatidia.

The fact that *CtBP*^{87De-10},*dan,danr*^{ex56} triple mutant clones are similar to *CtBP*^{87De-10}/*M* single mutant clones in being larger than either wild-type or *dan,danr*^{ex56} double mutant clones, is consistent with the fact that Dan and Danr are not expressed in anterior regions of the eye-antennal discs, and do not appear to have a role there (Emerald et al., 2003; Suzanne et al., 2003; Suzanne, 2004; Curtiss et al., 2007). Thus, the large size of *CtBP*^{87De-10},*dan,danr*^{ex56} clones is likely to be due solely to the effects of CtBP on proliferation.

CtBP functionally interacts with *dan* and *danr* during eye and antennal development

It has previously been shown that *dan* and *danr* have roles in eye and antennal specification (Emerald et al., 2003; Suzanne et al., 2003; Curtiss et al., 2007). Here we have demonstrated that CtBP can bind directly to Dan and Danr *in vitro*, that CtBP can interact in eye-antennal disc extracts with Dan (Fig 1) and that CtBP has a role in development of eye-antennal disc-derived structures (Fig 5,6). To test whether CtBP, Dan and Danr interact functionally during eye and antennal development, we constructed *dan^{ems3}, CtBP^{87De-10}* and *danr^{ex35}, CtBP^{87De-10}* double mutants, and *dan, danr^{ex56}, CtBP^{87De-10}* triple mutants. Because these double and triple mutant combinations are homozygous lethal, we used the *eyFLP/FRT* system together with the *Minute* technique to generate mosaic individuals in which the homozygous double and triple mutant clonal tissue comprised most of the eye-antennal-derived structures in the adult head (+/M; *CtBP^{87De-10}/M*; *dan^{ems3}/M*; *CtBP^{87De-10}, dan^{ems3}/M*; *danr^{ex35}/M*; *CtBP^{87De-10}, danr^{ex35}/M*; *dan, danr^{ex56}/M*; and *CtBP^{87De-10}, dan, danr^{ex56}/M*).

The effects on head bristles in these double and triple mosaic mutants appeared identical to those observed for the *CtBP^{87De-10}/M* mosaics (not shown), suggesting that CtBP is required for normal head bristle development but Dan and Danr are not. This is consistent with the fact that Dan and Danr are not expressed in the regions of the eye-antennal disc that give rise to these bristles, and that *dan* and *danr* mutants have no discernible effect on head bristle development (Emerald et al., 2003; Suzanne et al., 2003; Suzanne, 2004; Curtiss et al., 2007). In contrast, as described below, the *CtBP*, *dan*, and *danr* double and triple mutants had much stronger effects on antennal and eye development than any of the single mutants or the *dan, danr^{ex56}* double mutant.

CtBP, Dan and Danr interact genetically to promote antennal specification—

Strikingly, whereas *CtBP^{87De-10}/M*, *dan^{ems3}/M*, *danr^{ex35}/M* and *dan danr^{ex56}/M* mosaics have relatively minor defects in antennae, the *CtBP^{87De-10}, dan^{ems3}/M* and *CtBP^{87De-10}, danr^{ex35}* double and *CtBP^{87De-10}, dan, danr^{ex56}* triple mutant mosaics result in strong antenna-to-leg transformations. As previously described (Emerald et al., 2003), *dan^{ems3}* (Fig 7A) and *danr^{ex35}* (Fig 7B) single mutants, and the *dan danr^{ex56}* double mutant (Fig 7C) exhibit defects that indicate mild antenna-to-leg transformation of the distal antenna. These defects are characterized by a reduction in the size of the a3 antennal segment, the presence of bristles on the a3 antennal segment and an enlarged basal cylinder of the arista with bracted bristles, which are diagnostic of leg cuticle. As described above, *CtBP* mutants have bristle loss on a2 and defects in arista morphology, but no obvious changes in size of the basal cylinder of the arista, and no bracted bristles. In contrast, double and triple *dan/danr/CtBP* mutant clones show significant enlargement all along the length of the arista to form a structure that strongly resembles a leg; bracted bristles were observed on these structures, confirming leg identity (Fig 7D-F). These data provide evidence that *dan/danr/CtBP* interact functionally during antennal specification.

CtBP, Dan and Danr interact genetically during eye development—

As with the antenna, whereas *CtBP^{87De-10}/M*, *dan^{ems3}/M*, *danr^{ex35}/M* and *dan danr^{ex56}/M* mosaics have relatively minor defects in the eye, the *CtBP^{87De-10}, dan^{ems3}/M* and *CtBP^{87De-10}, danr^{ex35}* double and *CtBP^{87De-10}, dan, danr^{ex56}* triple mutant mosaics result in strong eye phenotypes (Fig 8). We counted ommatidia in the eyes of adult flies containing wild-type or *CtBP*, *dan* or *danr* homozygous single, double, and triple mutant clones generated via *eyFLP* and the *Minute* technique (Fig 8A), and detected a significant overall difference among the mean number of ommatidia of the eight genotypes (one-way ANOVA, $P < 0.0001$). A Tukey-Kramer post-hoc test revealed multiple significant differences among individual pairs of genotypes.

CtBP^{87De-10}/*M* mosaic eyes have a mean of 722 ommatidia (*N*=3), which is larger than control *+/M* mosaic eyes (mean=692 ommatidia; *N*=4) (compare Figs 8B,C), though not statistically significantly so. This result is consistent with the significantly larger eyes observed in *CtBP* transheterozygous combinations (Fig 5F-H), and supports the idea that *CtBP* is required to prevent eyes from growing too large. The fact that the *CtBP*^{87De-10}/*M* mosaic eyes are not statistically significantly larger than *+/M* mosaic eyes is likely to reflect the fact that these eyes are not entirely composed of homozygous *CtBP* mutant tissue.

Consistent with a previous description of *dan/danr* mutant phenotypes (Curtiss et al., 2007), the *dan*^{ems3}/*M* (Fig 8D) and *dan,danr*^{ex56}/*M* (Fig 8H) mosaic eyes have comparable numbers of ommatidia compared with control *+/M* mosaic eyes, and *danr*^{ex35}/*M* mosaic eyes (Fig 8F) have statistically significantly fewer ommatidia than the control. However, and strikingly, *CtBP*^{87De-10},*dan*^{ems3}/*M* (Fig 8E), *CtBP*^{87De-10},*dan,danr*^{ex56}/*M* (Fig 8I) and *CtBP*^{87De-10},*danr*^{ex35}/*M* (Fig 8G) mosaic eyes have statistically significantly fewer ommatidia and are much rougher compared to *dan*^{ems3}/*M*, *dan,danr*^{ex56}/*M*, or *danr*^{ex35}/*M* mosaic eyes, respectively.

Thus, although *CtBP*^{87De-10},*dan,danr*^{ex56} triple mutant clones ahead of the furrow in the larval eye disc are larger than wild-type clones (Fig 6), *CtBP*^{87De-10},*dan,danr*^{ex56}/*M* mosaic adult eyes are significantly smaller than either *CtBP*^{87De-10}/*M* single or *dan,danr*^{ex56}/*M* double mutant eyes. These results appear at first glance to be contradictory. However, as described above, it is most likely that the effect on triple mutant clone size far anterior to the furrow is due solely to loss of *CtBP*, since *Dan* and *Danr* are not expressed in this part of the disc (Curtiss et al., 2007). Therefore, although more cells are produced in the *CtBP*,*dan,danr* mutant clones, they either die instead of differentiating, or many fewer ommatidia are generated compared to *CtBP* single or *dan,danr* double mutant eyes. This genetic interaction suggests that, in addition to its early role in preventing overproliferation of eye precursors ahead of the furrow, *CtBP* also functions together with *Dan* and *Danr* at later stages to promote ommatidial cell survival and/or recruitment.

***CtBP*, *Dan* and *Danr* interact to promote formation of the founding R8 photoreceptor—**

To understand the reasons for the reduction in ommatidia in the *CtBP*/*dan/danr* double and triple mutants, we sectioned *CtBP*^{87De-10},*dan,danr*^{ex56}/*M* mosaic adult eyes to determine whether particular ommatidial cell types are affected in this genotype. In addition, we examined ommatidial development in *CtBP*^{87De-10},*dan,danr*^{ex56}/*M* mosaic eye-antennal imaginal discs using an antibody against the neuronal marker *Elav*, which stains photoreceptors. Apical sections of wild-type eyes reveal the stereotypic structure of a typical ommatidium (Fig 9A, inset): 6 “outer” photoreceptors (R1-R6) with large rhabdomeres surrounding 1 “inner” photoreceptor (R7) with a small rhabdomere. In more basal sections the R8 photoreceptor, which lies below R7, is found in the center of the outer photoreceptors and has a small rhabdomere (Wolff and Ready, 1993).

Previous studies have demonstrated that *dan,danr*^{ex56} double mutant ommatidia infrequently have the following defects: loss of one or more photoreceptors, two “inner” photoreceptors with small rhabdomeres rather than one, “inner” photoreceptors with a large rhabdomere (suggesting conversion of an “inner” photoreceptor to an “outer” photoreceptor fate, and gaps in the arrangement of ommatidia (Curtiss et al., 2007). Although *CtBP*^{87De-10},*dan,danr*^{ex56}/*M* eyes have significantly fewer ommatidia than *dan,danr*^{ex56} eyes (Fig 8A,H,I), sections reveal that *CtBP*^{87De-10},*dan,danr*^{ex56}/*M* eyes have similar defects in ommatidial structure compared to *dan,danr*^{ex56} double mutants (Fig 9A), albeit at a much higher frequency. Whereas many ommatidia have a normal complement of photoreceptors (black arrow), others show loss of photoreceptors (red and green arrows), gain of “inner” photoreceptors with small rhabdomeres (green arrow), apparent conversion

of “inner” to “outer” photoreceptor fate (yellow arrow), and gaps in the ommatidial array (white arrow). Consistent with the smaller size of *CtBP^{87De-10},dan,danr^{ex56}/M* eyes, *CtBP^{87De-10},dan,danr^{ex56}/M* triple mutant eyes have significantly more gaps in the ommatidial array than *dan,danr^{ex56}/M* double mutant eyes (not shown).

Accordingly, *CtBP^{87De-10},dan,danr^{ex56}/M* larval eye-antennal discs stained for the neuronal marker *Elav* consistently show 5-6 gaps in the developing ommatidial array (Fig 9D). Surprisingly, given that *CtBP^{87De-10}/M* adult eyes are larger than *+/M* adult eyes, *CtBP^{87De-10}/M* eye-antennal discs consistently have 1-2 gaps in the ommatidial array (Fig 9C, arrow). These results confirm that CtBP, Dan and Danr function together to promote ommatidial recruitment, and also suggest that loss of CtBP by itself affects ommatidial recruitment to a minor degree. The fact that *CtBP* mutant adult eyes have extra ommatidia despite a failure to differentiate a few ommatidia suggests that the effect of *CtBP* mutants on proliferation is even greater than is evident from the adult eye phenotype.

Ommatidial development depends on specification of the founding R8 photoreceptor, which subsequently recruits other photoreceptors and ommatidial accessory cells. R8 specification in turn depends on expression of the pro-neural gene *atonal* (*ato*), which encodes a bHLH transcription factor. Expression of the Ato protein initiates in all cells in a narrow band anterior to the morphogenetic furrow (e.g. Fig 9E', bracket). In wild-type eye-antennal discs, Ato is sequentially restricted to evenly spaced clusters of 8-10 cells referred to as intermediate groups, followed by groups of 2-3 cells that form an R8 equivalence group, and finally a single cell – the R8 precursor. Ato is expressed in R8 precursors for 2-3 rows, and subsequently fades (Jarman et al., 1994; Jarman et al., 1995; Baker et al., 1996; Dokucu et al., 1996; Frankfort and Mardon, 2002). The *senseless* (*sens*) gene is an Ato target whose expression is initiated in the R8 equivalence group, becomes restricted to the R8 and is also required for R8 development (Frankfort et al., 2001; Pepple et al., 2008).

The ommatidial phenotypes described for *dan,danr^{ex56}* mutants have previously been ascribed to a reduction in Ato expression anterior to the furrow leading to inefficient and incomplete R8 specification (Curtiss et al., 2007). Since it seemed plausible that the stronger phenotype in *CtBP^{87De-10},dan,danr^{ex56}/M* might result from an even stronger effect on Ato expression resulting from synergism among CtBP, Dan and Danr, we stained *CtBP^{87De-10}/M* and *CtBP^{87De-10},dan,danr^{ex56}/M* eye-antennal discs with anti-Ato.

Ato expression in *+/M* discs (Fig 9E) appeared identical to wild-type discs (not shown). Defects in *CtBP^{87De-10}/M* were variable and minor: in some cases the initial swath of cells expressing Ato ahead of the furrow appeared wider than in wild-type discs (compare the width of brackets in Fig 9E), and there were occasional gaps in the array of single R8 precursors (arrows in Fig 9F), consistent with the gaps in the array of developing ommatidia described above. In the *CtBP^{87De-10},dan,danr^{ex56}/M* mutant discs the initial swath of Ato-expressing cells ahead of the furrow is consistently wider (bracket in Fig 9G), and there were many gaps in the single R8 precursor array (arrows in Fig 9G). Expression of Ato in intermediate groups and R8 equivalence groups appeared normal in both *CtBP^{87De-10}/M* and *CtBP^{87De-10},dan,danr^{ex56}/M* mutant discs. We do not currently know what effect the increase in the number of Ato-expressing cells ahead of the furrow may have on R8 development, however, our data clearly shows that R8 establishment sometimes fails in *CtBP^{87De-10}/M* and even more strikingly in *CtBP^{87De-10},dan,danr^{ex56}/M* mutant discs.

To determine whether the gaps observed in the ommatidial array in *CtBP^{87De-10},dan,danr^{ex56}/M* and to a lesser extent in *CtBP^{87De-10}/M* eye-antennal discs in fact result from gaps in the formation of R8 precursors, we stained the discs with antibodies against the R8 marker *Sens*. In both *CtBP^{87De-10}/M* and *CtBP^{87De-10},dan,danr^{ex56}/M* discs

the gaps in the ommatidial array revealed by anti-Elav expression corresponded with gaps in R8 precursor formation (Fig 9I,J, arrows). This indicates that the gaps in ommatidia observed in these genotypes results from failure to establish R8 precursors. As with Ato, expression of Sens in R8 equivalence groups appeared normal in *CtBP^{87De-10}/M* and *CtBP^{87De-10},dan,danr^{ex56}/M* mutant discs.

Besides the gaps, we also noticed that, whereas in wild-type (not shown) or *+M* (Fig 9H, bracket) discs Sens expression in single R8 precursors remains strong for ~4 rows before fading slightly (Fig 9H', bracket), Sens expression in *CtBP^{87De-10}/M* and particularly in *CtBP^{87De-10},dan,danr^{ex56}/M* discs is strong for only ~2-3 rows before it fades (dramatically in *CtBP^{87De-10},dan,danr^{ex56}/M* discs; Fig 9I' and 9J', brackets) and finally rebounds somewhat by approximately the 8th row. This result suggests that CtBP, Dan, Danr are required to maintain high levels of Sens expression in R8 precursors, notably during the period when each R8 precursor is recruiting additional photoreceptors into the nascent ommatidial clusters. This may reflect a role for these factors in “locking” in the correct fate (perhaps via chromatin modification) following R8 specification, and likely explains the loss of photoreceptors and other defects observed in *CtBP^{87De-10},dan,danr^{ex56}/M* mutant eye sections (Fig 9A).

Importantly, the fact that early expression of Ato in intermediate groups and R8 equivalence groups, and of Sens in R8 equivalence groups, appears normal in both *CtBP^{87De-10}/M* and *CtBP^{87De-10},dan,danr^{ex56}/M* discs (Fig 9F-J), suggests that the roles of CtBP, Dan, Danr lie in the transition from the R8 equivalence group to a single R8 precursor. Taken together, our results strongly indicate that a complex containing CtBP, Dan and possibly Danr functions during ommatidial recruitment, promoting the establishment of R8 precursors from the R8 equivalence group, as well as the maintenance of the R8 fate that is required for proper recruitment of other cell types into ommatidia.

DISCUSSION

CtBP is a transcriptional co-regulator known to play many roles during *Drosophila* development. Here we demonstrate that CtBP can bind the probable transcription factors Dan and Danr *in vitro*, and that CtBP is part of a protein complex (or complexes) in the eye-antennal disc that includes Dan as well as the retinal determination factors Ey and Dac. As has been previously shown for the thorax, CtBP has a role in bristle development in the head capsule. In addition, CtBP regulates eye size, at least in part by limiting proliferation in eye precursors ahead of the morphogenetic furrow. We speculate that CtBP may interact with Ey to regulate proliferation of eye precursors at these early stages. Finally, CtBP synergizes with Dan and Danr for antennal specification and for maintenance/recruitment of eye precursors into ommatidia. Our results suggest that the CtBP co-regulator complexes with eye and antennal specification factors at multiple stages to regulate cell fate specification.

A role for CtBP in regulating proliferation in eye precursors

The eyes of several CtBP loss-of-function mutant combinations have statistically significantly more ommatidia than wild-type eyes, and *CtBP^{87De-10}* clones ahead of the furrow show a statistically significant increase in the number of cells undergoing mitosis per unit area. These results suggest a role for CtBP in downregulating proliferation of eye precursors, although we cannot currently exclude additional processes such as apoptosis that might contribute to the increase in eye size in *CtBP*- mutants.

The evidence presented here suggests that CtBP is required to downregulate proliferation of eye precursors ahead of the morphogenetic furrow: *CtBP*- clones are larger and show more mitotic figures than wild type clones in the most anterior regions of the eye field (Fig 6). As

described below, a number of factors are known to promote proliferation ahead of the furrow, and have been connected to CtBP in some way. Many of these factors can cause massive overgrowth when overexpressed. Thus, the role of CtBP may be to counteract the activity of one or more of these factors, to ensure that cells do not proliferate out of control.

Factors that have previously been linked to CtBP and are known to regulate proliferation of eye precursors ahead of the furrow include the Wingless (Treisman and Rubin, 1995; Lee and Treisman, 2001; Baonza and Freeman, 2002), Notch (Dominguez and de Celis, 1998; Papayannopoulos et al., 1998; Morel et al., 2001; Barolo et al., 2002; Nagel et al., 2005; Bray, 2006; Nagel et al., 2007; Kovall, 2008; Protzer et al., 2008; Borggreffe and Oswald, 2009; Fortini, 2009) and JAK/STAT (Chao et al., 2004; Dominguez and Casares, 2005; Reynolds-Kenneally and Mlodzik, 2005; Gutierrez-Avino et al., 2009) signaling pathways. We have not ruled out a possible interaction between CtBP and these signaling pathways in the context of eye precursor proliferation.

However, the combination of Hth, Tsh and Ey also regulates proliferation in ahead of the morphogenetic furrow (Bessa et al., 2002; Peng et al., 2009; Lopes and Casares, 2010), and we have demonstrated here that Ey and CtBP are part of a complex in eye-antennal disc cells, and that they interact genetically during eye development. Circumstantial evidence also suggests links between CtBP and Hth and Tsh. For instance, *Drosophila* and mouse Tsh homologs both contain a PxDLS motif, and have been shown to interact *in vitro* with *Drosophila* and mouse CtBP, respectively (Saller et al., 2002; Manfroid et al., 2004). In addition, the *Drosophila* Cdc25 homolog encoded by *string*, which triggers mitosis, appears to be a target of Hth, although it is not clear if it is a direct target (Lopes and Casares, 2010). DamID experiments with CtBP in Kc cells have also identified *string* as a potential direct CtBP target (Bianchi-Frias et al., 2004). Based on current data, we therefore propose that CtBP interacts with the Hth/Tsh/Ey complex in eye precursors ahead of the furrow.

CtBP and the transition from proliferation to differentiation in the *Drosophila* eye

CtBP^{87De-10} single mutant clones ahead of the furrow in the larval eye disc are larger than wild-type clones and show evidence of eye precursor overproliferation. Accordingly, adult eyes of *CtBP* transheterozygous combinations or of *CtBP^{87De-10}/M* mosaic individuals have more ommatidia than wild-type eyes. In contrast, whereas *CtBP^{87De-10}, dan, danr^{ex56}* triple mutant clones are similar to *CtBP^{87De-10}* single mutant clones in being larger than wild-type clones ahead of the furrow, *CtBP^{87De-10}, dan, danr^{ex56}* adults have small rough eyes. This suggests either that the *CtBP^{87De-10}, dan, danr^{ex56}* triple mutant cells fail to be efficiently recruited into ommatidia and/or eventually undergo apoptosis. In support of the former hypothesis, the phenotypic analysis shown in Fig. 9 demonstrates that recruitment of the R8 photoreceptor, which is required to recruit all other ommatidial cells, is affected by loss of *CtBP*, and is more strongly affected in the *CtBP, dan, danr* triple mutant.

Given the dynamic and overlapping expression patterns of the retinal determination factors, one intriguing hypothesis that fits our data is that a complex containing CtBP may have different members at different stages of eye development. For instance, a complex containing CtBP, Ey as well as possibly Tsh and Hth anterior to the furrow, might participate in maintaining a “poised” chromatin structure with respect to eye specific genes, in which genes involved in eye differentiation are not yet expressed and the cells are kept in a proliferative state. It has been suggested that vertebrate Pax6 is a “pioneering” factor for the lens lineage (Yang et al., 2006), and other “pioneering” factors have been shown to promote a “bivalent” state in which developmental genes are silenced, but “poised” for activation (e.g. (Bernstein et al., 2006). The down-regulation of Ey close to the furrow, and the initiation of Dan, Danr and Dac expression in the same region would be expected to

change the composition of the complex containing CtBP and lead to changes in transcription that reflect the transition from proliferation to differentiation.

At present we do not know what genes might be direct targets of the complexes containing CtBP and the eye specification factors. Some possibilities include the cell cycle regulator *string* (mentioned above), and the pre-proneural gene *atonal*, which is known to be regulated by Ey, So, Dan and Danr (Ey and So are direct regulators) (Zhang et al., 2006; Curtiss et al., 2007), and which plays a critical role in the transition from proliferation to differentiation of eye precursors. Thus, future work on *Drosophila* CtBP will shed light on the functions of this important transcriptional regulator, as well as on important transitions during development.

EXPERIMENTAL PROCEDURES

Alleles

The *dan^{ems3}*, *danr^{ex35}*, *dan danr^{ex56}* mutations have been described previously (Emerald et al., 2003; Curtiss et al., 2007). *CtBP^{87De-10}* and *CtBP⁰³⁴⁶³* were both obtained from the Bloomington *Drosophila* stock center. *CtBP^{87De-10}* (Hilliker et al., 1980) is reported to be a nonsense mutation that truncates the protein to 228 amino acids (Flybase). Using an antibody generated against amino acids 8-383 (Stern et al., 2009) (kind gift of Y. Nibu), we do not detect CtBP protein in *CtBP^{87De-10}* homozygous clones, suggesting that the truncated protein is not stable or that the precise epitope of this antibody lies between amino acids 228-383. This, together with the fact that the *eyFLP; CtBP^{87De-10}/M* phenotype resembles the *CtBP^{87De-10}/Df(3L)ry615* phenotype, suggests that *CtBP^{87De-10}* is a strong loss-of-function allele. *CtBP⁰³⁴⁶³* has a P-element insertion located 5' to the CtBP coding region (Poortinga et al., 1998).

P[GSV]^{A396} and *P[GSV]^{A132}* are bidirectional EP elements located in the first intron of *CtBP* (Toba et al., 1999; Fang et al., 2006) and were kind gifts from K. Cadigan.

Flies expressing FLAG-tagged Eyeless were a kind gift from R. Chen. The FLAG tag is a C-terminal fusion incorporated into a genomic construct containing the entire *eyeless* gene. The construct is inserted at chromosome region 68A, and rescues an *eyeless* mutant (R. Chen, personal communication).

GST Pulldowns

GST fusion proteins for Dan and Danr have been previously described (Curtiss and Mlodzik, 2000); GST-CtBP (Nibu et al., 1998b), and GST-SixHD (Kenyon et al., 2005b) were kind gifts from J. Posakony and F. Pignoni, respectively. Biotinylated *in vitro* translated proteins were generated using the TnT Quick-Coupled *in vitro* Transcription/Translation kit and Transcend Biotinylated tRNA (Promega). GST Pulldowns were performed using the MagnetGST Pull-Down System (Promega) according to the manufacturers protocol. Samples were analyzed by Western blotting, probing with HRP-conjugated streptavidin (1:10,000, Jackson ImmunoResearch). Chemiluminescent detection was performed and analyzed using a ChemiDoc XRX imaging system and Quantity One software (Biorad).

Western Blots and Immunoprecipitation

Western blots were performed as previously described (O'Keefe et al., 2009). Membranes were probed with rat anti-Dan (1:300), rat anti-Danr (1:500), mouse anti-Tubulin [1:500, Developmental Studies Hybridoma Bank (DSHB)], rabbit anti-Ey (1:100, gift of U. Walldorf), rabbit anti-CtBP (1:1500, gift of D. Arnosti) or mouse anti-Dac (1:200, DSHB),

washed and probed with HRP-conjugated secondary antibodies. Chemiluminescent detection and analysis was performed as described above.

Immunoprecipitations were performed by dissecting eye-antennal disk/brain complexes from 200 wild-type 3rd instar larvae. Eye-antennal disc/brain complexes were lysed in 500 μ L RIPA buffer containing protease inhibitors for 10 minutes with vigorous shaking at room temperature. Lysates were cleared by centrifugation at 10,000 rpm for 5 minutes at 4° C, and the supernatant was equally divided into two tubes. Anti-Dan, anti-CtBP (Stern et al., 2009) (kind gift of Y. Nibu) or anti-Tubulin (DSHB) (2 μ g for 300 μ L of lysate) was added and incubated for two hours at 4° C. 40 μ L of Protein G agarose beads (Invitrogen) was added and incubated for an additional two hours at 4° C. The beads were washed three times, shaking vigorously at room temperature in wash buffer (4.2mM Na₂HPO₄, 2mM KH₂PO₄, 140mM NaCl, 10mM KCl). Beads were then resuspended in 40 μ L 1x SDS-PAGE loading buffer (Biorad) and boiled 5 minutes, and 20 μ L of each sample was analyzed by Western blotting as described above.

For the anti-FLAG immunoprecipitations, 200 eye-antenna disc/brain complexes were lysed as above. The lysate volume was increased to 1 mL by the addition of 500 μ L lysis buffer (50 mM Tris pH7.4, 150 mM NaCl, 1mM EDTA, 1% Triton X-100). The lysate was then divided equally. 40 μ L anti-FLAG M2 beads (Sigma) were added to one aliquot, while 40 μ L protein G beads were added to the other. Both tubes were incubated overnight at 4° C, and then processed as described above.

Immunohistochemistry

Rabbit anti-Phospho-Histone H3 (1:400, Cell Signaling Technologies) stainings were performed according to the manufacturers protocol. Other primary antibodies used were guinea pig anti-Atonal (1:2000, gift from D. Marendt), guinea pig anti-Senseless (1:1000, gift from G. Mardon) and rat anti-Elav (1:100, Developmental Studies Hybridoma Bank). Fluorescent secondary antibodies (Jackson ImmunoResearch) were diluted 1:200. Images were obtained with a Zeiss Axioimager Z3 fluorescent microscope with Apotome and analyzed using Zeiss Axiovision, ImageJ and Adobe Photoshop software. Clonal and non-clonal areas were measured using Axiovision software.

Analysis of the adult phenotypes

For the analysis of the adult eye and vertex phenotypes, adult flies were dissected out of the pupal case (where necessary), frozen 20 minutes at -80° and then imaged with a Hitachi TM-1000 Tabletop Scanning Electron Microscope. For the analysis of the adult antenna phenotypes, antennae were dissected in PBS, transferred to methanol for 20 minutes, then mounted in 80% glycerol. Images were obtained and analyzed as described above.

Semi-thin sections of adult eyes were generated as previously described (O'Keefe et al., 2009).

Acknowledgments

We would like to thank Ken Cadigan, Graeme Mardon, Daniel Marendt, Yutaka Nibu, Francesca Pignoni, James Posakony, Uwe Walldorf, the Bloomington *Drosophila* Stock Center, and the Developmental Studies Hybridoma Bank for reagents. We would especially like to thank Rui Chen for sharing reagents prior to publication. Thanks to Kathryn Hanley for continuing patient help with statistics, and to Peter Cooke in the NMSU Electron Microscopy Laboratory for help with SEM. Thanks to Andreas Jenny and to Richard Cripps and to anonymous reviewers for insightful comments on the manuscript. This work was supported by NIH NIGMS GM08136.

Grant information: NIH NIGMS GM08136

REFERENCES

- Aihara, H.; Perrone, L.; Nibu, Y. Transcriptional Repression by the CtBP Corepressor in *Drosophila*. In: Chinnadurai, G., editor. CtBP Family Proteins. Georgetown. Landes Bioscience and Springer Science-Business Media; Texas: 2007. p. 18-26.
- Anderson J, Bhandari R, Kumar JP. A genetic screen identifies putative targets and binding partners of CREB-binding protein in the developing *Drosophila* eye. *Genetics*. 2005; 171:1655–1672. [PubMed: 15998717]
- Baker NE, Yu S, Han D. Evolution of proneural atonal expression during distinct regulatory phases in the developing *Drosophila* eye. *Curr Biol*. 1996; 6:1290–1301. [PubMed: 8939576]
- Baonza A, Freeman M. Control of *Drosophila* eye specification by Wingless signalling. *Development*. 2002; 129:5313–5322. [PubMed: 12403704]
- Barolo S, Stone T, Bang AG, Posakony JW. Default repression and Notch signaling: Hairless acts as an adaptor to recruit the corepressors Groucho and dCtBP to Suppressor of Hairless. *Genes Dev*. 2002; 16:1964–1976. [PubMed: 12154126]
- Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, Jaenisch R, Wagschal A, Feil R, Schreiber SL, Lander ES. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell*. 2006; 125:315–326. [PubMed: 16630819]
- Bessa J, Gebelein B, Pichaud F, Casares F, Mann RS. Combinatorial control of *Drosophila* eye development by eyeless, homothorax, and teashirt. *Genes Dev*. 2002; 16:2415–2427. [PubMed: 12231630]
- Bianchi-Frias D, Orian A, Delrow JJ, Vazquez J, Rosales-Nieves AE, Parkhurst SM. Hairly transcriptional repression targets and cofactor recruitment in *Drosophila*. *PLoS Biol*. 2004; 2:E178. [PubMed: 15252443]
- Biryukova I, Heitzler P. *Drosophila* C-terminal binding protein, dCtBP is required for sensory organ prepattern and sharpens proneural transcriptional activity of the GATA factor Pnr. *Dev Biol*. 2008; 323:64–75. [PubMed: 18773887]
- Bonifer C, Hoogenkamp M, Krysinska H, Tagoh H. How transcription factors program chromatin-- lessons from studies of the regulation of myeloid-specific genes. *Semin Immunol*. 2008; 20:257–263. [PubMed: 18579409]
- Borggreffe T, Oswald F. The Notch signaling pathway: transcriptional regulation at Notch target genes. *Cell Mol Life Sci*. 2009; 66:1631–1646. [PubMed: 19165418]
- Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*. 1993; 118:401–415. [PubMed: 8223268]
- Bray SJ. Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol*. 2006; 7:678–689. [PubMed: 16921404]
- Callaerts P, Leng S, Clements J, Benassayag C, Cribbs D, Kang YY, Walldorf U, Fischbach KF, Strauss R. *Drosophila* Pax-6/eyeless is essential for normal adult brain structure and function. *J Neurobiol*. 2001; 46:73–88. [PubMed: 11153010]
- Casares F, Mann RS. Control of antennal versus leg development in *Drosophila*. *Nature*. 1998; 392:723–726. [PubMed: 9565034]
- Cavodeassi F, Diez del Corral R, Campuzano S, Domínguez M. Compartments and organising boundaries in the *Drosophila* eye: the role of the homeodomain Iroquois proteins. *Development*. 1999; 126:4933–4942. [PubMed: 10529412]
- Chao JL, Tsai YC, Chiu SJ, Sun YH. Localized Notch signal acts through eyg and upd to promote global growth in *Drosophila* eye. *Development*. 2004; 131:3839–3847. [PubMed: 15253935]
- Chen R, Amoui M, Zhang Z, Mardon G. Dachshund and Eyes Absent proteins form a complex and function synergistically to Induce ectopic eye development in *Drosophila*. *Cell*. 1997; 91:893–903. [PubMed: 9428513]
- Chinnadurai G. Transcriptional regulation by C-terminal binding proteins. *Int J Biochem Cell Biol*. 2007; 39:1593–1607. [PubMed: 17336131]

- Cirillo LA, Lin FR, Cuesta I, Friedman D, Jarnik M, Zaret KS. Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. *Mol Cell*. 2002; 9:279–289. [PubMed: 11864602]
- Curtiss J, Burnett M, Mlodzik M. distal antenna and distal antenna-related function in the retinal determination network during eye development in *Drosophila*. *Dev Biol*. 2007; 306:685–702. [PubMed: 17493605]
- Curtiss J, Mlodzik M. Morphogenetic furrow initiation and progression during eye development in *Drosophila*: the roles of *decapentaplegic*, *hedgehog*, and *eyes absent*. *Development*. 2000; 127:1325–1336. [PubMed: 10683184]
- Dokucu ME, Zipursky SL, Cagan RL. Atonal, rough and the resolution of proneural clusters in the developing *Drosophila* retina. *Development*. 1996; 122:4139–4147. [PubMed: 9012533]
- Dominguez M, Casares F. Organ specification-growth control connection: new in-sights from the *Drosophila* eye-antennal disc. *Dev Dyn*. 2005; 232:673–684. [PubMed: 15704149]
- Dominguez M, de Celis JF. A dorsal/ventral boundary established by Notch controls growth and polarity in the *Drosophila* eye. *Nature*. 1998; 396:276–278. [PubMed: 9834035]
- Dong PD, Dicks JS, Panganiban G. Distal-less and homothorax regulate multiple targets to pattern the *Drosophila* antenna. *Development*. 2002; 129:1967–1974. [PubMed: 11934862]
- Dong PDS, Chu J, Panganiban G. Coexpression of the homeobox genes *Distal-less* and *homothorax* determines *Drosophila* antennal identity. *Development*. 2000; 127:209–216. [PubMed: 10603339]
- Duncan DM, Burgess EA, Duncan I. Control of distal antennal identity and tarsal development in *Drosophila* by spineless-aristapedia, a homolog of the mammalian dioxin receptor. *Genes Dev*. 1998; 12:1290–1303. [PubMed: 9573046]
- Emerald BS, Curtiss J, Mlodzik M, Cohen SM. Distal antenna and distal antenna related encode nuclear proteins containing pipsqueak motifs involved in antenna development in *Drosophila*. *Development*. 2003; 130:1171–1180. [PubMed: 12571108]
- Escudero LM, Freeman M. Mechanism of G1 arrest in the *Drosophila* eye imaginal disc. *BMC Dev Biol*. 2007; 7:13. [PubMed: 17335573]
- Fang M, Li J, Blauwkamp T, Bhambhani C, Campbell N, Cadigan KM. C-terminal-binding protein directly activates and represses Wnt transcriptional targets in *Drosophila*. *Embo J*. 2006; 25:2735–2745. [PubMed: 16710294]
- Ferres-Marco D, Gutierrez-Garcia I, Vallejo DM, Bolivar J, Gutierrez-Avino FJ, Dominguez M. Epigenetic silencers and Notch collaborate to promote malignant tumours by Rb silencing. *Nature*. 2006; 439:430–436. [PubMed: 16437107]
- Fortini ME. Notch signaling: the core pathway and its posttranslational regulation. *Dev Cell*. 2009; 16:633–647. [PubMed: 19460341]
- Frankfort BJ, Mardon G. R8 development in the *Drosophila* eye: a paradigm for neural selection and differentiation. *Development*. 2002; 129:1295–1306. [PubMed: 11880339]
- Frankfort BJ, Nolo R, Zhang Z, Bellen H, Mardon G. senseless repression of rough is required for R8 photoreceptor differentiation in the developing *Drosophila* eye. *Neuron*. 2001; 32:403–414. [PubMed: 11709152]
- Gehring WJ. New perspectives on eye development and the evolution of eyes and photoreceptors. *J Hered*. 2005; 96:171–184. [PubMed: 15653558]
- Gehring WJ, Ieko K. *Pax-6* mastering eye morphogenesis and eye evolution. *Trends Genet*. 1999; 15:371–377. [PubMed: 10461206]
- Giot L, Bader JS, Brouwer C, Chaudhuri A, Kuang B, Li Y, Hao YL, Ooi CE, Godwin B, Vitols E, Vijayadamodar G, Pochart P, Machineni H, Welsh M, Kong Y, Zerhusen B, Malcolm R, Varrone Z, Collis A, Minto M, Burgess S, McDaniel L, Stimpson E, Spriggs F, Williams J, Neurath K, Ioime N, Agee M, Voss E, Furtak K, Renzulli R, Aanensen N, Carrola S, Bickelhaupt E, Lazovatsky Y, DaSilva A, Zhong J, Stanyon CA, Finley RL Jr. White KP, Braverman M, Jarvie T, Gold S, Leach M, Knight J, Shimkets RA, McKenna MP, Chant J, Rothberg JM. A protein interaction map of *Drosophila melanogaster*. *Science*. 2003; 302:1727–1736. [PubMed: 14605208]
- Gutierrez-Avino FJ, Ferres-Marco D, Dominguez M. The position and function of the Notch-mediated eye growth organizer: the roles of JAK/STAT and four-jointed. *EMBO Rep*. 2009; 10:1051–1058. [PubMed: 19662079]

- Halder G, Callaerts P, Flister S, Walldorf U, Kloter U, Gehring WJ. Eyeless initiates the expression of both *sine oculis* and *eyes absent* during *Drosophila* compound eye development. *Development*. 1998; 125:2181–2191. [PubMed: 9584118]
- Halder G, Callaerts P, Gehring WJ. Induction of ectopic eyes by targeted expression of the *eyeless* gene in *Drosophila*. *Science*. 1995; 267:1788–1792. [PubMed: 7892602]
- Hildebrand JD, Soriano P. Overlapping and unique roles for C-terminal binding protein 1 (CtBP1) and CtBP2 during mouse development. *Mol Cell Biol*. 2002; 22:5296–5307. [PubMed: 12101226]
- Hilliker AJ, Clark SH, Chovnick A, Gelbart WM. Cytogenetic analysis of the chromosomal region immediately adjacent to the rosy locus in *Drosophila melanogaster*. *Genetics*. 1980; 95:95–110. [PubMed: 6776006]
- Huang DH, Chang YL, Yang CC, Pan IC, King B. pipsqueak encodes a factor essential for sequence-specific targeting of a polycomb group protein complex. *Mol Cell Biol*. 2002; 22:6261–6271. [PubMed: 12167718]
- Hussain MA, Habener JF. Glucagon gene transcription activation mediated by synergistic interactions of pax-6 and cdx-2 with the p300 co-activator. *J Biol Chem*. 1999; 274:28950–28957. [PubMed: 10506141]
- Iwahara J, Kigawa T, Kitagawa K, Masumoto H, Okazaki T, Yokoyama S. A helix-turn-helix structure unit in human centromere protein B (CENP-B). *Embo J*. 1998; 17:827–837. [PubMed: 9451007]
- Janody F, Lee JD, Jähren N, Hazelett DJ, Benlali A, Miura GI, Draskovic I, Treisman JE. A mosaic genetic screen reveals distinct roles for trithorax and polycomb group genes in *Drosophila* eye development. *Genetics*. 2004; 166:187–200. [PubMed: 15020417]
- Jarman AP, Grell EH, Ackerman L, Jan LY, Jan YN. Atonal is the proneural gene for *Drosophila* photoreceptors. *Nature*. 1994; 369:398–400. [PubMed: 8196767]
- Jarman AP, Sun Y, Jan LY, Jan YN. Role of the proneural gene, atonal, in formation of *Drosophila* chordotonal organs and photoreceptors. *Development*. 1995; 121:2019–2030. [PubMed: 7635049]
- Johnston LA, Ostrow BD, Jasoni C, Blochlinger K. The homeobox gene cut interacts genetically with the homeotic genes proboscipedia and Antennapedia. *Genetics*. 1998; 149:131–142. [PubMed: 9584091]
- Kenyon KL, Li DJ, Clouser C, Tran S, Pignoni F. Fly SIX-type homeodomain proteins Sine oculis and Optix partner with different cofactors during eye development. *Dev Dyn*. 2005a; 234:497–504. [PubMed: 15937930]
- Kenyon KL, Yang-Zhou D, Cai CQ, Tran S, Clouser C, Decene G, Ranade S, Pignoni F. Partner specificity is essential for proper function of the SIX-type homeodomain proteins Sine oculis and Optix during fly eye development. *Dev Biol*. 2005b; 286:158–168. [PubMed: 16125693]
- Kobayashi M, Nishikawa K, Suzuki T, Yamamoto M. The homeobox protein Six3 interacts with the Groucho corepressor and acts as a transcriptional repressor in eye and forebrain formation. *Dev Biol*. 2001; 232:315–326. [PubMed: 11401394]
- Kovall RA. More complicated than it looks: assembly of Notch pathway transcription complexes. *Oncogene*. 2008; 27:5099–5109. [PubMed: 18758478]
- Kumar JP. The molecular circuitry governing retinal determination. *Biochim Biophys Acta*. 2009; 1789:306–314. [PubMed: 19013263]
- Kumar JP, Jamal T, Doetsch A, Turner FR, Duffy JB. CREB binding protein functions during successive stages of eye development in *Drosophila*. *Genetics*. 2004; 168:877–893. [PubMed: 15514061]
- Kumar V, Carlson JE, Ohgi KA, Edwards TA, Rose DW, Escalante CR, Rosenfeld MG, Aggarwal AK. Transcription corepressor CtBP is an NAD(+)-regulated dehydrogenase. *Mol Cell*. 2002; 10:857–869. [PubMed: 12419229]
- Lee JD, Treisman JE. The role of Wingless signaling in establishing the anteroposterior and dorsoventral axes of the eye disc. *Development*. 2001; 128:1519–1529. [PubMed: 11290291]
- Lopes CS, Casares F. hth maintains the pool of eye progenitors and its downregulation by Dpp and Hh couples retinal fate acquisition with cell cycle exit. *Dev Biol*. 2010; 339:78–88. [PubMed: 20036228]
- Lopez-Rios J, Tessmar K, Loosli F, Wittbrodt J, Bovolenta P. Six3 and Six6 activity is modulated by members of the groucho family. *Development*. 2003; 130:185–195. [PubMed: 12441302]

- Lours C, Bardot O, Godt D, Laski FA, Couderc JL. The *Drosophila melanogaster* BTB proteins bric a brac bind DNA through a composite DNA binding domain containing a pipsqueak and an AT-Hook motif. *Nucleic Acids Res.* 2003; 31:5389–5398. [PubMed: 12954775]
- Manfroid I, Caubit X, Kerridge S, Fasano L. Three putative murine Teashirt orthologues specify trunk structures in *Drosophila* in the same way as the *Drosophila* teashirt gene. *Development.* 2004; 131:1065–1073. [PubMed: 14973285]
- Mani-Telang P, Arnosti DN. Developmental expression and phylogenetic conservation of alternatively spliced forms of the C-terminal binding protein corepressor. *Dev Genes Evol.* 2007; 217:127–135. [PubMed: 17120023]
- Mann RS. The developmental and molecular biology of genes that subdivide the body of *Drosophila*. *Annu. Rev. Cell Dev. Biol.* 2000; 16:243–271. [PubMed: 11031237]
- Morel V, Lecourtois M, Massiani O, Maier D, Preiss A, Schweisguth F. Transcriptional repression by suppressor of hairless involves the binding of a hairless-dCtBP complex in *Drosophila*. *Curr Biol.* 2001; 11:789–792. [PubMed: 11378391]
- Nagel AC, Krejci A, Tenin G, Bravo-Patino A, Bray S, Maier D, Preiss A. Hairless-mediated repression of notch target genes requires the combined activity of Groucho and CtBP corepressors. *Mol Cell Biol.* 2005; 25:10433–10441. [PubMed: 16287856]
- Nagel AC, Wech I, Schwinkendorf D, Preiss A. Involvement of co-repressors Groucho and CtBP in the regulation of single-minded in *Drosophila*. *Hereditas.* 2007; 144:195–205. [PubMed: 18031354]
- Nardini M, Spano S, Cericola C, Pesce A, Massaro A, Millo E, Luini A, Corda D, Bolognesi M. CtBP/BARS: a dual-function protein involved in transcription co-repression and Golgi membrane fission. *Embo J.* 2003; 22:3122–3130. [PubMed: 12805226]
- Newsome TP, Asling B, Dickson BJ. Analysis of *Drosophila* photoreceptor axon guidance in eye-specific mosaics. *Development.* 2000; 127:851–860. [PubMed: 10648243]
- Nibu Y, Zhang H, Bajor E, Barolo S, Small S, Levine M. dCtBP mediates transcriptional repression by Knirps, Kruppel and Snail in the *Drosophila* embryo. *Embo J.* 1998a; 17:7009–7020. [PubMed: 9843507]
- Nibu Y, Zhang H, Levine M. Interaction of short-range repressors with *Drosophila* CtBP in the embryo. *Science.* 1998b; 280:101–104. [PubMed: 9525852]
- Niimi T, Seimiya M, Kloter U, Flister S, Gehring WJ. Direct regulatory interaction of the Eyeless protein with an eye-specific enhancer in the *sine oculis* gene during eye induction in *Drosophila*. *Development.* 1999; 126:2253–2260. [PubMed: 10207149]
- O’Keefe DD, Gonzalez-Nino E, Burnett M, Dylla L, Lambeth SM, Licon E, Amesoli C, Edgar BA, Curtiss J. Rap1 maintains adhesion between cells to affect Egfr signaling and planar cell polarity in *Drosophila*. *Dev Biol.* 2009; 333:143–160. [PubMed: 19576205]
- Palacios D, Puri PL. The epigenetic network regulating muscle development and regeneration. *J Cell Physiol.* 2006; 207:1–11. [PubMed: 16155926]
- Papayannopoulos V, Tomlinson A, Panin VM, Rauskolb C, Irvine KD. Dorsal-ventral signaling in the *Drosophila* eye. *Science.* 1998; 281:2031–2034. [PubMed: 9748163]
- Peng HW, Slattey M, Mann RS. Transcription factor choice in the Hippo signaling pathway: homothorax and yorkie regulation of the microRNA bantam in the progenitor domain of the *Drosophila* eye imaginal disc. *Genes Dev.* 2009; 23:2307–2319. [PubMed: 19762509]
- Pepple KL, Atkins M, Venken K, Wellnitz K, Harding M, Frankfort B, Mardon G. Two-step selection of a single R8 photoreceptor: a bistable loop between senseless and rough locks in R8 fate. *Development.* 2008; 135:4071–4079. [PubMed: 19004852]
- Pignoni F, Hu B, Zavitz KH, Xiao J, Garrity PA, Zipursky SL. The eye-specification proteins So and Eya form a complex and regulate multiple steps in *Drosophila* eye development. *Cell.* 1997; 91:881–891. [PubMed: 9428512]
- Poortinga G, Watanabe M, Parkhurst SM. *Drosophila* CtBP: a Hairy-interacting protein required for embryonic segmentation and hairy-mediated transcriptional repression. *Embo J.* 1998; 17:2067–2078. [PubMed: 9524128]
- Protzer CE, Wech I, Nagel AC. Hairless induces cell death by downregulation of EGFR signalling activity. *J Cell Sci.* 2008; 121:3167–3176. [PubMed: 18765565]

- Punzo C, Kurata S, Gehring WJ. The eyeless homeodomain is dispensable for eye development in *Drosophila*. *Genes Dev.* 2001; 15:1716–1723. [PubMed: 11445545]
- Quiring R, Walldorf U, Kloter U. Homology of the *eyeless* gene of *Drosophila* to the *Small eye* gene in mice and *Aniridia* in humans. *Science.* 1994; 265:785–789. [PubMed: 7914031]
- Reynolds-Kenneally J, Mlodzik M. Notch signaling controls proliferation through cell-autonomous and non-autonomous mechanisms in the *Drosophila* eye. *Dev Biol.* 2005; 285:38–48. [PubMed: 16039641]
- Saller E, Kelley A, Bienz M. The transcriptional repressor Brinker antagonizes Wingless signaling. *Genes Dev.* 2002; 16:1828–1838. [PubMed: 12130542]
- Schaeper U, Boyd JM, Verma S, Uhlmann E, Subramanian T, Chinnadurai G. Molecular cloning and characterization of a cellular phosphoprotein that interacts with a conserved C-terminal domain of adenovirus E1A involved in negative modulation of oncogenic transformation. *Proc Natl Acad Sci U S A.* 1995; 92:10467–10471. [PubMed: 7479821]
- Shi Y, Sawada J, Sui G, Affar el B, Whetstone JR, Lan F, Ogawa H, Luke MP, Nakatani Y. Coordinated histone modifications mediated by a CtBP co-repressor complex. *Nature.* 2003; 422:735–738. [PubMed: 12700765]
- Siegmund T, Lehmann M. The *Drosophila* Pipsqueak protein defines a new family of helix-turn-helix DNA-binding proteins. *Dev Genes Evol.* 2002; 212:152–157. [PubMed: 11976954]
- Silver SJ, Davies EL, Doyon L, Rebay I. Functional dissection of eyes absent reveals new modes of regulation within the retinal determination gene network. *Mol Cell Biol.* 2003; 23:5989–5999. [PubMed: 12917324]
- Silver SJ, Rebay I. Signaling circuitries in development: insights from the retinal determination gene network. *Development.* 2005; 132:3–13. [PubMed: 15590745]
- Stern MD, Aihara H, Cho KH, Kim GT, Horiguchi G, Roccaro GA, Guevara E, Sun HH, Negeri D, Tsukaya H, Nibu Y. Structurally related Arabidopsis ANGUSTIFOLIA is functionally distinct from the transcriptional corepressor CtBP. *Dev Genes Evol.* 2007; 217:759–769. [PubMed: 17972097]
- Stern MD, Aihara H, Roccaro GA, Cheung L, Zhang H, Negeri D, Nibu Y. CtBP is required for proper development of peripheral nervous system in *Drosophila*. *Mech Dev.* 2009; 126:68–79. [PubMed: 18992810]
- Sutrias-Grau M, Arnosti DN. CtBP contributes quantitatively to Knirps repression activity in an NAD binding-dependent manner. *Mol Cell Biol.* 2004; 24:5953–5966. [PubMed: 15199149]
- Suzanne M. Expression analysis of the *Drosophila* pipsqueak family members fernandez/distal antenna and hernandez/distal antenna related. *Dev Dyn.* 2004; 230:361–365. [PubMed: 15162514]
- Suzanne M, Estella C, Calleja M, Sanchez-Herrero E. The hernandez and fernandez genes of *Drosophila* specify eye and antenna. *Dev Biol.* 2003; 260:465–483. [PubMed: 12921746]
- Toba G, Ohsako T, Miyata N, Ohtsuka T, Seong KH, Aigaki T. The gene search system. A method for efficient detection and rapid molecular identification of genes in *Drosophila melanogaster*. *Genetics.* 1999; 151:725–737. [PubMed: 9927464]
- Treisman JE, Rubin GM. wingless inhibits morphogenetic furrow movement in the *Drosophila* eye disc. *Development.* 1995; 121:3519–3527. [PubMed: 8582266]
- Turner J, Crossley M. Cloning and characterization of mCtBP2, a co-repressor that associates with basic Kruppel-like factor and other mammalian transcriptional regulators. *Embo J.* 1998; 17:5129–5140. [PubMed: 9724649]
- Turner J, Crossley M. The CtBP family: enigmatic and enzymatic transcriptional co-repressors. *BioEssays.* 2001; 23:683–690. [PubMed: 11494316]
- Wang H, Hartwood E, Finnegan DJ. Pogo transposase contains a putative helix-turn-helix DNA binding domain that recognises a 12 bp sequence within the terminal inverted repeats. *Nucleic Acids Res.* 1999; 27:455–461. [PubMed: 9862965]
- Wolff T, Ready DF. Cell death in normal and rough eye mutants of *Drosophila*. *Development.* 1991; 113:825–839. [PubMed: 1821853]
- Wolff T.; Ready, DF. Pattern formation in the *Drosophila* retina. In: Martinez-Arias, MBA., editor. The development of *Drosophila melanogaster*. Cold Spring Harbor Press; Cold Spring Harbor: 1993. p. 1277-1326.

- Xu T, Rubin GM, Greenwood S, Struhl G. Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development*. 1993; 117:1223–1237. [PubMed: 8404527]
- Yang Y, Stopka T, Golestaneh N, Wang Y, Wu K, Li A, Chauhan BK, Gao CY, Cveklova K, Duncan MK, Pestell RG, Chepelinsky AB, Skoultchi AI, Cvekl A. Regulation of alphaA-crystallin via Pax6, c-Maf, CREB and a broad domain of lens-specific chromatin. *Embo J*. 2006; 25:2107–2118. [PubMed: 16675956]
- Zhang T, Ranade S, Cai CQ, Clouser C, Pignoni F. Direct control of neurogenesis by selector factors in the fly eye: regulation of atonal by Ey and So. *Development*. 2006; 133:4881–4889. [PubMed: 17108002]
- Zhu CC, Dyer MA, Uchikawa M, Kondoh H, Lagutin OV, Oliver G. Six3-mediated auto repression and eye development requires its interaction with members of the Groucho-related family of co-repressors. *Development*. 2002; 129:2835–2849. [PubMed: 12050133]

Figure 1.

Dan and Danr interact with CtBP *in vitro* and *in vivo*. (A) Alignments of partial predicted protein sequences for Danr (top) and Dan (bottom) orthologs in *Drosophila* species for which genome sequences are available. The *Drosophila melanogaster* sequence is boxed horizontally in gray. The highly conserved CtBP-binding PEDLS motif is boxed vertically in black. The single *Aedes aegypti* (mosquito) ortholog (bottom-most sequence in the Dan alignment) also contains a PEDLS sequence (boxed). (B) GST-pulldowns for *in vitro* translated Dan (top panel) or Danr (bottom panel). GST-CtBP, GST-Danr, and GST-Dan interact with both Dan and Danr, but GST and GST-Six HD (a GST construct containing the Six domain and the homeodomain of Sine oculis) do not. (C) Anti-Dan, but not anti-Tubulin, is capable of immunoprecipitating CtBP and Danr from eye-antennal disc extracts. (D) Anti-Dan detects an ~90 kD band in extracts from wild-type eye-antennal discs. In *dan, dan^{ex56}* homozygous eye-antennal discs this band is absent. The non-specific band at ~175 kD serves as a loading control. (E) Anti-CtBP, but not anti-Tubulin, is capable of immunoprecipitating Dan from eye-antennal disc extracts.

Figure 2.

Overexpression of CtBP under *ey-Gal4* control causes defects in eye and head capsule.

(A,B) Scanning electron micrographs of eyes from *ey-Gal4/+; P[GSV]^{A396}/+* flies. Inset in B is an enlargement of the boxed region in the larger picture. The *ey>CtBP* eyes are smaller than wild type, contain extra interommatidial bristles (arrows in inset in B) and are occasionally split by cuticular structures (arrow in B). (C,D) Scanning electron micrographs of dorsal views of heads from wild-type (A) and *ey-Gal4/+; P[GSV]^{A396}/+* (B) flies. The heads of *ey>CtBP* flies are occasionally split by a cleft (black arrow in D) and contain abnormalities in pattern such as the extra orbital bristles denoted by the white arrow in D.

Figure 3.

Dan and Danr are required for CtBP function, and vice versa. (A-H) Scanning electron micrographs of eyes from the following genotypes: (A,B) *ey-Gal4/+; P[GSV]^{A396}/+*; (C) *ey-Gal4/+; P[GSV]^{A396}/dan^{ems3}*; (D) *ey-Gal4/+; P[GSV]^{A396}/danr^{ex35}*; (E) *ey-Gal4, UAS-dan/+*; (F) *ey-Gal4, UAS-dan/CtBP^{87De-10}*; (G) *ey-Gal4, UAS-danr/+*; (H) *ey-Gal4, UAS-danr/CtBP^{87De-10}*. Inset in B is an enlargement of the boxed region in the larger picture, showing the extra interommatidial bristles found when CtBP is overexpressed (arrows). A single copy of either the *dan^{ems3}* (C) or the *danr^{ex35}* (D) mutations suppresses the small eye phenotype resulting from CtBP overexpression (A,B). Conversely, a single copy of the *CtBP^{87De-10}* mutation suppresses the small eye phenotype resulting from overexpression of either *dan* (compare E,F) or *danr* (compare G,H)

Figure 4.

CtBP interacts in eye-antennal discs with Dan, and Ey, and interacts genetically with *ey* and *dac*, but not with *eya* or *so*. (A) Anti-Dan, but not anti-Tubulin, is capable of immunoprecipitating Ey and Dac from eye-antennal disc extracts. (B) Sepharose beads conjugated to anti-FLAG are capable of pulling down Dan and CtBP from extracts generated from eye-antennal discs expressing FLAG-tagged Ey; sepharose beads alone do not pull down CtBP from the same extracts. (C) Anti-CtBP, but not anti-Tubulin, is capable of immunoprecipitating Ey from eye-antennal disc extracts. (D-L) Scanning electron micrographs of eyes from the following genotypes: (D,G) *ey-Gal4/+; P[GSV]^{A396}/+*; (E) *ey-Gal4/+; P[GSV]^{A396}/+; ey²/+*; (F) *ey-Gal4/dac⁴; P[GSV]^{A396}/+; ey-Gal4/+; P[GSV]^{A396}/+*; (H) *ey-Gal4/eya²⁰¹⁵; P[GSV]^{A396}/+*; (I) *ey-Gal4/so³; P[GSV]^{A396}/+*; (J,K) *ey-Gal4, UAS-ey/+*; (L) *ey-Gal4, UAS-ey/CtBP^{87De-10}*. A single copy of the *ey²* (E) or *dac⁴* (F) mutations suppress the small eye phenotype resulting from CtBP overexpression (D,G). Conversely, a single copy of *eya²⁰¹⁵* (H) or of *so³* (I) enhances or has no effect on the CtBP overexpression phenotype, respectively. A single copy of the *CtBP^{87De-10}* mutation represses the effects of *eyeless* overexpression (compare L to J,K).

Figure 5.

CtBP mutants affect bristle development on the head capsule and antennae, and also affect eye size and development of interommatidial bristles. (A-C) Scanning electron micrographs of dorsal views of heads from (A) *eyFLP; FRT82 M/FRT82*, (B) *eyFLP; FRT82 M/FRT82 CtBP^{87De-10}* and (C) *CtBP^{87De-10}/CtBP⁰³⁴⁶³* flies. Panel (A) shows the normal pattern of bristles on the dorsal side of the head (fro=frontal; f-or=fronto-orbital; ioc=interocellar; oc=ocellar; orb=orbital; pv=post-vertical; ver=vertical). In this example of a stronger *CtBP* mutant phenotype (B), several of the vertical bristles are duplicated (arrows), most of the frontal bristles are missing, and all of the ocellar, interocellar and post-vertical bristles are missing. In this example of a weaker *CtBP* phenotype (C), vertical bristles and one of the orbital bristles are duplicated (arrows), and some of the fronto-orbital and interocellar bristles are missing. (D,E) Light micrographs of wild-type (D) and *CtBP^{87De-10}/Df(3R)ry615* (E) antennae. In the *CtBP*-mutant, a2 and a3 appear bigger than wild type, fewer bristles are found on the 2nd antennal segment, and the arista is shorter and misshapen compared to wild type. (F,G) Scanning electron micrographs of wild-type (F) and *CtBP^{87De-10}/CtBP⁰³⁴⁶³* (G) eyes. Insets are enlargements of the boxed region shown in the larger picture. The *CtBP*-mutant eye is larger and is missing interommatidial bristles (inset). (H) Bar graph showing the mean number of ommatidia for each of the indicated genotypes. Error bars show standard error of the mean. Genotypes were assigned to statistical groups using the Tukey-Kramer post hoc test. Groups sharing at least one letter are not significantly different; groups not sharing any letter are significantly different ($P < 0.05$).

Figure 6.

CtBP^{87De-10} clones are larger than wild type clones. (A-D) Fluorescence micrographs of eye-antennal discs containing wild-type (A); *CtBP^{87De-10}* (B); *CtBP^{87De-10},dan,danr^{ex56}* (C) and *dan,danr^{ex56}* (D) clones. (E) Bar graph showing the mean ratio of prefurrow clone area divided by prefurrow total area for each of the indicated genotypes. Error bars show standard error of the mean. Genotypes were assigned to statistical groups using the Tukey-Kramer post hoc test. Groups sharing at least one letter are not significantly different; groups not sharing any letter are significantly different ($P < 0.05$). (F) Bar graph showing the mean ratio of number of anti-PH3-stained cells in prefurrow clones divided by total area of prefurrow clones, for each of the indicated genotypes. Error bars show standard error of the mean.

Figure 7.

CtBP interacts genetically with *dan* and *danr* for antennal development. (A-F) Light micrographs of the following genotypes: (A) *eyFLP; FRT82 dan^{ems3}/FRT82 M*; (B) *eyFLP; FRT82 danr^{ex35}/FRT82 M*; (C) *eyFLP; FRT82 dan danr^{ex56}/FRT82 M*; (D) *eyFLP; FRT82 CtBP^{87De-10}, dan^{ems3}/FRT82 M*; (E) *eyFLP; FRT82 CtBP^{87De-10}, danr^{ex35}/FRT82 M*; (F) *eyFLP; FRT82 CtBP^{87De-10}, dan danr^{ex56}/FRT82 M*. (A-C) Single *dan* (A) and *danr* (B) or double *dan danr* (C) mutants show mild transformation of distal antenna to leg, as demonstrated by the smaller relative size of a3 and the fact that a3 contains bristles (white arrow), by the enlarged a5 with bracted bristles (black arrow), and by the defects in aristae. (D-F) Double *CtBP, dan* (D), and *CtBP, danr* (E), and triple *CtBP, dan danr* mutants show a dramatically stronger antennal-to-leg transformation, with a strong enhancement in size of a5 (black arrows) and corresponding shortening of the arista. For wild type and *CtBP* single mutant, see Fig 2D,E).

Figure 8.

CtBP interacts genetically with *dan* and *danr* for eye development. (A) Bar graph showing the mean number of ommatidia for each of the indicated genotypes. Error bars show standard error of the mean. Genotypes were assigned to statistical groups using the Tukey-Kramer post hoc test. Groups sharing at least one letter are not significantly different; groups not sharing any letter are significantly different ($P < 0.05$). (B-I) Scanning electron micrographs of eyes of the following genotypes: (B) *eyFLP*; *FRT82/FRT82* M; (C) *eyFLP*; *FRT82 CtBP^{87De-10}/FRT82* M; (D) *eyFLP*; *FRT82 dan^{ems3}/FRT82* M; (E) *eyFLP*; *FRT82 CtBP^{87De-10}, dan^{ems3}/FRT82* M; (F) *eyFLP*; *FRT82 danr^{ex35}/FRT82* M; (G) *eyFLP*; *FRT82 CtBP^{87De-10}, danr^{ex35}/FRT82* M; (H) *eyFLP*; *FRT82 dan danr^{ex56}/FRT82* M; (I) *eyFLP*; *FRT82 CtBP^{87De-10}, dan danr^{ex56}/FRT82* M. Eyes mosaic for the double *CtBP*, *dan* mutant are significantly smaller than those mosaic for single *CtBP* or *dan* mutants; eyes mosaic for the double *CtBP*, *danr* mutant are significantly smaller than those mosaic for single *CtBP* or *danr* mutants; and eyes mosaic for the triple *CtBP*, *dan* *danr* mutant are significantly smaller than those mosaic for single *CtBP* or the double *dan* *danr* mutants.

Figure 9.

CtBP, Dan, Danr function together during recruitment of the founding R8 photoreceptor. (A) Apical section of an *eyFLP; FRT82 CtBP^{87De-10}, dan danr^{ex56}/FRT82 M* adult eye at the R7 level. Inset shows a single wild-type ommatidium, enlarged, and numbers mark rhabdomeres of the individual R1-R7 photoreceptors. Black arrow: normal-appearing ommatidium, white arrow: gap in the ommatidial array, red arrow: ommatidium with too few photoreceptors, green arrow: ommatidium with too few outer photoreceptors and two inner photoreceptors, yellow arrow: ommatidium containing an inner photoreceptor with a large rhabdomere. (B) Wild-type late third instar eye-antennal disc stained with anti-Elav (red). (C-J) Eye-antennal discs containing wild-type (+/M; E,H), *CtBP^{87De-10}* (*CtBP^{87De-10}/M*; C,F,I) or *CtBP^{87De-10}, dan danr^{ex56}* (*CtBP^{87De-10}, dan danr^{ex56}/M*; G,J) clones of cells (β-galactoside negative) generated using the FLP/FRT system together with *eyFLP* and the *Minute* technique. Discs were stained with anti-β-gal (green), anti-Elav (red) and either anti-Ato (E-G) or anti-Sens (H-J). Gaps occur in the array of developing ommatidia in *CtBP^{87De-10}/M* and *CtBP^{87De-10}, dan danr^{ex56}/M* discs (arrows in C,D). In *CtBP^{87De-10}/M* discs, initial Ato expression ahead of the furrow is wider in some places than in wild-type or +/M discs (brackets in E',F'), and there are gaps in the array of R8 precursors expressing Ato (arrows in E', F'). In *CtBP^{87De-10}, dan danr^{ex56}/M* discs, initial Ato expression ahead of the furrow is consistently wider than in wild-type or +/M discs (bracket in G'), and there are large gaps in the array of R8 precursors expression Ato (arrows in G'). Whereas Sens expression in R8 equivalence groups (asterisks in H'-J') appears normal, gaps in single R8 Sens expression correspond with loss of ommatidial differentiation (arrows in H, H', I, I', J, J'). Furthermore, the number of single R8 cells with strong Sens expression is reduced in *CtBP^{87De-10}/M* and *CtBP^{87De-10}, dan danr^{ex56}/M* discs (compare width of brackets in H'-J').

Table 1

ey>CtBP/+ 25°C	w ¹¹¹⁸	<i>dan^{ems3}/+</i>	<i>dan^{ex35}/+</i>		
% smaller than WT	31% N=35	16% N=19	13% N=30		
ey><i>dan</i>/+ 30°C	w ¹¹¹⁸	<i>CtBP^{87De-10}/+</i>	<i>CtBP^{rev19}/+</i>		
% smaller than 50% WT size	36% N=14	9% N=11	17% N=12		
ey><i>dan</i>/+ 30°C	w ¹¹¹⁸	<i>CtBP^{87De-10}/+</i>			
% smaller than WT	90% N=58	55% N=22			
ey>CtBP/+ 25°C	w ¹¹¹⁸	<i>ey²/+</i>	<i>ey^R/+</i>	<i>dac^P/+</i>	<i>dac^d/+</i>
% smaller than WT size	46% N=24	35% N=20	53% N=17	39% N=56	46% N=13
Size range	60-80% WT size	80-100% WT size	80-95% WT size	60-80% WT size	60-80% WT size
ey><i>ey</i>/+ 30°C	w ¹¹¹⁸	<i>CtBP^{87De-10}/+</i>			
% smaller than WT size	100% N=63	54% N=26			