Erratum

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The first online version of this manuscript, which was published on July 14, contained the following error.

In the Summary, the full name for the gene *sey* was given as *scratched eyes*. This was incorrect. The full name is *small eyes*.

This error was corrected in the second online version, published on July 21. The final online version and the print version are correct.

We apologise to the authors and readers for this mistake.

Functional divergence between *eyeless* and *twin of eyeless* in *Drosophila melanogaster*

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Summary

***Pax6* genes encode transcription factors with two DNA-binding domains that are highly conserved during evolution. In *Drosophila*, two *Pax6* genes function in a pathway in which *twin of eyeless* (*toy*) directly regulates *eyeless* (*ey*), which is necessary for initiating the eye developmental pathway. To investigate the gene duplication of *Pax6* that occurred in holometabolous insects like *Drosophila* and silkworm, we used different truncated forms of *toy* and *small eyes* (*sey*), and tested their capacity to induce ectopic eye development in an *ey*-independent manner. Even though the Paired domains of TOY and SEY have DNA-binding properties that differ from those of the Paired domain of EY, they all are capable of inducing ectopic eye development in an *ey* mutant background. We also show that one of the main functional differences between *toy* and *ey* lies in the C-terminal region of their protein products, implying differences in their transactivation potential. Furthermore, we show that only the homeodomain (HD) of EY is able to downregulate the expression of *Distal-less* (*Dll*), a feature that is required during endogenous eye development. These results suggest distinct functions of the two DNA-binding domains of TOY and EY, and significant evolutionary divergence between the two *Drosophila Pax6* genes.**

Key words: *ey*, *toy*, *sey*, PD, HD, *Drosophila*

Introduction

During *Drosophila* embryogenesis, the segmentation genes establish a repetitive pattern of body segments, whereas the HOX genes specify the identity of each segment. Subsequentially, another class of selector gene determines the different appendages or organs within a given segment. The combinatorial or exclusive expression of this later class gives each organ its specific identity. Several *Drosophila* genes have been classified as selector genes according to their ability to induce ectopic appendages when misexpressed. *Vestigial* (*vg*) is essential for wing and haltere identity (Kim et al., 1996), *Dll* for leg identity and, in combination with *extradenticle* (*exd*) and *homothorax* (*hth*), for antenna determination (Casares and Mann, 1998; Gonzalez-Crespo et al., 1998). The selector genes for eye identity in *Drosophila* are the two *Pax6* homologs *ey* and *toy* (Quiring et al., 1994; Halder et al., 1995; Czerny et al., 1999). Recent studies suggest that transformation of the eye disc into antennal identity occurs during the mid to late second instar, indicating a crucial time window for the eye and antennal selector gene action to determine the cell fate of their respective discs (Kumar and Moses, 2001). Although these selector genes are required to maintain and specify disc identity during larval disc development, they are first expressed during embryogenesis, when they specify, within a given segment, the few epidermal cells that invaginate to form the precursor cells of the future imaginal disc.

The eye disc is fused to the antennal disc and forms a compound eye-antennal disc. The eye-antennal disc grows during the three larval stages and, towards the end of the third instar, the cells of the eye disc start to differentiate. Differentiation is marked by a wave of morphological changes, reflected by the morphogenetic furrow (MF), which moves from the posterior to the anterior of the disc (reviewed by Wolff and Ready, 1991). Cells anterior to the furrow are still undifferentiated, whereas cells posterior to the furrow start to form clusters of hexagonal arrays, which reflect the shape of an adult ommatidium. Only the undifferentiated cells anterior to the MF express the two *Drosophila Pax6* genes *ey* and *toy*, whereas in the differentiated cells posterior to the MF, both mRNAs seem to be downregulated (Czerny et al., 1999); however, TOY protein is still detected posterior to the MF (U. Walldorf, personal communication). Although differentiation of the eye disc only starts at the third larval stage, its cells become determined during the second instar, when the eye specifying genes *ey* and *toy*, and their early downstream target genes *sine oculis* (*so*), *eyes absent* (*eya*) and *dachshund* (*dac*), are expressed (Kumar and Moses, 2001). Interestingly, during first to mid-second instar stages of disc development, *ey*, the

selector gene for eye identity, is also expressed in the antennal disc (Kenyon et al., 2003). Because cells specifying the eye-antennal disc invaginate together and form a compound disc, selector genes are found to be expressed in both eye and antennal discs at an early stage, and only later do they become restricted to either the eye or the antennal part when determination sets in. Consistent with this, recent studies have shown that ectopic *ey* is able to repress DLL, an antenna specific gene (Kurata et al., 2000). Furthermore, it has been shown that this repression is mediated by the homeodomain (HD) of *ey* (Punzo et al., 2001). The ability of selector genes to downregulate each other can therefore lead to an exclusive territorial expression, and to the specification of the identity of a given disc (Benassayag et al., 2003).

The existence of two *Pax6* genes in *Drosophila* raises the question of whether they have a redundant function or whether they have diverged to control different sets of target genes. A recent characterization of new alleles of *ey* and *toy* mutants by Kronham et al. (Kronham et al., 2002), and by others (S. Flister, U. Kloter, M.S., C.P., L. Michaut and W.J.G., unpublished), suggests a functional divergence, with a partial redundancy remaining. Epistasis studies showed that *toy* lies upstream of *ey*, because ectopic *toy* is capable of inducing ectopic *ey* but not vice versa (Czerny et al., 1999). Additionally, *toy* cannot induce ectopic eyes in an ey^2 mutant background whereas *ey* can (Czerny et al., 1999). The regulation of *ey* by *toy* is due to a direct binding of the TOY-PD to the *ey*-enhancer, which is located in the second intron of the *ey* gene. The EY-PD contains a glycine at position 14, whereas the TOY-PD has an asparagine at that same position. This difference allows TOY to regulate *ey* through the *ey*-enhancer, whereas EY cannot regulate itself (Czerny et al., 1999). Complementary experiments showed that ectopic expression of a HD-deleted version of the EY protein did not induce the endogenous full-length gene, and therefore confirmed the lack of an auto-regulatory feedback loop for *ey* (Punzo et al., 2001). Endogenous *ey* can only be induced by misexpression of the three downstream genes *eya*, *so* and *dac*, or by *toy* (Halder et al., 1998; Czerny et al., 1999). Interestingly, the mouse *Pax6* gene *sey* also has an asparagine at position 14 of the PD and has, therefore, the same DNA-binding properties as *toy* (Hill et al., 1991; Czerny et al., 1999). Moreover, *sey* and *toy* have multiple stretches of conserved amino acids in their C termini, whereas the C terminus of *ey* diverged. Thus, ectopic *sey* is able to induce ectopic *ey* but it does not induce ectopic *toy* (Czerny et al., 1999). This suggests that the auto-regulatory feedback loop found in the vertebrate *Pax6* gene evolved into a hetero-regulatory interaction in *Drosophila* with *toy* regulating *ey* expression (Gehring and Ikeo, 1999). Overall these data indicate that not only the PDs, but also the cis-regulatory sequences of the two *Drosophila Pax6* genes, have diverged to control different sets of target genes. This hypothesis is further supported by the fact that only *toy* is expressed in the ocelli territory of the eye disc but both *toy* and *ey* regulate the eye-specific enhancer of the *so* gene, by binding partly to the same and partly to different binding sites, and by discriminating between eye and ocelli development during larval stages (Punzo et al., 2002).

The PD and the HD are the most conserved regions within the Pax6 proteins, indicating evolutionary constraints imposed to maintain specific binding to target genes. We therefore investigated to what extent the PD of TOY and SEY, which diverged in their DNA binding properties from the PD of EY, were able to induce the eye developmental pathway independently of *ey*. Moreover, we determined whether only the HD of *ey* was able to downregulate *Dll* expression, as previously shown by Punzo et al. (Punzo et al., 2001), and whether this function is required during endogenous eye development to specify the eye territory. We have tested these hypotheses by generating deletion constructs of *sey* similar to those described for *ey* and *toy* (Punzo et al., 2001; Punzo et al., 2002), as well as EY-TOY chimeric proteins, and scored for ectopic eye formation. Furthermore, we rescued the *ey* null mutant $ey^{J5.71}$ (Punzo et al., 2001) by transferring the genomic region of the *ey* gene onto the third chromosome. This allowed us to analyze mutant *ey* clones in a wild-type background. We found that both *sey* and *toy* were able to activate eye development in an *ey*-independent manner, and that one of the main differences between *toy* and *ey*, besides their DNA-binding properties, lies in their C-terminal region, and therefore mainly in their transactivation potential. This suggests that most of the differences reside in their capacity to interact with different sets of proteins. We also show that only the HD of *ey* is able to downregulate DLL expression in an ectopic situation, and that this downregulation is required during endogenous eye development.

Materials and methods

Fly strains and histology

Flies were reared on standard medium at 25°C. Lines used: *so*10-*lacZ* (Niimi et al., 1999), *so-lacZ* (Cheyette et al., 1994), dpp^{blink}-Gal4 (Staehling-Hampton and Hoffmann, 1994), *ey*-Gal4 (Halder et al., 1998), UAS-*ey* (Halder et al., 1995), UAS-*toy* (Czerny et al., 1999), UAS-*sey* (Halder et al., 1995), ey^2 (Quiring et al., 1994), UAS-*eyΔPD* and UAS-*eyΔHD* (Punzo et al., 2001), UAS-*toyΔPD* and UAS-*toyΔHD* (Punzo et al., 2002), UAS-P35 (Zhou et al., 1997), *Dll-lacZ* (Gorfinkiel et al., 1997), *yw*; TM6Tb/82B FRT P(smo^+,hsp70-GFP) and *yw*; $Dp(1:4)1021y^+$/ $Dp(1:4)1021y^+$ (Methot and Basler, 1999), $ey^{J5.71}$ (S. Flister, U. Kloter and W.J.G., unpublished).

Specific genotypes generated for this publication were: (1) UAS-*sey*/UAS-*sey*; ey^2/ey^2, (2) UAS-*seyΔPD*/UAS-*seyΔPD*; ey^2/ey^2, (3) UAS-*seyΔHD*/UAS-*seyΔHD*; ey^2/ey^2, (4) UAS-*toy*/UAS-*toy*; ey^2/ey^2, (5) UAS-*toyΔPD*/UAS-*toyΔPD*; ey^2/ey^2, (6) UAS-*toyΔHD*/UAS-*toyΔHD*; ey^2/ey^2, (7) UAS-P35/UAS-P35; UAS-*ey*/UAS-*ey*; ey^2/ey^2, (8) UAS-P35/UAS-P35; UAS-*eyΔPD*/UAS-*eyΔPD*; ey^2/ey^2, (9) UAS-P35/UAS-P35; UAS-*eyΔHD*/UAS-*eyΔHD*; ey^2/ey^2, (10) UAS-P35/UAS-P35; ey^2/ey^2, (11) *so*10-*lacZ*/*so*10-*lacZ*; TM6Tb/dpp^{blink}-Gal4, (12) CyO/*so-lacZ*; TM6Tb/dpp^{blink}-Gal4; ey^2/ey^2, (13) CyO/*Dll-lacZ*; TM6Tb/dpp^{blink}-Gal4; (14) *ey*-Gal4/*ey*-Gal4; ey^2/ey^2. For the clonal analysis the following lines were generated: (15) *ywhsflp*; 82B FRT/82B FRT; $Dp(1;4)1021y^+$/$Dp(1;4)1021y^+$, (16) *ywhsflp*; 82B FRT/82B FRT; $ey^{J5.71}/ci^D$, (17) *yw*; TM6Tb/82B FRT *P*(smo^+,*hsp70-GFP*) *p6.3-ey*; $Dp(1:4)1021y^+$/$Dp(1:4)1021y^+$, (18) *yw*; TM6Tb/82B FRT *P*(smo^+,*hsp70-GFP*) *p6.3ey*; $ey^{J5.71}/ci^D$. The crosses between the lines 15 and 16, and between the lines 17 and 18, respectively, give the two lines that were used to cross together for the clonal analysis (Fig. 7B).

Transgenic lines were generated by P element-mediated germline transformation in yw^{1118}. New lines generated for this study: UAS-*seyΔPD*, UAS-*seyΔHD*, UAS-*eyPD-TOYBB*, UAS-*toyPD-EYBB*, UAS-*EYBB-toyCT*, UAS-*TOYBB-eyCT* and p6.3*ey*/TM6Tb (genomic rescue line).

Antibody staining on discs was performed according to Halder et al. (Halder et al., 1998). β-Galactosidase staining and antibody

stainings on cryosections were performed as desribed in Ashburner (Ashburner, 1989). Dilution of antibodies was as follows: rabbit α-EY, 1:3000 (for clonal analysis); mAb α-DLL, 1:20; and α-GFP, 1:1000 (Torrey Pines Biolab).

Cloning procedure and western blots

Both *sey* PD and HD deletion constructs were made by standard recombinant PCR techniques, deleting only the PD or the HD, respectively. PCR was performed directly on the cDNA that was cloned in pUAST (Halder et al., 1995). The newly generated *sey* cDNAs were directly ligated into a PCR cloning vector, sequenced and then cloned back into pUAST with *Not*I-Asp718. All four chimera constructs were made by recombinant PCR techniques, switching exactly the PD or the C terminus at the end of the HD of the corresponding protein. PCR was performed directly on the cDNAs, which were cloned in pBSK. The newly generated cDNAs were directly ligated into pUAST using *Bgl*II and *Xba*I. Both sites were inserted into the primers used for the recombinant PCR. All four constructs were confirmed by sequencing. Detailed descriptions of the primers used are available upon request. To rescue the *ey* null mutant $ey^{J5.71}$, we first screened a genomic P-element library with *ey* cDNA (Tamkun et al., 1992). Positive clones were further analyzed by means of restriction digest and PCR analysis to confirm the presence of all exons, and to map the length of the 5′ and 3′ region of the rescue clones. Two clones containing the entire genomic area were injected: one with a 2 kb extension at the 5′ end of exon 1 and a 15 kb extension at the 3′ end of exon 9 (referred to as clone p6*ey*), and one with a 5 kb extension at the 5′ end of exon 1 and an 8 kb extension at the 3′ end of exon 9 (referred to as clone p14*ey*). The cosmid p6*ey* was able to rescue the $ey^{J5.71}$ mutant. As only one line carrying p14*ey* was obtained we cannot exclude at present that this genomic region would also be sufficient to rescue the $ey^{J5.71}$ mutant. Embryonic and pupal lethality, developmental delay, as well as eye phenotypes were restored by p6*ey*. Sterility was restored for males but not for females, indicating that regulatory sequences required for *ey* expression in the female sexual organs were not present within the rescue cosmid p6*ey*. Western blot experiments were carried out as described by Punzo et al. (Punzo et al., 2001). The rabbit α-quail-PD (Carriere et al., 1993) was used at a dilution of 1:200, the rabbit α-EY at a dilution of 1:200, in which the antibody was pre-absorbed with larval tissue. The anti-β-Galactosidase antibody was used at a dilution 1:2000 (Promega).

Results

Induction of ectopic eyes by the TOY- and SEY-PD in an *ey* mutant background

It has been shown that, during embryonic stages of eye development, *toy* directly regulates *ey* expression, and that toy cannot induce ectopic eyes in a selected ey^{2} mutant background (Czerny et al., 1999). Furthermore, it has been shown that, although *toy* and *ey* have different DNA-binding properties in their PD (Czerny et al., 1999), both were able to regulate the eye-specific enhancer element of *so*, a gene required for the development of both ocelli and compound eyes (Punzo et al., 2002). These findings, and the fact that target gene activation during eye development seems to depend only on the PD of *ey* (Punzo et al., 2001), led us to investigate to what extent the PD of *toy* is still able to activate genes that are required during eye development in *Drosophila*.

We made use of the previously published PD and HD deletion constructs of *ey* and *toy* (Punzo et al., 2001; Punzo et al., 2002), and, in addition, generated a PD- and HD-deleted version of the mouse *Pax6* gene *sey*. Because *sey* has the same in vitro DNA-binding specificity as *toy*, it is also able to induce ectopic *ey* expression and ectopic eyes when misexpressed in a wild-type background, but it is not able to induce ectopic *toy* (Czerny et al., 1999). We used the UAS/Gal4 system (Brand and Perrimon, 1993) to drive our different UAS-deletion constructs by dpp^{blink}-Gal4 (Staehling-Hampton and Hoffmann, 1994). First, we confirmed that the HD-deleted versions of *toy* (*toyΔHD*) and *sey* (*seyΔHD*) were still able to induce ectopic eye development in a wild-type background (data not shown). This result was in agreement with data showing that the TOY-PD and the SEY-PD regulate *ey* through the *ey* enhancer (Czerny et al., 1999).

To test to what extent the PD of *sey* and *toy* could still activate the eye developmental cascade in an *ey*-independent manner, we misexpressed both *toyΔHD* and *seyΔHD* in an *ey* mutant background. Unexpectedly, both *toyΔHD* and *seyΔHD* were able to induce ectopic eye development in an ey^{2} and in an *ey* null ($ey^{J5.71}$) (Punzo et al., 2001) (S. Flister, U. Kloter, M.S., C.P., L. Michaut and W.J.G., unpublished) mutant background (Fig. 1B,D; data not shown for $ey^{J5.71}$). In addition, in the control cross, in which both full-length proteins were misexpressed in the same genetic background, ectopic eyes were also induced (Fig. 1A,C), whereas the PD-deleted versions (*toyΔPD* and *seyΔPD*) did not lead to any phenotype in either a wild-type or *ey* mutant background (data not shown). This result shows that the induction of ectopic eyes by *toyΔHD* and *seyΔHD* in an *ey* mutant background is not due to the absence of the HD. Moreover, we conclude that *toy* and *sey* can activate all target genes required for eye development in the absence of endogenous *ey*, despite their different DNA-binding properties.

To further confirm that early direct or late indirect *Pax6*-regulated target genes of eye development are activated upon misexpression of *toyΔHD* or *seyΔHD*, we monitored the expression of *so* and *rhodopsin 1* (*ninaE* – FlyBase). Earlier reports have shown that the eye-specific enhancer of *so* can be directly activated by the TOY-PD (Punzo et al., 2002). Therefore, we monitored endogenous *so* expression by the use of an enhancer-trap line (Cheyette et al., 1994). β-Galactosidase staining revealed ectopic induction of *so-lacZ* upon misexpression of *toy*, *toyΔHD*, *sey* and *seyΔHD* in an ey^{2} mutant background (Fig. 1E-H). No induction was observed in the absence of the PD (data not shown). This shows that *toy* and *sey* are both able to directly activate *so* transcriptionally through their PD in vivo, and further explains why ectopic eyes can be induced by *toy* and *sey* in an *ey* mutant background.

rhodopsin 1 has been suggested to be directly regulated by the HD of *ey* (Sheng et al., 1997). We have previously shown for *ey* that ectopic eye development is independent of the EY-HD and that ectopic eyes generated in an *ey* mutant background still express *rhodopsin 1* (Punzo et al., 2001). The same was found to be true for *toy* and *sey* (Fig. 1I-L), suggesting that ectopic eyes induced in an *ey* mutant background by *toy*, *toyΔHD*, *sey* and *seyΔHD* complete their developmental program. It further strengthens the hypothesis that *rhodopsin 1* induction is independent of the *Pax6* HD, as observed in mice where Pax6 is not expressed in differentiating rods or cones (reviewed by Pichaud et al., 2001).

Effects of different expression levels of *Pax6*

We wondered whether the difference observed in our experiments, in which we were able to induce ectopic eyes in

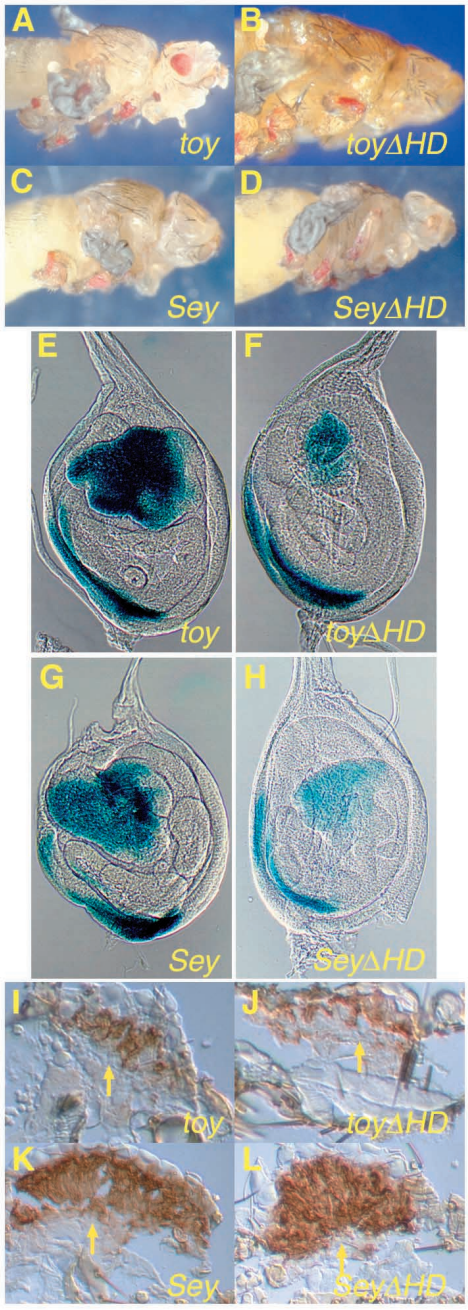

Fig. 1. Ectopic eyes in an ey^2 mutant background. For each panel (A-L), the UAS transgene that is driven by the dpp^{blink}-Gal4 transgene in an ey^2 mutant background is indicated in the lower right of the panel. (A-D) Phenotype of adult flies carrying the UAS transgene indicated. Misexpression of *toy* (A), *toyΔHD* (B), *sey* (C) and *seyΔHD* (D) leads to the induction of ectopic eyes in an ey^2 mutant background. (E-L) Early and late marker genes are induced during ectopic eye development in the absence of endogenous *ey*. (E-H) *lacZ* expression of third instar leg discs carrying the *so*-enhancer trap, in addition to the dpp^{blink}-Gal4 transgene and the UAS-construct indicated in an ey^2 mutant background. All four transgenes were able to induce *lacZ* expression. (I-L) Rhodopsin 1 expression in ectopic eyes. Rhodopsin 1 was monitored with an α-Rhodopsin 1 antibody on cryosections of ectopic eyes induced by the UAS transgene indicated driven by dpp^{blink}-Gal4 in an ey^2 mutant background. Rhodopsin 1 was found to be expressed in ectopic eyes induced by all four transgenes. Arrows indicate the retina.

an *ey* mutant background by *toy* and *sey* and their HD-deleted versions, might be due to dosage dependence when compared with previously published data (Czerny et al., 1999). We noticed in the course of this work that several independent transgenic deletion lines of *ey* (*eyΔPD* and *eyΔHD*) showed strong variations in expressivity when crossed to dpp^{blink}-Gal4 (Fig. 2A,B; data not shown for *eyΔPD*). Western blot experiments using an α-EY antibody revealed a clear correlation between the phenotypes induced and the amount of protein expressed (Fig. 2C). This result shows that not only the presence, but also the amount of ectopically induced Pax6 protein is crucial to induce ectopic eye morphogenesis. In order to compare the phenotypes of individual transgenic lines of either the same or of a homologous protein, the expression levels of the corresponding proteins in the individual lines have to be compared too. To re-examine the different results obtained in our experiments and the earlier ones of Czerny et al. (Czerny et al., 1999), we made use of the temperature sensitivity of the Gal4 system (Jarrett, 2000). We repeated the misexpression experiment with *toy* in an ey^2 mutant background using the line that gave us ectopic eyes and reared

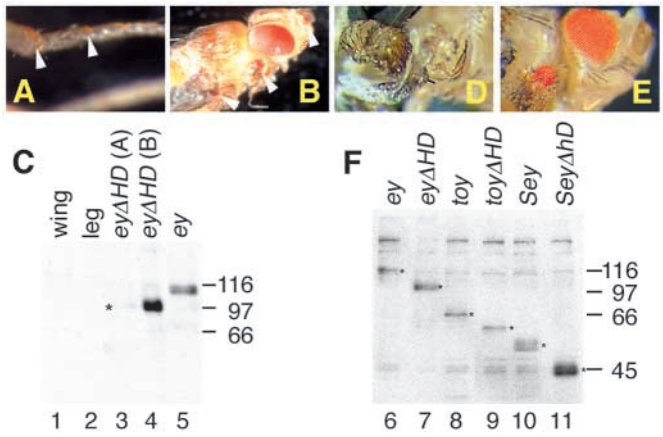

Fig. 2. Dosage dependence of the phenotype. (A,B) Flies carrying a UAS-*eyΔHD* transgene in addition to the dpp^{blink}-Gal4 transgene. A and B are two independent transgenic lines of the same transgene showing ectopic eyes (arrowheads) obtained to a different degree of strength. (C) Western blot analysis using a rabbit α-EY antibody on third instar leg discs expressing the transgenes used for the phenotypes obtained in panels A and B. The strength of the phenotype correlates with the amount of ectopically induced protein. Asterisk marks the protein expressed at lower level corresponding to the ectopic eyes in panel A. Molecular weight marker is indicated at the right. Lanes 1 and 2, *yw* control wing and leg discs, respectively; lanes 3-5, leg discs expressing the various *ey* constructs by dpp^{blink}-Gal4. (Lane 3) Misexpression of *eyΔHD*, transgenic line of panel A. (Lane 4) Misexpression of *eyΔHD*, transgenic line of panel B. (Lane 5) Misexpression of full-length *ey*. (D-F) Temperature dependence of the Gal4 system. (D,E) Flies carrying the UAS-*toy* transgene and the dpp^{blink}-Gal4 transgene in an ey^2 mutant background raised either at 18°C (D) or at 25°C (E). Ectopic eyes were never observed on flies raised at 18°C, in contrast to flies raised at 25°C. (F) Western blot analysis with a rabbit α-quail-PD antibody of third instar leg discs expressing the various *Pax6* transgenes under the control of the dpp^{blink}-Gal4 transgene in a wild-type background. Pax6 proteins are marked by asterisks. Molecular weight marker is indicated at the right. (Lanes 6-11) Misexpression of *ey* (lane 6), *eyΔHD* (lane 7), *toy* (lane 8), *toyΔHD* (lane 9), sey*ΔHD* (lane 10) and sey*ΔHD* (lane 11).

the flies at 18°C and 25°C. Flies developing at 18°C showed appendage truncation without ectopic eyes as previously described by Czerny et al. for misexpression of *toy* in an ey^2 mutant background (Czerny et al., 1999). By contrast, the flies developing at 25°C exhibited ectopic eyes (Fig. 2D,F). Western blot experiments on leg discs of third instar larvae with *ey*, *toy*, *sey* and their HD-deleted versions confirmed that our ectopically induced proteins were expressed at the expected molecular weight and at comparable levels (Fig. 2F). This allows us to conclude that our *toy*, *toyΔHD*, *sey* and *seyΔHD* lines are able to induce ectopic eyes in an *ey* mutant background, when expressed at comparable levels to those required for ectopic eye induction by *ey*. Overall, these experiments show that the ectopically induced protein levels are crucial for inducing phenotypic changes.

Functional differences between *toy* and *ey*

The two *Drosophila Pax6* genes *ey* and *toy* share a high sequence similarity in their two DNA-binding domains, which may explain why *toy* is still able to induce ectopic eyes in an *ey* mutant background. By contrast, their C termini (CT) show a higher degree of divergence. To further investigate functional differences between *toy* and *ey*, we created chimeric proteins by swapping the PD or the CT of the two proteins. We generated four UAS constructs, which we refer to as *toy*-paired-domain-*ey*-backbone (*toyPD-EYBB*), EY-backbone-*toy*-c-terminus (*EYBB-toyCT*), TOY-backbone-*ey*-c-terminus (*TOYBB-eyCT*) and *ey*-paired-domain-*toy*-backbone (*eyPD-TOYBB*) (Fig. 3A). The failure of *toy* to induce ectopic eyes on the antenna when misexpressed by dpp^{blink}-Gal4 (U. Walldorf, personal communication) allowed us to distinguish between *toy*- and *ey*-specific functions, whereas the functional constructs could be identified by ectopic eye induction on the legs, which works for both *toy* and *ey* misexpression. As expected, all four chimeras were able to induce ectopic eyes on the legs when driven by dpp^{blink}-Gal4 in a wild-type background (data not shown). Nevertheless, the chimeras containing the TOY-CT (*eyPD-TOYBB*; *EYBB-toyCT*) poorly induced ectopic eyes on legs, a phenotype that mimics full-length *toy* expression but contrasts with full-length *ey*. Scoring for ectopic eye induction on the antenna revealed that only the chimeric proteins containing the EY-CT (*toyPD-EYBB*; *TOYBB-eyCT*) were able to induce ectopic eye development on the antennae, whereas the proteins containing the TOY-CT (*eyPD-TOYBB*; *EYBB-toyCT*) did not (Fig. 3A, column 1). This suggests a functional difference between the C terminus of EY and TOY. By contrast, the PD seems to have diverged to a lesser extent with respect to ectopic eye morphogenesis during larval stages.

To further corroborate this finding, we made use of eye-specific enhancer of the *so* gene (*so*10), which contains five *toy* and three *ey* binding sites, and is very sensitive to over expression of both *ey* and *toy* (Punzo et al., 2002). β-Galactosidase staining of leg and antennal discs of larvae carrying the *lacZ* gene under the control of the *so*10 enhancer and co-expressing the different constructs in the dpp^{blink} domain showed results that are consistent with the eye phenotypes described above. *so*10-*lacZ* expression was always detected in leg discs, confirming our results at the transcriptional level (data not shown). In antennal discs, *so*10-*lacZ* expression was only detected when EY-CT was present

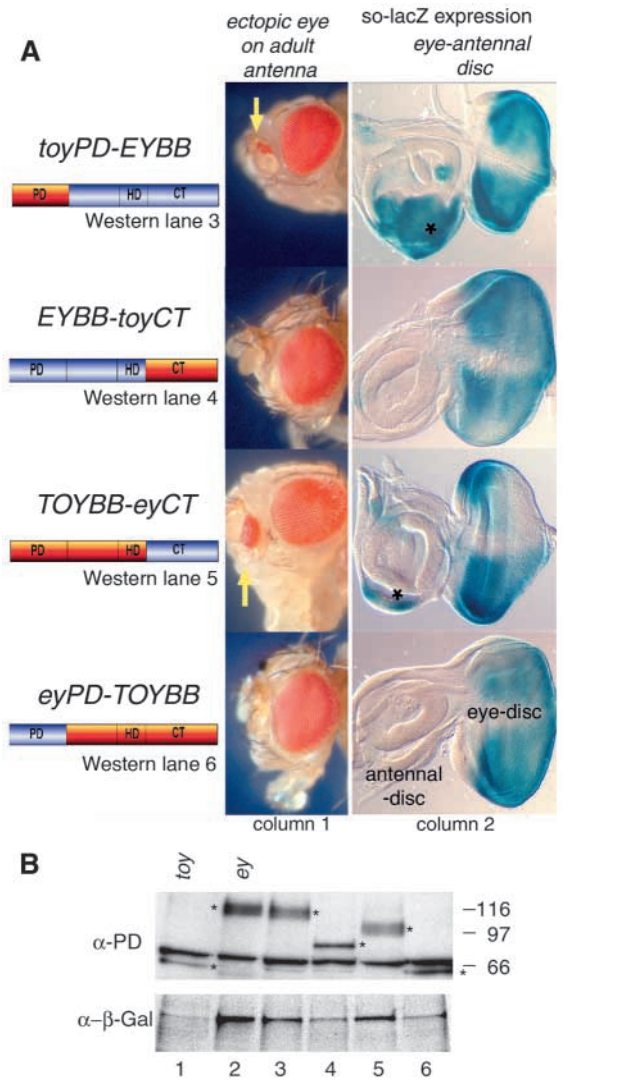

Fig. 3. Ectopic eyes on the antenna depend on the C terminus of EY. (A) Column 1 shows the head region, including the antenna of flies carrying the dpp^{blink}-Gal4 transgene and the corresponding transgene (marked on the left of each row) in a wild-type background. Ectopic eyes (arrows) on the antenna were induced only by transgenes containing the EY C terminus. Column 2 shows *so*10-*lacZ* expression on eye-antennal discs of third instar larval stage. In addition to the *so*10-*lacZ* transgene, they express the same transgenes as the flies in column 1. β-Galactosidase expression was detected only in antennal discs where ectopic eyes on the adult antenna were also observed. Asterisks mark the area of ectopic staining. (B) Western blot analysis of third instar leg discs carrying the *so*10-*lacZ*, the dpp^{blink}-Gal4 transgenes and the different *ey/toy* chimeras in a wild-type background. Molecular weight marker is indicated at the right. The western blot was probed once with a rabbit α-quail-PD antibody (upper) and once with an anti-β-Galactosidase antibody (lower). The various Pax6 proteins are indicated by an asterisk. (Lanes 1-6) Misexpression of *toy* (lane 1), *ey* (lane 2), *toyPD-EYBB* (lane 3), *EYBB-toyCT* (lane 4), *TOYBB-eyCT* (lane 5) and *eyPD-TOYBB* (lane 6). Lanes 3 and 5 show a higher amount of β-Galalactosidase protein, consistent with the fact that both transgenes inducing β-Galalactosidase contain the *ey* C terminus.

in the chimeric protein (Fig. 3A, column 2). As we have previously shown that the amount of protein expressed is

crucial to obtain a reproducible phenotype, we performed western blot analysis on leg discs carrying the different *ey* and *toy* transgenes, as well as the *so*10-*lacZ* and the *dpp^{blink}*-Gal4 transgenes. We probed the western blot twice: first with α-Pax6 PD-specific antibody to detect the ectopically expressed chimeras, and subsequently with anti-β-Galactosidase-specific antibody in order to visualize the strength of induction of the reporter construct. The blots show that the different proteins have the expected molecular weight (Fig. 3B). The proteins containing the EY-CT were expressed at slightly higher levels than the one containing the TOY-CT, but this cannot account for the difference in phenotype, as in fact *toy*, which is expressed at the lowest level (Fig. 3B, lane 1), was still able to induce ectopic eyes and *so-lacZ* staining on leg discs (data not shown). Furthermore, the strength of induction of β-Galactosidase clearly correlates with the presence of the EY-CT. These results suggest that the C termini of *ey* and *toy* differ significantly in their transactivation properties. This may be due to interactions with a different set of co-factors to increase DNA-binding specificity and transactivation potential.

Dll repression by the HD of *ey* is required during endogenous eye development

We have previously shown that the HD of *ey* is involved in repressing the selector gene *Dll*, as monitored by antibody staining, in an ectopic situation leading to leg truncation (Punzo et al., 2001). Interestingly, the PD-deleted versions of *sey* and *toy* did not lead to appendage truncation when misexpressed (data not shown). Consistent with this observation, the *Dll-lacZ* enhancer was downregulated by misexpression of *eyΔPD* only, and not by misexpression of *seyΔPD* or *toyΔPD* (data not shown). Although all three proteins share the same crucial amino acids at positions 50 and 51 of the recognition helix of their HD, this difference may be attributed either to a specific interaction of the *ey* C terminus with other cofactors, or to protein-protein interactions between the homeodomains of *ey* and *Dll*. Therefore, we asked whether the repression of DLL by EY-HD might have any endogenous function during eye development. First, we analyzed *Dll* expression in third instar eye discs of *ey^2* mutants, assuming that, if *ey* is required to repress *Dll*, a lack of *ey* would lead to an activation of *Dll*. Antibody staining with an α-DLL antibody did not reveal any DLL expression in *ey^2* mutant eye discs (data not shown). However, cell death is increased in those mutant eye discs because of the absence of EY (Halder et al., 1998) and, therefore, the lack of DLL expression could also be due to apoptosis. To overcome this problem, we prevented cell death by expressing the anti-apoptotic protein P35 of baculovirus with the *ey* enhancer (*ey*-Gal4) in *ey^2* mutant eye discs. Expression of P35 in the eye discs of *ey^2* mutants leads to a duplication of the antennal disc in almost 100% of the offspring and also to a duplication of the *Dll*-expressing domain (Fig. 4E,F). Consequently, the adult fly replaces the normal eye with an additional antenna, resulting in an imago with four antennae (Fig. 4G,H). In the absence of *ey*, when cell death is prevented, DLL remains active and the cells differentiate into the fate dictated by *Dll* and form an antenna.

It was previously shown by Gehring that removing the eye part of an eye-antennal disc and in vivo culturing results in overproliferation of the antennal disc (Gehring, 1966). After

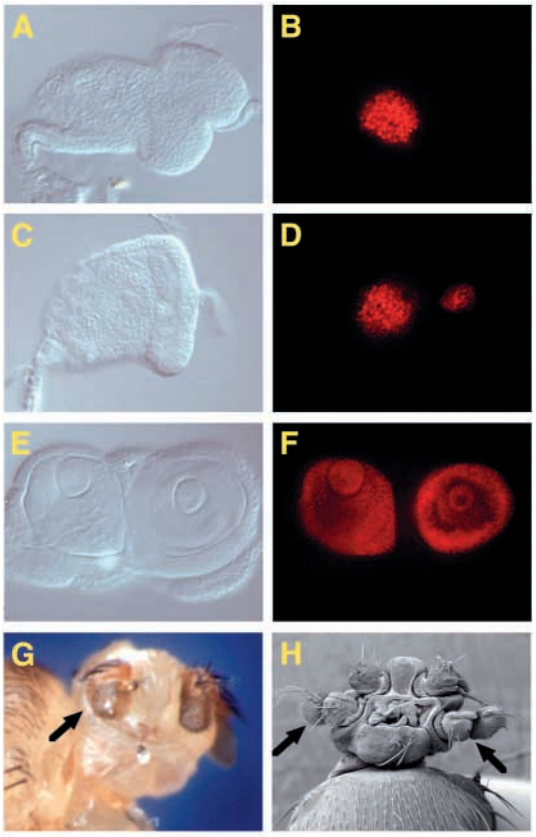

Fig. 4. Antenna duplication by DLL induction. (A-F) Eye discs stained with an α-DLL antibody (B,D,F) expressing UAS-P35 under the control of *ey*-Gal4 in an *ey^2* mutant background. (A,C,E) Bright-field microscopy pictures of eye discs shown in B,D and F, respectively. (A,B) Late second instar, (C,D) early third and (E,F) late third instar eye discs. DLL is gradually induced (D), independently of endogenous DLL expression in the antenna, and leads to a transformation of the eye disc into an antennal disc (F). (G,H) The duplication of the antennal disc leads to a duplication of the adult antenna with all segments present, and the duplicated antenna in the location of the missing eye (arrows in G,H).

reaching a critical size, the disc splits into two, leading to a duplication of the antennal disc. To distinguish between such a regeneration event and change in cell fate during development, we repeated the experiment, but analyzed earlier larval stages. Antibody staining of second and early third instar larvae revealed a group of cells expressing DLL in the center of the eye disc. This group of cells expands, changes the fate of the eye disc, and results in a duplication of the antennal disc (Fig. 4A-F). We therefore conclude that the adult phenotype observed here is not due to regeneration of the eye disc, but to a change in cell fate caused by the lack of *ey* expression, leading to a derepression of *Dll* and a transdetermination to antenna.

To mimic the endogenous situation more closely, we prevented cell death by expressing the different *ey* deletion constructs, hence rescuing the *ey^2* mutant, instead of expressing the anti-apoptotic protein P35. We repeated previously published rescue experiments in which *ey*, *eyΔPD* or *eyΔHD* were expressed by *ey*-Gal4 in an *ey^2* mutant background (Punzo et al., 2001) and checked for DLL expression. Rescuing the *ey^2* mutant by *ey* or *eyΔHD* leads to ectopic DLL induction

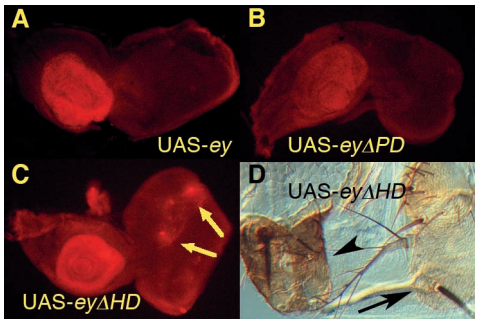

Fig. 5. Induction of *Dll* expression in the eye disc. (A-C) Antibody staining with α-DLL antibody on late third instar eye discs carrying the *ey*-Gal4 and the UAS-*ey* (A), the UAS-*eyΔPD* (B) and the UAS-*eyΔHD* (C) transgenes in an ey^2 mutant background. DLL is induced in small patches of cells only in the absence of the EY-HD (arrows in C). (D) Antennal-like outgrowth from the adult eye of a fly carrying the *ey*-Gal4 and the UAS-*eyΔHD* transgene in an ey^2 mutant background. The arrow indicates the regular antenna and the arrowhead the ectopic antenna.

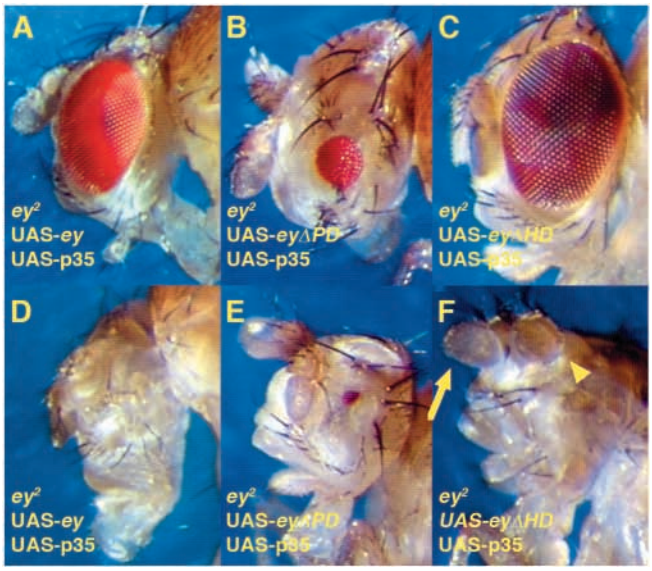

Fig. 6. Repression of antennal identity by the EY-HD. (A-F) Flies carrying the *ey*-Gal4, the UAS-P35 and the UAS-*ey* (A,D), the UAS-*eyΔPD* (B,E) or the UAS-*eyΔHD* (C,F) transgenes in an ey^2 mutant background. A and C show a full rescue of the ey^2 mutant eye phenotype by a PD containing EY protein. The percentage of flies rescued by *ey* or *eyΔHD* are 50% or 79%, respectively, for a rescue of at least 80% of the wild-type eye size (Punzo et al., 2001). D and F show the cases where the rescue did not work at all, even though the PD was present. In those cases, the co-expression of P35 led to a duplication of the antenna (arrowhead, duplicated antenna; arrow, wild-type antenna) only in the absence of the EY-HD, indicating that the HD is able to repress antennal identity. B and E show two different eye sizes of an ey^2 mutant, where no rescue is observed because of the expression of a PD-deleted EY protein, but, in addition, no duplication of the antenna is observed because of the presence of the HD.

in the absence of the HD, whereas in the presence of the HD DLL expression is completely inhibited (Fig. 5A-C). DLL is induced only in some cells in around 5% of the discs. This explains why antenna-like outgrowths were obtained in a small percentage of flies (around 2%) that showed an incomplete rescue with *eyΔHD* (Fig. 5D); these outgrowths were never observed when *ey* was expressed. These results led us to the following interpretation: the EY-PD is required to induce the eye developmental pathway preventing cell death, whereas the HD is required, maybe in conjunction with other genes, to enhance the switch between antennal fate and eye fate by repressing one or several antennal-specific genes.

To corroborate our findings that prevention of cell death in the eye disc derepresses DLL if the cells do not enter the eye developmental pathway, we co-expressed the various truncated forms of *ey* with P35 in ey^2 mutant eye discs. This experiment also allowed us to see whether the EY-HD is involved in repressing antennal identity. Expression of *eyΔPD* and P35 did not lead to antennae duplication, indicating that the HD of *ey* is able to repress antennal identity, as P35 prevents cell death and *eyΔPD* does not induce eye development (Fig. 6B,E). Co-expressing either *ey* and P35, or *eyΔHD* and P35, rescued compound eye development in both cases (Fig. 6A,C). Interestingly, in the few cases where eye development was not rescued at all (Fig. 6D,F), only the co-expression of *eyΔHD* and P35 led to a duplication of the antenna (Fig. 6F). This shows that the presence of the EY-HD is sufficient to repress antennal fate identity. Finally, a control experiment, in which P35 was expressed in the eye disc of wild-type flies, did not show any antenna duplication, confirming that it does not actively transform the eye into an antenna (data not shown). We therefore conclude from our genetic experiments that the induction of the eye developmental pathway is mediated through the PD of EY, and that the repression of antennal identity in the eye disc is partially due to the HD of EY. Moreover, repression of antennal identity is not required, because changing cell fate towards eye morphogenesis is sufficient to repress *Dll* by other mechanisms of the eye developmental program.

As a further test of our hypothesis, we performed clonal analysis, inducing *ey*-mutant cell clones in a wild-type eye disc (Xu and Rubin, 1993). We expected that cells lacking *ey* expression would express *Dll* if they neither undergo apoptosis nor enter the eye developmental pathway. Because *ey* is located on the fourth chromosome, which is not suitable for mitotic recombination experiments, we transferred a genomic segment of the *ey* gene onto the third chromosome. We screened a genomic P-element library (Tamkun et al., 1992) and isolated two clones harboring all the *ey* exons but differing at the 5′ and 3′ extensions of the gene. Both clones were injected and crossed into an $ey^{J5.71}$ mutant background. Only one of the two clones fully rescued the *ey* null mutant phenotype, with the exception of female sterility, which, in contrast to male sterility, was not rescued. The rescue clone starts 2kb upstream of the first exon and ends 15 kb downstream of exon 9 (Fig. 7A). The insertion of the rescue clone was mapped to the chromosomal location 96E-F, and was recombined with FRT sequences located at position 82B on a chromosome that also carries a heat shock-GFP P(smo^+, hsp70-GFP) marker on the same chromosome arm (3R). Because female sterility was not rescued, flies were kept balanced over a ci^D fourth chromosome containing a wild-type *ey* allele. To distinguish the homozygous from heterozygous $ey^{J5.71}$ mutant larvae, we made use of a translocation from the

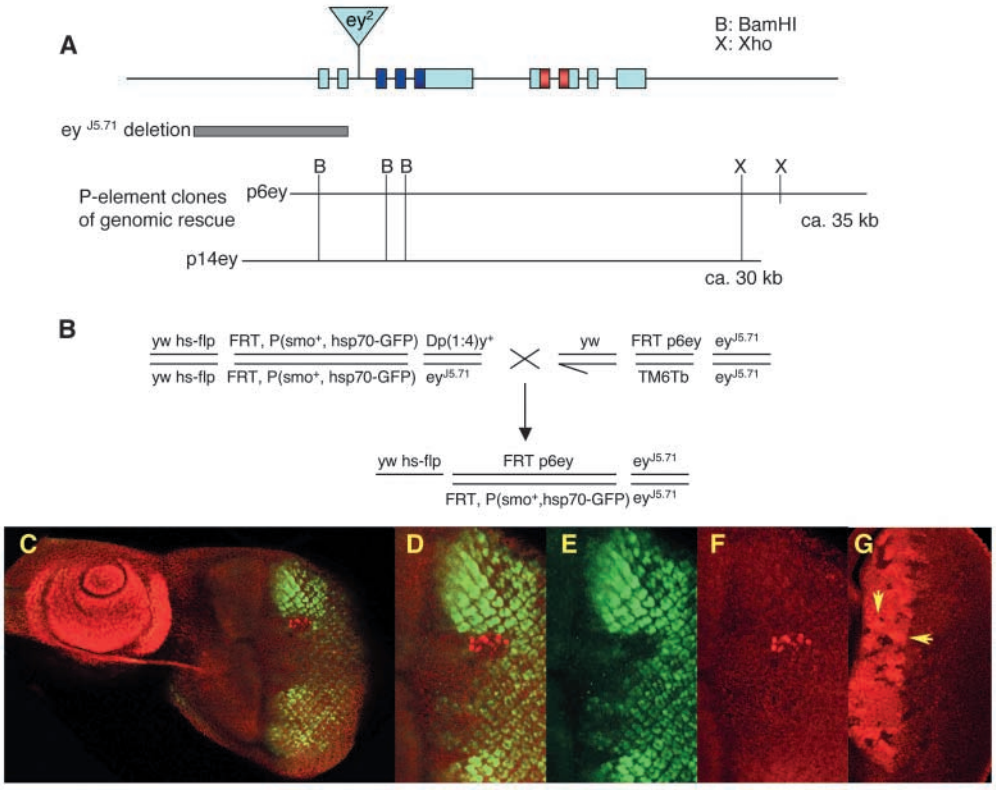

Fig. 7. *ey* mutant clones in the eye disc express DLL. (A) Genomic organization of the *ey* region. The nine exons of the *ey* gene are indicated as turquoise boxes. The areas coding for the PD are highlighted in blue and the areas coding for the HD in red. The extent of the $ey^{J5.71}$ deletion is indicated by a gray box. The DOC-element insertion of the ey^{2} mutant into the second intron of the *ey* gene is indicated by a triangle. The two genomic P-element clones and the respective genomic areas they cover are marked below. (B) Flies generated to perform the cross for the clonal analysis. Larvae that were y^{-} (25%) were dissected for antibody staining. (C-F) Antibody staining on eye discs where mutant clones were induced. DLL expression is shown in red, GFP expression in green recapitulates the *smo* enhancer expression domain in the posterior part of the disc. Posterior mutant clones are marked by a lack of GFP expression. Only clones visualized in the posterior domain of the eye disc are shown. DLL expression was never detected in cells present in the *ey* domain (anterior part of the eye disc) in $ey^{-/-}$ cells. Anterior clones were detected by the use of an *ey* antibody. (C) Shows the entire eye-antennal disc with DLL expression in the antenna and a clone of DLL-expressing cells in the eye disc. (D-F) Magnification of the image shown in C. (D) Merged image of GFP (E) and DLL (F) expression. (G) Antibody staining with the rabbit α-EY antibody where clones were randomly induced. Lack of EY protein is only visible in the anterior part of the eye disc (where EY is expressed). Brighter twin clones have two copies of the *ey* transgene (arrows).

first to the fourth chromosome containing the *yellow* (y^{+}) gene. Therefore, all y^{+} larvae are heterozygous for the $ey^{J5.71}$ mutation (see Fig. 7B). Antibody staining of eye discs in which mutant clones were randomly induced using heat-shock-flipase (*hs-flp*) during late second early third larval stage revealed ectopic induction of *Dll* in around 5% of the mutant clones (Fig. 7C-F). *Dll* induction was only observed posterior to the MF or within the *decapentaplegic* (*dpp*: secreted morphogen homolog of BMP2/4) domain (Fig. 5C), and not anterior although clones were present on both sides. Presence of anterior clones was visualized by the lack of EY protein, confirming at the same time that system is working and that the clones really lack EY protein (Fig. 7G). We then analyzed the phenotype of the *ey* clones in the mature eye. Clones were marked by the absence of the mini white gene in flies that are y^{-}. No fly displayed an obvious eye phenotype, nor were any antenna-like outgrowths observed. We compared adult eye sections of wild type, *ey* null ($ey^{J5.71}$) mutants and *ey* mutant clones but found no difference. This is in agreement with the fact that *ey* null mutants have a variable eye phenotype, most likely due to the redundancy of *toy*. Taken together with our previous experiments, these results support our hypothesis that one of the functions of the EY-HD during normal eye development is to downregulate *Dll* expression, thereby contributing to the subdivision of the eye-antennal disc into an eye and an antenna-specific region.

Discussion

The paired domains of eyeless, twin of eyeless and small eye

The evolutionary conservation of the PD and HD of Pax6 proteins points towards strong evolutionary constraints to maintain the DNA-binding specificity of these transcription factors. Nevertheless, TOY and SEY differ significantly from EY by an amino acid substitution at position 14 of the PD, from N to G, which changes the DNA-binding specificity. This change was shown to be significant because it prevents EY from binding to its own enhancer (Czerny et al., 1999). As the DNA-binding specificity of EY has been changed, the cis-regulatory sequences must also have diverged. This is illustrated by the finding that SEY (N14) is able to induce ectopic expression of *ey*, but not that of *toy*. Also, *toy* is expressed in the ocelli region of the eye disc, whereas *ey* is not (Czerny et al., 1999; Punzo et al., 2002). This suggests that the auto-regulatory feedback loop found in vertebrate *Pax6* genes has evolved upon gene duplication into a hetero-regulatory interaction in which *toy* regulates *ey* expression (Gehring and Ikeo, 1999).

As the PD of EY and TOY have diverged in evolution, we have analyzed to what extent the PD of TOY would be able to initiate eye development in an *ey*-independent manner. In the analysis we also included the mouse *Pax6* gene *sey*, which

functionally is more closely related to *toy* than to *ey*. Regardless of the differences in DNA-binding specificity between *toy* and *sey* versus *ey*, all three genes retain the ability to induce ectopic eyes, indicating that they bind to the cis-regulatory elements of *Pax6* target genes. This is supported by our analysis of the *sine oculis* enhancer *so*10, which contains five Pax6-binding sites, all of which bind TOY, but only three of them also interact with EY (Punzo et al., 2002).

Dependence on expression levels

In mammals *Pax6* mutations are haploinsufficient and in heterozygotes eye development is critically affected. However, our recent analysis of *ey* and *toy* mutants ($ey^{15.71}$ and $toy^{G3.39}$) and deletions indicate that *ey* and *toy* are completely recessive (Flister et al., unpublished). Nevertheless, overexpression of ey^{+} in the eye disc leads to a reduced eye phenotype (Curtiss and Mlodzik, 2000), indicating that expression levels are important.

Because of its variable expressivity, the ey^{2} mutant has been considered to be a hypomorph. However, we have found that neither *ey* mRNA nor EY protein can be detected in ey^{2} eye discs and the embryonic eye-anlagen (Quiring et al., 1994; Punzo et al., 2001). These findings strongly suggest that ey^{2} is a null mutation with respect to eye development. Thus, the variable eye size observed in ey^{2} flies may be due to redundant functions of *ey* and *toy*. This interpretation is supported by recent analyses of *ey* and *toy* mutants (Kronhem et al., 2002) (Flister et al., unpublished), which indicates partial complementation between the two genes. In a previous study, we had observed that *toy* cannot induce ectopic eyes in a strongly selected ey^{2} background with a high penetrance and expressivity of the eyeless phenotype. However, the data presented here show that higher expression levels of TOY protein are capable of inducing ectopic eyes in an ey^{2} background. This is in line with our finding that both *ey* and *toy* directly activate *sine oculis* (*so*) (Punzo et al., 2002) by binding to its *so*10 enhancer, so that the prior activation of *ey* by *toy* is not absolutely required for initiation of the genetic cascade leading to eye development.

Finally, we would like to issue a caveat concerning the use of the UAS/Gal4 system for ectopic expression experiments, because it is difficult to ensure that the expression levels are in the range of physiological conditions.

The C terminus of *ey* and *toy*

To determine more precisely how the two *Drosophila* Pax6 proteins achieve functional specificity, we swapped the PDs and the C termini of both proteins. We show here that the C-terminal domains of EY and TOY differ considerably, and imply functional differences between the two proteins. This is suggested by the fact that we were able to induce ectopic eyes on the antenna only when the C terminus of *ey* was present within the Pax6 protein, suggesting that the PD does not play a decisive role in this respect. Thus, the EY-CT may interact with a different subset of transcription factors and co-factors to increase DNA-binding specificity, functional activity and transactivation potential. Interestingly, on the western blot of the chimeric constructs (Fig. 3B), all of the proteins harboring the EY-CT show a more diffuse band than the proteins harboring the TOY-CT. This is typically seen for phosphorylated proteins and suggests that *ey* function may also be regulated post-transcriptionally through multiple phosphorylation sites. At the CT of Pax6, there are two highly conserved domains (Glardon et al., 1998) that are present in SEY and TOY but absent from EY, which may account for the observed differences in function.

The results obtained on the C terminus complement previous findings on *ey* and *toy*, where it has been shown that the same binding site (e.g. *so*10 enhancer) (Punzo et al., 2002) can be bound by both, but depending either on the cellular context, the presence of co-factors, protein kinases or phospatases, the activity of *ey* and/or *toy* may be modulated in order to obtain the correct cellular response.

The Eyeless-homeodomain in endogenous eye development

We have shown previously that *eyΔPD* can downregulate *Dll* expression at the transcriptional level in an ectopic situation leading to leg truncation (Punzo et al., 2001), whereas *toyΔPD* and *seyΔPD* do not, even though all three HDs have the same amino acids at positions conferring DNA-binding specificity. These functional differences between EY and TOY most likely reside in the CT of EY, which differs significantly from that of TOY and SEY. Although our previous findings showed that DNA binding of the HD is required for the downregulation of DLL (Punzo et al., 2001), the C terminus of EY appears to confer the functional specificity of the *Dll* repression.

Several lines of evidence point to the fact that the induction of *Dll* is not directly controlled by *ey* but rather by a secondary late event of postmitotic differentiation. First, in ey^{2} mutants *Dll* is normally not expressed. Only in very rare cases do those mutants show a transdifferentiation from eye to antenna (Lindsley and Zimm, 1992). Second, over expression of P35 in ey^{2} mutants does not lead to Dll induction until the third larval stage when differentiation sets in. Those *Dll*-expressing cells reside at the posterior tip of the eye disc, where differentiation starts with the onset of MF movement. Third, rescuing the ey^{2} mutant by *eyΔHD* (Fig. 5C) leads to normal eye development and not to uniform up-regulation of *Dll*. Only in rare cases was *Dll* found to be expressed in those eye discs and in even fewer cases showed antenna like outgrowth. These results are in line with the clonal analysis, where only a small percentage of clones show induction of *Dll*, but no clone displayed an adult eye phenotype. Thus, only rarely might the size of *Dll*-expressing clones be big enough the lead to a transdifferentiation. Additionally, the ability of *toy* to function redundantly to *ey* (Punzo et al., 2002) may account for those observations. Fourth, the co-expression experiment of the various *ey* constructs with P35 (Fig. 6) in the ey^{2} mutant strongly suggests that only the repression of *Dll* is *ey* dependent, not the induction, as P35 in conjunction with *eyΔPD*, which does not initiate eye development, completely abolishes antenna duplication. Antenna duplications are only observed in those rare cases where P35, in conjuction with *eyΔHD*, does not rescue eye development and thus fails to instruct the cells to enter the eye developmental pathway.

Taken together, these findings suggest that expressing a PD-containing Pax6 protein is sufficient to prevent *Dll* activation. By contrast, the EY-HD clearly confers downregulation of *Dll*. A more profound study with double mutant clones of *ey* and *toy* preventing the presence of any Pax6-PD containing protein may be more conclusive. The downregulation of *Dll* by *ey* may

be direct or indirect, but the activation is *ey* independent. Recent studies by Kenyon et al. showed that *dpp* is required for the activation of *Dll* in the antenna primodium (Kenyon et al., 2003). This may explain why in the absence of *ey*, *Dll* is only activated in cells located in or behind the MF that fail to differentiate to photoreceptors, cells that have already seen *dpp* and reside therefore normally in the posterior part of the eye disc or within the range of *dpp* signaling.

Here, we show that *Dll* repression is required in the normal eye disc to prevent antennal development and to install the eye development program. The failure to repress *Dll* in the eye primordia leads to a transdetermination from eye to antennal structures, and the formation of an additional antenna in the eye field. The downregulation of *Dll* in the eye region of the eye-antennal discs depends on the EY-HD and the EY-CT, whereas the EY-PD (and the PD of *toy*) are required to install the eye development program, mainly by activation of the subordinate target genes.

Divergent functions of EY and TOY

Our results strongly suggest that the functional differences between *ey* and *toy* are not only due to their different DNA-binding specificities and changes in the cis-regulatory sequences of their PDs, but also to interactions with different co-factors through their C termini. Recent studies showed that the transcriptional activator *Pax5* is converted into a repressor by interaction with the *groucho* protein through its C terminus and its octapeptide (Eberhard et al., 2000). Similarly, the EY-CT, which differs strongly from that of TOY, is likely to interact with a different set of co-factors to confer specific activation or repression of target genes. This hypothesis is supported by the analysis of the CT. Only the EY-CT, and not that of TOY, is capable of inducing ectopic eyes on the antenna, and only the EY-HD with an EY-CT is able to confer DLL repression, which is required for normal eye development. Thus, our experiments provide new insights into the evolutionary divergence of the two Pax6 genes in *Drosophila*, and their role in eye and head development.

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