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Partner specificity is essential for proper function of the SIX-type homeodomain proteins Sine oculis and Optix during fly eye development

Kristy L. Kenyon[1], Donghui Yang-Zhou, Chuan Qi Cai, Susan Tran, Chris Clouser, Gina Decene, Swati Ranade, Francesca Pignoni*

Department of Ophthalmology, Harvard Medical School and Massachusetts Eye and Ear Infirmary, Boston, MA 02114, USA

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Abstract

The development of the *Drosophila* visual system utilizes two members of the highly conserved Six-Homeobox family of transcription factor, Sine oculis and Optix. Although in vitro studies have detected differences in DNA-binding and interactions with some co-factors, questions remain as to what extent the activity for these two transcriptional regulators is redundant or specific in vivo. In this work, we show that the So^D mutation within the Six domain does not abolish DNA–protein interactions, but alters co-factor binding specificity to resemble that of Optix. A mutation in the same region of Optix alters its activity in vivo. We propose that the dominant mutant phenotype is primarily due to an alteration in binding properties of the Sine oculis protein and that distinct partner interactions is one important mechanism in determining significant functional differences between these highly conserved factors during eye development.

Keywords: Six-co-factors; Drl; So^{Drl}; So^D; Dominant negative; Six3; Six6; Six1; Six2

Introduction

Much of the fly head, including the eye, develops from an epithelium called 'eye-antennal imaginal disc'. Formation of the eye begins in the second larval stage (L2) with the specification of an eye primordium. Several transcription factors that are linked in a genetic cascade play a critical role in this process and are encoded by the genes *eyeless* (*ey*), *sine oculis* (*SO*), *eyes absent* (*eya*), and *dachshund* (*dac*) (Bonini et al., 1993, 1997; Cheyette et al., 1994; Quiring et al., 1994; Halder et al., 1995, 1998; Mardon et al., 1994; Chen et al., 1997; Pignoni et al., 1997). Early in L2 and in response to signaling by the BMP4-related factor Decapentaplegic (Dpp), Ey induces *eya* expression (Halder et al., 1998; Chen et al., 1999; Kenyon et al., 2003). This is soon followed by the induction of SO, and then Dac. The resulting co-expression of Ey, Eya, SO, and Dac is thought to establish a pool of eye progenitor cells within the epithelium (Fig. 1A). Early in the third larval stage (L3), signaling by Dpp and Hedgehog (Hh) initiates development of the photoreceptor neuron array. During L3, a wave of morphogenesis sweeps anteriorly across the eye disc leaving in its wake clusters of differentiating photoreceptor neurons (Fig. 1B). The front of this wave is marked by a furrow (morphogenetic furrow, MF); cells in and around the MF express Dpp. Ahead of the MF, cells stop dividing and transition into a 'preproneural' stage marked by the transient expression of the transcription factor Hairy (H) (Brown et al., 1991; Greenwood and Struhl, 1999; Bessa et al., 2002). This step is followed by induction of the 'proneural' gene for photoreceptor neurons, *atonal* (*ato*) (Jarman et al., 1994). Broad expression of this gene is first observed within the MF and marks the start of neurogenesis. Soon after, Ato expression becomes restricted to a single cell called R8, the first photoreceptor neuron of each eye unit or ommatidium. Finally, seven more photoreceptor neurons emerge around the R8 cell and begin to differentiate. Posterior to the MF, developing neurons can be visualized by the expression of the pan-neural marker ELAV. Eye development and the

* Corresponding author. Fax: +1 617 573 4290.
E-mail address: francesca_pignoni@meei.harvard.edu (F. Pignoni).
[1] Current address: Department of Biology, Hobart and William Smith Colleges, Geneva, NY 14456, USA.

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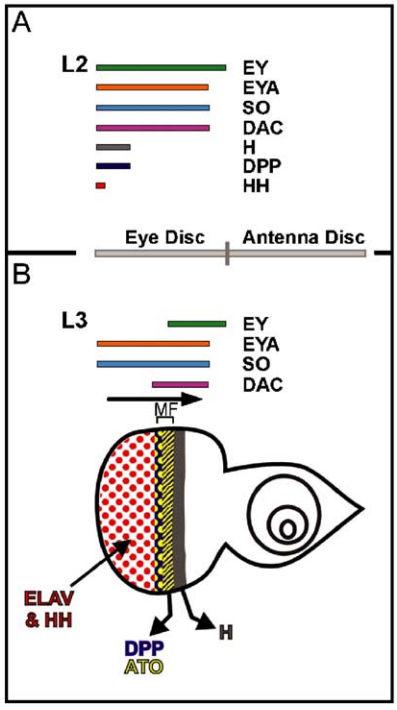

Fig. 1. Molecular markers at the L2 (A) and L3 (B) stages and schematic diagram of eye disc development in L3 (B). Bars indicate extent of protein expression within the disc. MF is marked with a bracket in panel B. The antennal disc is continuous with the eye disc, but its development is not addressed in this work. (A) By the end of L2, the eye disc is poised for onset of neurogenesis in early L3. A late L2 disc displays broad co-expression of Ey, Eya, SO, and Dac marking eye progenitor cells and expression of *h*, *dpp*, and *hh* along the posterior margin of the disc. (B) During L3, expression of Ey and Dac changes as both genes are downregulated posterior to the MF; Ey sharply at the MF, Dac more gradually posterior to it. In addition, *h* and *dpp* expression becomes very dynamic and is linked to the migrating MF; *h* is expressed ahead of the MF, and *dpp* within it. As neurogenesis proceeds, *ato* is also transiently expressed within the MF first broadly and then within few cells. Staining with the pan-neural marker Elav highlights the neuronal clusters forming posterior to the MF. Hh is expressed by neuronal cells posterior to the MF and diffuses anteriorly where it induces progenitor cells to initiate neuronal morphogenesis.

expression of molecular markers in L2 and L3 are summarized in Fig. 1 (see Pappu and Mardon, 2004; Silver and Rebay, 2005 for recent reviews).

The Six class homeobox transcription factor Sine oculis (SO) plays a fundamental role in this process. SO is expressed in eye progenitor cells in L2 and L3 discs and continues to be expressed in the developing neural epithelium posterior to the MF during L3 (Cheyette et al., 1994; Serikaku and O'Tousa, 1994). Its function is required at multiple stages, including eye primordium formation, MF initiation, MF progression, and neuronal differentiation (Pignoni et al., 1997). A second Six class homeobox transcription factor, Optix/dSix3, also appears to be involved in eye development based on its expression in the eye disc and its ability to induce ectopic eye formation (Seimiya and Gehring, 2000). However, the loss-of-function phenotype of Optix has not been characterized; therefore, its role in normal eye development is not known. Optix is expressed throughout the eye field in L2 and continues to be expressed in progenitor cells, i.e. anterior to the MF, in the L3 disc. Gene expression is downregulated at the MF and the Optix protein is not detected within the developing neuronal array posterior to the MF (Seimiya and Gehring, 2000; Kenyon et al., 2005). Although related at the molecular level, SO and Optix play somewhat different roles in eye development. Based on expression pattern, both proteins function anterior to the MF; only SO, however, functions during neuronal development (posterior to the MF).

Additional differences suggest that SO and Optix play significantly different roles in progenitor cells. First, the SO and Optix homeobox DNA-binding domains (HD) differ at 20 of 60 aa residues. In fact, they are much more closely related to vertebrate Six genes of the same subfamily than to each other. The HD of SO is nearly identical to the mouse Six1/2 type HD, displaying only 3 conservative changes when compared to Six 1 or Six 2 plus 1 non-similar substitution with Six 1 (Seo et al., 1999). The Optix HD is most similar to the mouse Six3 HD (Six3/6 subfamily) from which it differs at only 3 positions, one change being conservative (Seo et al., 1999). Although the DNA-binding specificity of the SO and Optix HDs has not been characterized, information is available about the DNA-binding specificity of the related mouse factors. Based on homology within the HD, we would predict that SO binds to a CG rich motif identified as a putative site for Six1/2 factors (Spitz et al., 1998), whereas Optix likely binds to an ATTA-type motif recognized by the Six 3 protein (Zhu et al., 2002). Thus, differences in the DNA-binding domains suggest that these proteins control non-overlapping, if not completely distinct, sets of transcriptional targets.

SO and Optix also display differences in protein–protein interactions mediated by their Six domains (SD). Specifically, they share some but not all binding partners thus far characterized. In the L3 eye disc, the co-factors Groucho (Gro), Eya, and Obp are expressed in domains that overlap with both SO and Optix. However, only SO can bind the co-activator Eya and only Optix interacts with the transcription factor Obp, whereas both proteins can bind the general repressor Gro (Pignoni et al., 1997; Seimiya and Gehring, 2000; Silver et al., 2003; Kenyon et al., 2005). Sbp, another factor able to bind both SO and Optix, can only partner SO in vivo due to its restricted expression pattern (Kenyon et al., 2005). Thus, SO and Optix regulate gene expression at least in part through distinct transcription factor complexes. In summary, differences in their expression patterns, DNA-binding, and co-factor recruitment strongly suggest that SO and Optix fulfill non-redundant roles during eye development. However, direct gene targets of either SO or Optix have not been identified and differential protein interactions have only been documented in yeast or in vitro. It is not known if or to what extent each of these mechanisms contributes to functional specificity in vivo.

In this paper, we present evidence that specificity in co-factor recruitment is indeed critical to the function of SO

and Optix. In analyzing the dominant mutant allele SO^D (*syn. Droplet, Drl*) (Heitzler et al., 1993; Cheyette et al., 1994), we have identified a single amino acid change within the SD that alters the protein–protein interaction properties of the Six domain. Specifically, the mutation appears to cause a switch in co-factor specificity such that SO^D is able to recruit an Optix co-factor. We propose that binding to inappropriate partners is the molecular mechanism underlying the SO^D mutant phenotype. Our findings support the view that specificity in partner recruitment is critical in determining distinct functions of the SO and Optix transcription factors during eye development.

Materials and methods

Genetics

The following fly lines were used: SO^1, SO^3, SO^5, SO^9, SO^D, and *so-lacZ(SO^7)* (Cheyette et al., 1994; Heitzler et al., 1993), *hs-SO* (Cheyette et al., 1994); *UAS-SO* (Pignoni et al., 1997); *UAS-Optix* (Kenyon et al., 2005); *ato^{EARLY}-lacZ* (Zhang and Pignoni, unpublished); *ato5′-lacZ* (Sun et al., 1998). Lines carrying *ey-gal4*, *pGMR-gal4*, *hs-Gal4*, *dpp-gal4*, *dpp-lacZ*, and *wg-lacZ* were obtained from the Bloomington Stock Center and are described at http://flybase.bio.indiana.edu. The following constructs were made by introducing single bp changes by site directed mutagenesis: *hs-SO$^{G>A}$*, *hs-SO$^{V>D}$*, *hs-SO$^{G>A\ +\ V>D}$*, *UAS-SO$^{V>D}$*, *UAS-SO$^{G>D}$*, *UAS-SO$^{L>P}$*, *UAS-SO$^{V>DL>P}$*, *UAS-Optix$^{D>G}$*. Multiple transgenic lines were obtained and the *Gal4* binary expression system (Brand and Perrimon, 1993) was used to drive expression in the eye and ectopically. Under the control of *ey-gal4* (Hazelett et al., 1998), the responder transgene is not only over-expressed within eye progenitor cells in L2 and L3 but it is also expressed at the earlier L1 stage when endogenous SO or Optix is not yet expressed. In addition, expression continues in cells posterior to the MF albeit at lower levels than in progenitor cells. Under the control of the *pGMR-gal4* driver (Freeman, 1996), high levels of responder transgene expression occur exclusively posterior to the morphogenetic furrow (i.e. in differentiating eye tissue). Transgene expression levels were assessed by using the *dpp-Gal4* driver (Staehling-Hampton et al., 1994) which induces expression in other imaginal discs where SO is not normally expressed (antenna, wing, leg). The level of protein expression promoted by each transgene was monitored by staining discs with the SO antibody and assessing expression in the antenna and/or wing discs. In comparing the effects of different transgenes, genetic combinations showing similar levels of expression were compared. This is particularly relevant in the case of the *UAS-SO$^{L>P}$* and *UAS-SO$^{V>D\ +\ L>P}$* transgenes, which do not induce a visible dominant effect. Expression was confirmed by detecting ectopically expressed $SO^{L>P}$ or *SO$^{V>D+L>P}$* and comparing it to the level of expression driven by three different *UAS-SO$^{V>D}$* lines (lines F1, 22, and 63) that produce dominant phenotypes. The *UAS-SO$^{V>D}$* lines were more effective than *UAS-SO$^{G>D}$* lines in inducing the dominant phenotype when expressed anterior to the MF. We attribute this to a combination of lower expression by the *UAS-SO$^{G>D}$* transgenes, but we also believe that the *SO$^{G>D}$* protein may have somewhat weaker dominant activity than *SO$^{V>D}$*. When expressed posterior to the MF under the control of the strong *pGMR-Gal4* driver, *UAS-SO$^{V>D}$* and *UAS-SO$^{G>D}$* lines induced similarly rough eye phenotypes. We generated and used a double *UAS-SO$^{G>D}$* line to induce effects comparable to a strong *UAS-SO$^{V>D}$* line under the control of *ey-gal4*. *UAS-Optix$^{D>G}$* transgenes also induce a rough eye phenotype when driven posterior to the MF by *pGMR-gal4*.

Molecular analysis of SO^D and SO^5 mutant alleles

In order to sequence the SO^D allele, *lacZ*-negative homozygous mutant embryos (*SO^D/SO^D*) from a cross of *SO^D/SO-lacZ* flies were identified by single embryo PCR. *SO* exons were PCR amplified from several single embryos and independently sequenced. For SO^5, genomic DNA was from homozygous flies.

Protein–protein interactions

Yeast 2-hybrid testing was carried out using bait constructs containing the SD and HD of SO fused to the Gal4 DNA-binding domain (vector pGBKT7) in the yeast strain AH109. Prey clones were in the pGAD or the pACT2 vectors and are described by Pignoni et al. (1997) and Kenyon et al. (2005). Positive interactions were identified by nutritional selection (markers HIS3 and ADE2) and by a colorimetric assay for an enzymatic marker (MEL1/alpha-gal). To increase the stringency of the test, the inhibitor 3-AT was added to selection plates. Only positives for all three tests were considered positive for protein–protein interaction. For pull-down assay, a DNA fragment encoding Gal4AD-HA-Obp was released from the pACT2-OBP yeast 2-hybrid clone with *Hin*dIII, and subcloned into the expression vector pRmHa3 (pRmHa3-G4-HA-Obp). *Drosophila* S2 cells (2×10^6/ml) were transfected for 6 h with 10 μg pRmHa3-HA-Obp by the calcium phosphate method (Pascal and Tjian, 1991). After 24 h of recovery, expression was induced with 1 mM $CuSO_4$. Cells were harvested 24 h later and lysed by sonication in PBS containing protease inhibitors (Roche). Cell lysate were cleared by centrifugation (12000 rpm, 30 min, 4°C), mixed with anti-HA affinity matrix (Roche) and incubated overnight at 4°C. Beads were washed 3 times with PBS 0.03% Triton-X100, 3 times with PBS/protease inhibitor, and resuspended in PBS/protease inhibitor. Beads containing bound HA-Obp or unbound beads were incubated for 2 h at room temperature with in vitro transcribed/translated ^{35}S-SO^D (TnT T7 Coupled Reticulocyte Lysate System; Promega). After washing 5 times with PBS, 0.03% Triton-X, and 4 times with PBS,

beads were resuspended in SDS buffer, incubated 3 min at 95°C, and run on a 10% polyacrylamide gel.

Gel shift assays

GST control protein and GST-SOSDHD fusion protein were produced in *Escherichia coli* strain BL 21 and purified by MicroSpin™ GST Purification Module (Amersham Pharmacia). The target DNA was generated by PCR from the plasmid Mef3(×6)-pGL3 (Spitz et al., 1998; Kenyon, unpublished) using high fidelity polymerase Pfx (Invitrogen). After gel purification, the 178 bp fragment, which contains 6 repeats of the consensus sequence TCAGGTT, was non-radioactively (DIG) labeled and tested for protein binding according to the protocol of the DIG Gel Shift Kit (Roche). Unlabeled target DNA was used as competitor inhibitor; unlabeled control oligonucletides 39mer, containing the binding site for Oct2A and provided by the kit, were used as non-competitor inhibitor DNA. The binding reactions (1.5 μg GST or GST-fusion proteins; 0.155 fmol target DNA) were incubated at room temperature for 1 h and electrophoresis was performed through 10% TBE Ready Gels (BIO-RAD). After electrophoresis, the gels were blotted to positively charged nylon membranes and the membranes were then cross-linked by UV Stratalinker (Stratagene), followed by chemiluminescent detection using anti-Digoxigenin-AP Conjugate and CSPD substrate.

Transactivation assay

The pRmHa3-*flag-eya*, pRmHa3-*myc-so*, and *ARE-luciferase* plasmids were kindly provided by Ilaria Rebay (Silver et al., 2003). A pRmHa3-*myc-so*^{D} plasmid was generated by site-directed mutagenesis (Promega) using pRmHa3-*myc-so* as template. Transient transfections with calcium phosphate were as described in Silver et al. (2003) and Pascal and Tjian (1991). Cells were transfected for 6 h with a total of 16 μg DNA, including 10 μg *ARE-luciferase*, 1 μg pcDNA3.1/His/lacZ (Invitrogen), 2.5 μg of each desired pRmHa3-expression construct and pBS-SK DNA as needed. After 24 h, protein(s) expression was induced with 1 mM $CuSO_4$. Cells were harvested 24 h later and luciferase activity was measured using the Luciferase Assay System (Promega). β-galacosidase activity was quantified using the Galacto-Star System (Tropix/Applied Biosystems). Three independent transfections were performed for each condition. Luciferase activity was normalized relative to the β-galactosidase activity. Data were graphed in Microsoft Excel; error bars indicate one standard deviation above and below the mean.

Histology

Larvae were dissected and discs were stained for reporter gene (*dpp-lacZ*, *wg-lacZ*, *so-lacZ*, *ato-lacZ*), protein (Elav, Eya, Dac, SO) or mRNA (*h*) expression by standard protocols. Antibodies used were: mouse MAb anti-Elav, 1:50 (Robinow and White, 1991); mouse MAb anti-Dac, 1:500 (Mardon et al., 1994); mouse MAb anti-Eya, 1:80 (Bonini et al., 1993); mouse anti-SO, 1:200 (Cheyette et al., 1994). Secondary antibodies were used at 1:200, including goat anti-mouse HRP (Biorad); goat anti-mouse Cy3 (Jackson Lab); goat anti-mouse Cy2 (Jackson Lab).

Results

SO function is required for eye primordium formation in L2 and maintenance in L3

The recessive mutant alleles SO^{1}, SO^{3}, SO^{9}, and SO^{5} (Table 1) were used to generate genetic backgrounds in which SO function is progressively diminished. In increasingly more severe mutant backgrounds ($SO^{5}/SO^{9} < SO^{9}/SO^{9} < SO^{9}/SO^{3} < SO^{1}/SO^{1}$), the adult fly eye becomes progressively reduced in size until it is lost completely (not shown). This loss of eye results from reduced neuronal development during L3 as shown by the progressive reduction in the size of the Elav positive field in the developing disc (Fig. 2A; not shown). Reduced neurogenesis is preceded and accompanied by reduced expression of *dpp* and the early acting eye specification factors Eya and Dac (Figs. 2A and B and not shown).

The effects of complete loss of SO function during eye development can be seen in the SO^{1} mutant background. Unlike the recessive lethal null allele SO^{3}, SO^{1} is an eye-specific null allele and affects gene expression in the eye disc but not in embryos (Cheyette et al., 1994). Similarly to SO^{3} mutant tissue, SO^{1} mutant clones over-proliferate and do not develop as eye; rather, they give rise to cuticle or die (Table 1; not shown). In homozygous SO^{1} mutant discs, *dpp* and Eya are expressed in L2, i.e. prior to neurogenesis (Pignoni et al., 1997; Halder et al., 1998). However, their expression is progressively lost and little or no expression of either factor can be detected by late L3 (Fig. 2A; Pignoni et al., 1997). Expression of Dac is also affected by the SO^{1} allele. Dac expression initiates as expected along the very posterior border of the L2 disc (Fig. 2C). However, the expansion of its expression domain away from the margins and towards the center of the disc does not take place and, by late L3, SO^{1} mutant discs do not displays significant Dac staining (Pignoni et al., 1997). Finally, SO^{1} mutant tissue does not display any of the changes in gene expression associated with the start of neuronal morphogenesis, as shown by the failure to induce expression of the preproneural gene *h* (Fig. 2C; not shown) and the proneural gene *ato* (Jarman et al., 1994) which normally precede expression of neuronal markers.

In summary, the gradual loss of SO function during eye development results in a progressive reduction in the size of the eye primordium and in neuronal morphogenesis (Figs. 2A and B). Ultimately, in a null background, an eye

Table 1
SO mutant alleles used in this study

	SO^3/SO^3	SO^9/SO^9	SO^5/SO^5	SO^1/SO^1	SO^D
Allele type	Null/amorph recessive	Hypomorph recessive	Hypomorph recessive	Eye-specific null recessive	Dominant eye phenotype recessive lethal
Molecular lesion	Small deletion in coding DNA	Deletion of the 5′ regulatory region	D217N	1199 BP DELETION (BP 3983–5181 OF THE LAST INTRON)	V200D (IN SD) G66A (NO EFFECT ON PHENOTYPE)
Phenotype adults	Embryonic lethal	Viable, small eyes, reduced ocelli	Viable, slightly reduced eyes; normal ocelli	Viable; small eye or eyeless	Dominant eyeless L1 RECESSIVE LETHAL
Phenotype discs (L3)	na	REDUCED EYE FIELD, PROGRESSIVE ARREST OF NEUROGENESIS FIRST ALONG THE MARGINS AND THEN IN THE CENTER	SLIGHTLY REDUCED EYE FIELD	Small disc size; few or no Elav positive cells LOSS OF *EYA, DAC, DPP, STRING, H, ATO* BUT NOT *EY* EXPRESSION IN L3; ECTOPIC WG IN LATE L3 BUT NOT IN LATE L2.	SO^D/+ DISCS ARE SMALLER THAN WT BUT LARGER THAN SO^1/SO^1. THEY SHOW LOSS OF *ATO* BUT NOT *EY, EYA, DAC, DPP, HH OR H* EXPRESSION. DOMAINS OF *H, DPP* AND *HH* EXPRESSION ARE EXPANDED. ECTOPIC WG EXPRESSION IS SEEN ALONG THE POSTERIOR MARGIN IN LATE L3 BUT NOT LATE L2 DISCS. SO^D MUTANT PHENOTYPE IF ONLY WEAKLY SUPPRESSED IN SO^D WG^{LACZ}/SO^+ WG^{TS} DISCS AT 25°C
Phenotype in clones (L3)	Non-autonomous over-proliferation phenotype; loss of *eya, dac*, *dpp* but not *ey* expression	nd	nd	OVER-PROLIFERATION PHENOTYPE AND LACK OF NEURONAL DEVELOPMENT IN L3, CUTICLE FORMATION IN ADULT EYE	Clones show normal expression of eye specification factors
References	1, 2, 4	1, 8 (Fig. 1)	1, 2, 8 (M and M, and not shown)	1, 3, 4, 5, 6, 8 (Fig. 1, not shown)	1, 2, 7, 8 (Fig. 2, not shown)

[1]Cheyette et al., 1994; [2]Heitzler et al., 1993; [3]Jarman et al., 1994; [4]Pignoni et al., 1997; [5]Halder et al., 1998; [6]Niimi et al., 1999; [7]Roeder et al., 2005; [8]this study, information in small caps.

primordium (Eya/SO/Dac co-expression domain) is not properly established in L2 and cannot be maintained during L3 (Fig. 2C). However, mutant tissue does not loose its 'eye disc' identity, as shown by the continued expression of the upstream regulator Ey (Pignoni et al., 1997). Thus, lowering SO activity using recessive mutant alleles affects every stage of eye development including eye specification, MF initiation, and MF progression.

The SO^D mutant phenotype is not consistent with a simple reduction in SO function

SO^D/+ mutant flies display little or no eye, whereas SO^D homozygous or SO^D over recessive alleles of *SO* is lethal (Heitzler et al., 1993). Since the *SO* locus is not haplo-insufficient (*SO+/Df* flies have normal eyes), the dominant effect of SO^D reflects the production of a mutant SO protein. In addition, the SO^D phenotype can be partially rescued in a dosage-dependent manner by increasing copies of the wild-type *SO* allele. The severity of the phenotype follows the order: SO^D/+ > SO^D/++ > SO^D/++/+ (Heitzler et al., 1993). Repeated induction of a *hs-*SO^{WT} transgene in SO^D/+ larvae has a similar effect (Cheyette et al., 1994). These data suggest that the SO^D protein lowers the activity of wild-type SO by functioning in a dominant negative fashion. To investigate this possibility, we characterized the SO^D mutant phenotype during eye development. We hypothesized that if the SO^D allele functioned purely as a dominant negative then its developmental phenotype should be comparable to that of partial or complete loss-of-function alleles of *SO*. Specifically, since the SO^D mutation results in adult flies with few or no ommatidia, we expected SO^D/+ mutant discs to show a phenotype similar to SO^1/SO^1 or SO^9/SO^3 (Fig. 2).

Contrary to our prediction, a developmental analysis shows that the SO^D phenotype differs significantly from recessive loss-of-function mutant backgrounds. Unlike SO^9/SO^3 or SO^1/SO^1, SO^D/+ mutant discs display normal eye primordium specification and maintenance: Eya, *SO-lacZ*, and Dac are still broadly and robustly expressed in late L3 (Fig. 3A and not shown; Roederer et al., 2005; Kumar et al., 2004; Hu, 1997). Moreover, eye progenitor cells transition through the preproneural state as evidenced by robust induction of *hairy* expression and downregulation along the very posterior hedge of the disc (Fig. 3B). Unlike SO^1/SO^1, late L3 SO^D/+ mutant discs display expanded expression of the reporter lines *dpp-lacZ* and *hh-lacZ* (Figs. 3C and D). However, the acquisition of a proneural state is impaired since *ato* induction is absent or very weak (Fig. 3E; not shown). When present, *ato-lacZ* expression is limited to small patches of cells some distance from the posterior margin (arrowhead in Fig. 3E) and

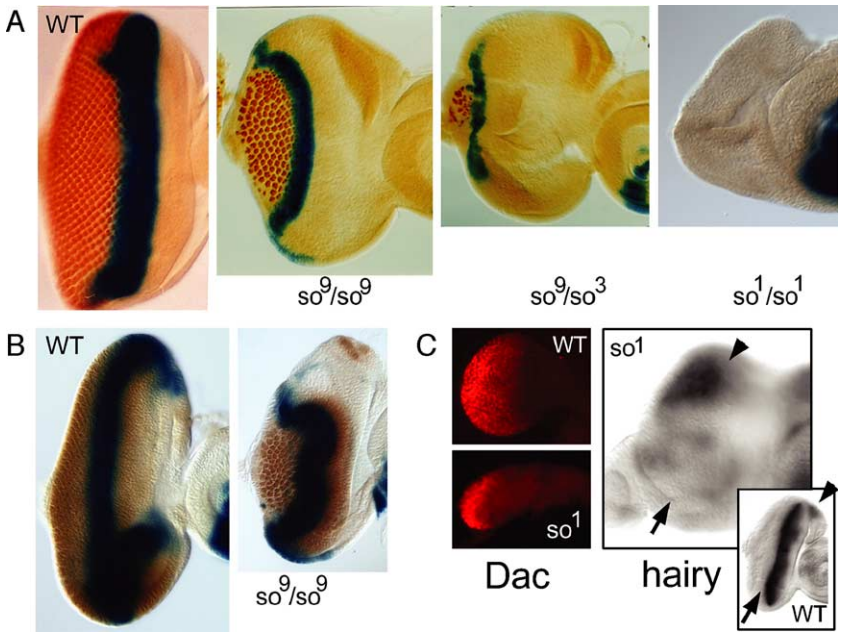

Fig. 2. (A) Phenotypic series showing the effect of decreasing SO function: WT (left) through null mutant background, SO^1/SO^1 (right). All discs show Elav expression in developing neuronal cluster (brown) and *dpp-lacZ* expression within the MF (blue). (B) WT and SO^9/SO^9 L3 disc stained for *dpp-lacZ* (blue) and Eya (brown). (C) Left panels: Dac expression (red) in WT and SO^1/SO^1 mutant discs in late L2; Right panels: *h* expression (black) detected by in situ hybridization in WT and SO^1/SO^1 mutant discs in late L3. The MF-associated expression of *h* is missing (arrow) in late L3 mutant disc, whereas expression of *h* in the ocellar region is expanded (arrowhead).

ahead of few Elav positive neurons (arrow in Fig. 3E). These results show that, in contrast to SO^1 and other strong recessive loss-of-function alleles, $SO^D/+$ mutant eye discs are arrested at the time of neuronal morphogenesis and severely affect relatively late steps in eye development without impairing earlier stages.

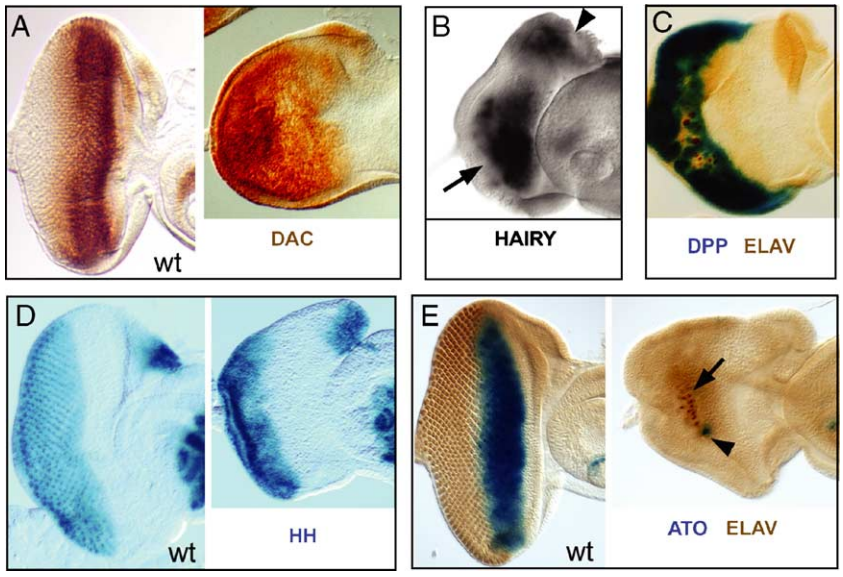

Fig. 3. All discs are from late L3 larvae. Unmarked discs in panels A, B, C, and D are $SO^D/+$, wild-type discs are marked wt. (A) WT and $SO^D/+$ mutant discs stained for Dac (brown). (B) *h* expression in $SO^D/+$ mutant disc (black); the ocellar (arrowhead) and MF-associated (arrow) domains of *h* gene expression are present and expanded. Compare to wild-type patterns in Fig. 2C. (C) Elav (brown) and *dpp-lacZ* (blue) expression in $SO^D/+$ mutant disc. Compare to wild-type patterns in Fig. 2A. In $SO^D/+$, *dpp-lacZ* is expressed in an expanded domain within the posterior region of the disc. (D) WT and $SO^D/+$ mutant discs stained for *hh-lacZ* (blue). In the WT eye disc, *hh-lacZ* expression is detected in few cells along the posterior margin prior to MF initiation, and in the differentiating neuronal cluster during MF progression. In $SO^D/+$ mutant discs, *hh-lacZ* is expressed in an expanded domain along the posterior margin. (E) WT and $SO^D/+$ mutant discs showing ato^{EARLY}-*lacZ* expression (blue). Expression of *ato* in a broad band (early pattern) and then in cluster of cells and eventually R8 alone (late pattern) is controlled from separate enhancer regions (Sun et al., 1998). Expression of the late *ato5′-lacZ* reporter (Sun et al., 1998) was similarly absent or strongly reduced (not shown).

Finally, unlike strong recessive *SO* mutant alleles, SO^D is not embryonic lethal. Lethality of SO^D homozygous animals occurs in L1. Thus, the SO^D allele, in absence of any wild-type SO protein, can provide sufficient function to offset embryonic lethality.

These findings show that SO^D does not merely lower the activity of the wild-type SO protein and lead us to conclude that SO^D is not a simple antimorphic allele.

The gain-of-function activity of the SO^D protein is due to a single amino acid change within the conserved Six domain

In order to understand how SO^D disrupts normal eye development, we set out to identify the molecular defect responsible for its mutant phenotype. Genomic DNA corresponding to each exon of the *SO* gene was PCR amplified from SO^D/SO^D mutant embryos. Sequencing revealed two point mutations: a G to C transversion causing a Glycine to Alanine substitution at amino acid 66 and a T to A transversion resulting in the substitution of a non-charged Valine with a negatively charged Aspartate at position 200 (an aa change also identified by Roederer et al., 2005). The amino acid residue 66 falls in a non-conserved region of the SO protein. On the contrary, position 200 falls in the highly conserved Six domain.

In order to establish which of these amino acid changes caused the dominant phenotype, we introduced cDNAs with the above mutations back into flies. Constructs that expressed proteins mutant at one or both sites under the control of the hsp70 promoter (*hs-*$SO^{G>A}$, *hs-*$SO^{V>D}$, and *hs-*$SO^{G>A+V>D}$) were transformed into flies. In absence of heat shock, transgenic flies carrying these constructs were phenotypically indistinguishable from wild type. Following heat shock, the *hs-*$SO^{G>A}$ transgenic flies behaved exactly like transgenic flies carrying a *hs-*SO^+ transgene, i.e. expression of the $SO^{G>A}$ allele in a wild-type background did not disrupt eye development. In addition, it completely rescued the eyeless phenotype of SO^1 homozygotes and also partially rescued the SO^D eye phenotype in a dosage-dependent manner (not shown). The eyes of heat-shock treated *hs-*$SO^{V>D}$ or *hs-*$SO^{G>A+V>D}$ flies, however, were markedly reduced or completely absent, a phenotype similar to SO^D/+ (Figs. 4B and C; not shown).

From these results, we conclude that the $^{G>A}$ change does not detectably alter SO protein function, whereas the $^{V>D}$ substitution is solely responsible for the dominant phenotype. The Valine at position 200 corresponds to the 98th aa of the conserved SD (domain limits based on Seo et al., 1999). We refer to this aa substitution as the V98D mutation because its position within the SD appears to be significant (see below).

The SO^D mutation does not prevent binding to DNA but it alters the interaction profile of the Six domain

Since the V98D mutation falls within the protein–protein interaction domain, it is likely to alter the interaction of SO with other co-factors. In this case, we predicted that the SO^D mutant protein would still bind DNA and that DNA-binding may, in fact, be required to express the dominant phenotype. To investigate these hypotheses, we set out to compare the DNA-binding and protein–protein interaction properties of the SO^{WT} and $SO^{V>D}$ mutant proteins.

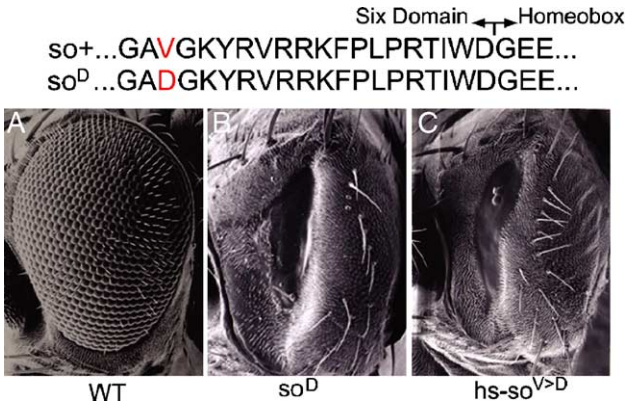

Fig. 4. The SO^D dominant phenotype is due to a single amino acid substitution ($V > D$) within the Six domain. Sequence shown includes the last 20 aa of the SD and the first 3 aa of the HD. SEM images of wt (A), SO^D/+ (B), and *hs-*$SO^{V>D}$ (C) adult fly heads. Larvae carrying the *hs-*$SO^{V>D}$ transgene were heat shocked three times for 2-h (37°C) from late L2 through mid L3.

Although a *bona fide* binding site for SO is not known, a DNA target site for the closely related Six1 vertebrate homologue has been identified (Spitz et al., 1998). We made use of this site (TCAGGTT) to test for SO–DNA interactions. In gel shift experiments, both wild-type SO (GST-SD$^+$HD fusion) and $SO^{V>D}$ mutant (GST-SD$^{V>D}$ HD fusion) can specifically shift a DNA fragment containing six tandem copies of the target site (Fig. 5A). This result suggests that the V98D change does not prevent the SO^D mutant protein from binding its gene targets in vivo.

To test whether DNA-binding was in fact required, we assayed the ability of a 'DNA-binding deficient' SO^D protein to induce the dominant phenotype. To impair DNA-binding, we introduced a single amino acid change within the HD, an L to P substitution at aa 257 (Treisman et al., 1989) generating the *UAS-*$SO^{L>P}$ and *UAS-*$SO^{V>D+L>P}$ transgenes. The L257P mutation did affect wild-type SO function, since *hs-Gal4* driven expression of *UAS-*SO^{WT}, but not *UAS-*$SO^{L>P}$, can rescue the eyeless phenotype of SO^1 homozygous mutant flies (not shown). The L41P substitution also affected the activity of the $SO^{V>D}$ protein and suppressed the dominant phenotype due to the V98D mutation. Specifically, *hs-Gal4* driven expression of *UAS-*$SO^{V>D}$ but not *UAS-*$SO^{V>D+L>P}$ was able to induced the dominant phenotype (not shown), and transgenic flies expressing the $SO^{V>D+L>P}$ double mutant protein are indistinguishable from wild type. Therefore, a functional DNA-binding domain appears to be necessary to induce the dominant mutant phenotype. Based on this evidence, we propose that the $SO^{V>D}$ mutant protein retains its DNA-binding activity and most likely functions by directly regulating gene transcription.

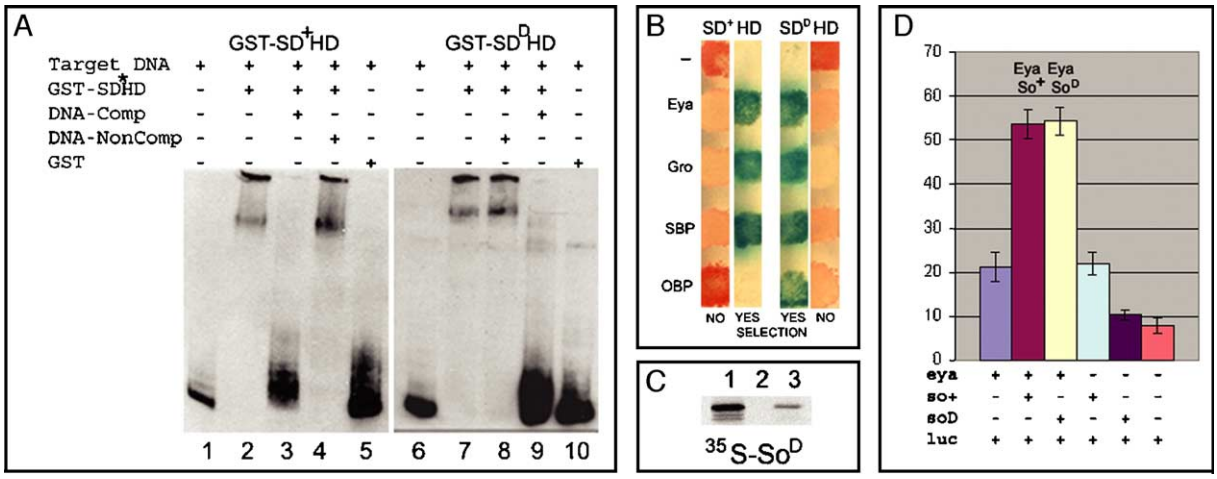

Fig. 5. (A) Electrophoretic mobility shift assay: target DNA is shifted by either GST-SD^{+}HD (lane 2) or GST-SD^{D}HD (lane 7); unlabeled competitor DNA abolishes the shifts (lanes 3 and 9). DNA-Com = competitor DNA; DNA-NonComp = non-competitor DNA. (B) Testing interactions with SO and $SO^{V>D}$ in the yeast 2-hybrid system. Non-selective plates lack Trp and Leu to ensure cells contained both prey and bait plasmids but did not select for prey–bait interactions. Selective plates also lack Ade and contain X-α-gal. Growth on Ade minus plates (yes columns) and α-galactosidase activity (blue) reflects an interaction between bait and prey proteins. Baits: SDHD = pGBKT7-SDHD and SD^{D}HD = pGBKT7-SD^{D}HD. (C) Direct binding between SO^{D} and Obp. Lane 1: 10% of ^{35}S-SO^{D} input. Lane 2: pulldown from ^{35}S-SO^{D} incubated with unbound anti-HA beads. Lane 3: pulldown from ^{35}S-SO^{D} incubated Obp-HA-bound anti-HA beads. (D) Co-expression of Eya with either wild-type SO (purple bar) or SO^{D} (yellow bar) results in similar levels of ARE-luciferase transcription. The *y*-axis shows relative luciferase activity.

We next investigated whether the $SO^{V>D}$ mutation affects the protein–protein interaction properties of the SD. Among putative SO partners, Eya, Gro, and Sbp were tested for their ability to interact with an SD containing the V98D substitution. In addition, we also tested the Zn-finger transcription factor Obp, a putative partner of Optix (Kenyon et al., 2005). Testing was carried out in yeast using baits containing the Six and Homeobox domains, SD^{+}HD (wild-type bait) and SD^{D}HD (mutant bait). The SD^{+}HD bait interacted strongly with Eya, Gro, and Sbp, but not with Obp, whereas the SD^{D}HD mutant bait interacted robustly with all four factors (Fig. 5B). Since ^{35}S-SO^{D} can be pulled down in vitro using HA-tagged-OBP, the interaction detected in yeast likely reflects direct binding between SO and OBP (Fig. 5C). Moreover, in trans-activation assays, both mutant and wild-type SO proteins synergize similarly with Eya to induce expression of a luciferase reporter containing the related ARE binding sites (Fig. 5D) (Silver et al., 2003). Thus, the $^{V>D}$ mutation does not affect the transactivating activity of the Eya–SO^{D} complex and modifies some but not all protein–protein interactions mediated by the Six domain.

These results strongly suggest that the V98D change alters the interaction profile of the Six domain and may lead to the abnormal recruitment by SO^{D} of a putative partner of Optix and/or other unknown factors that do not normally associate with SO.

The presence of a D residue, and not the loss of V, causes the SO^{D} phenotype

Sequence alignment of the Six domains of SO and Optix shows that the V98D substitution occurs in a region of the SD that is very highly conserved with the corresponding region in Optix. Strikingly, in Optix, the conserved Valine at position 98 is followed by a D at position 99 (Fig. 6A). This intriguing observation raises the possibility that it is the introduction of a charged D residue in this region of the protein and not the loss of the Valine that gives rise to the dominant phenotype.

In order to test this hypothesis, we changed the G at position 99 of the Six domain of SO to a D ($UAS\text{-}SO^{G>D}$), thus generating a match to the Optix sequence in this region. The $SO^{G>D}$ protein induced similar dominant effects as $SO^{V>D}$ when driven by *ey-Gal4* (Fig. 6B) and other Gal4 drivers (see materials and methods). Since the V at position 98 is unchanged in the $SO^{G>D}$ protein, this result excludes the loss of the conserved V residue as a critical factor in generating the dominant mutant phenotype and establishes a link between the introduction of a D residue in this region of SO and the dominant loss of eye structures characteristic of the SO^{D} mutant allele.

The 99D residue within the Optix SD is critical for normal protein function

These findings strongly suggested that the specific aa sequence in this region of the Six domain is important for some protein–protein interactions. We therefore predicted that the presence of a D residue in Optix is essential to at least some aspects of Optix function. To investigate this hypothesis, we introduced a D to G substitution in Optix ($UAS\text{-}Optix^{D>G}$) thereby making the Optix SD identical to SO in this region. We assayed the effect of this change by misexpressing either the wild-type protein or Optix$^{D>G}$ under the control of *ey-Gal4* and comparing the induced

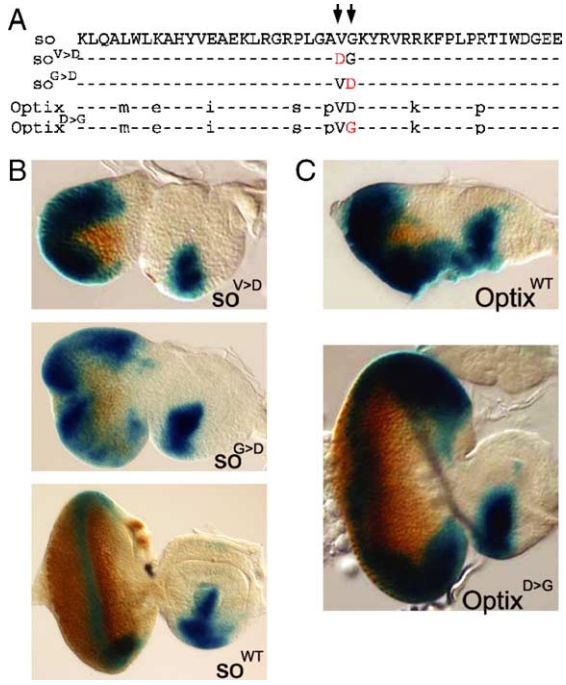

Fig. 6. (A) The C-terminal portion of the Six domains of SO and Optix is highly conserved. Sequence shown includes the last 42 aa of the SD and the first 3 aa of the HD. Amino acid residues 98 and 99 within the SD are marked by arrows and shown in upper case. Changes from the wild-type sequence at these positions are shown in red. Sequence identities relative to SO are indicated by hyphens, all other aa changes are shown in lower case regardless of similarity. (B) Panels show mid to late L3 discs stained for *dpp-lacZ* (blue) and Eya (brown); *ey-gal4* driven expression of *UAS-SO*$^{V>D}$ (top panel) or *UAS-SO*$^{G>D}$ (middle) results in smaller discs and impaired eye development; expression of *UAS-SO*^{WT} (bottom panel) has little of no effect. (C) Panels show early to mid L3 discs stained for *dpp-lacZ* (blue) and Eya (brown); *ey-Gal4* driven expression of *UAS-Optix*^{WT} (top panel) results in smaller discs; expression of *UAS-Optix*$^{D>G}$ (bottom panel) does not.

phenotypes. Expression of the wild-type protein interfered strongly with normal eye development leading to an arrest of development prior to the onset of neurogenesis (Fig. 6C top panel). The mutant Optix$^{D>G}$ did not show the same effect and eye discs expressing this protein developed normally (Fig. 6C bottom panel). This outcome was not due to a complete loss of activity by the Optix$^{D>G}$ protein, since misexpression of Optix$^{D>G}$ under the control of *pGMR-Gal4*, i.e. posterior to the MF, produces a rough eye phenotype in the adult, an effect similar to wild-type Optix (not shown). Thus, Optix protein function is altered, but not abolished, by the D99G substitution.

This finding is consistent with the hypothesis that presence or absence of the D residue in this region of the Six domain plays an important role in differentiating the protein–protein interaction properties of the Six domains of SO and Optix.

Discussion

In this paper, we report a detailed analysis of a dominant eyeless mutant allele of the SO transcription factor. We show that the *SO*^{D} mutant phenotype is significantly different from the recessive *SO* loss-of-function phenotype. In null mutant *SO*^{1} discs, the eye primordium fails to be properly specified. Onset of early eye primordium-related gene expression such as *dpp* and Eya occurs normally; however, proper expression of the downstream factor Dac fails and onset of *hairy* does not occur (this work; Bonini et al., 1993; Pignoni et al., 1997). Over time, expression of Eya, *dpp,* and Dac ceases completely (Pignoni et al., 1997). On the contrary, *SO*^{D} mutant discs show normal eye-primordium specification and maintenance including expression of Eya, *SO* itself, and Dac in late L3 discs (this work; Roederer et al., 2005). In addition, expression of *hairy* is present indicating that the L3 disc is primed for the start of neurogenesis. It is at this point that eye development fails to proceed as the onset of *atonal* expression does not take place.

We identify the critical mutation in the *SO*^{D} allele as a single amino acid change within the Six domain. As the *SO*^{D} mutation occurs in the protein–protein interaction domain, changes in this region may be expected to influence the direct interaction of SO with one or more of its partners. However, due to its close proximity to the homeodomain (18 aa upstream), this mutation may also influence DNA-binding. Indeed, a study of DNA-binding by the mouse Six 4 protein shows that the Six domain contains sequences required for DNA-binding specificity (Kawakami et al., 1996). Two lines of evidence argue against the involvement of a D residue in determining the specificity of DNA-binding. First, we did not detect any differences in DNA-binding between GST-SD^{+}HD and GST-SD$^{V>D}$HD fusion proteins. Indeed, both protein fragments give similar shifts that can be specifically abolished by competitor DNA. Second, the SO protein has been shown to regulate transcription through the Six 4 binding site, the ARE motif, in cell culture (Silver et al., 2003). Since Six 4, similarly to Six 3, has a D residue in this region (position 99), the presence or absence of this residue does not appear to dictate differential DNA-binding specificity. In fact, SO and SO^{D} can similarly transactivate expression of an ARE-luciferase reporter in the presence of the co-factor Eya. Although we cannot exclude that changes in SO-DNA interactions contribute to the *SO*^{D} phenotype, our analysis indicates that at least some aspects of DNA-binding specificity remain intact. Moreover, we show that the DNA-binding activity of SO is required to induce the dominant effect. Hence, *SO*^{D} likely functions by misregulating transcription of SO target genes.

In contrast to our observations on DNA-binding, we did find evidence of an altered protein–protein interaction. In particular, we identify a putative partner of the related Optix transcription factor, Obp, as a co-factor that may be abnormally recruited in vivo by the *SO*$^{V>D}$ protein. As Obp is a transcription factor of unknown function (Kenyon et al., 2005), it is difficult to speculate on the significance of

the $SO^{V>D}$–Obp interaction for the generation of the dominant phenotype. Additional evidence suggests that recruitment of Obp is not the only change in the protein–protein binding activity of $SO^{V>D}$. In fact, in vivo expression of the $SO^{V>D}$ mutant protein can interfere with eye development not only prior to neuronal development but also at later stages when recruitment and differentation of photoreceptor neurons and accessory cells are in progress (not shown). Since *Obp* mRNA is not expressed behind the MF (Kenyon et al., 2005), recruitment of this Optix co-factor is less likely to be involved in these late effects. Nonetheless, our findings raise the possibility that interactions with other proteins, not normally partnered by SO, lead to interference in eye development.

Throughout the last third of the Six domain, the SO and Optix proteins show a remarkable degree of conservation, differing at only 8 positions out of 42 (3/8 changes are conservative; Identity, I = 81%; Similarity, S = 88%) (Fig. 6A; Seo et al., 1999). This high level of sequence conservation holds true not only for SO and Optix but generally for members of the Six1/2 and Six3/6 subfamilies (Seo et al., 1999). By contrast, SO and Optix are far less conserved in the first two thirds of the SD (I = 44%; S = 66%). Moreover, the amino acid at position 99 of the SD is highly conserved appearing as a G in Six1/2-type genes and as a D in Six3/6-type factors (only in zebrafish Six7, a member of the Six3/6 subfamily, a conservative change to E is observed) (Seo et al., 1999). Thus, the V98D change in the SO protein introduces a negatively charged residue in a highly conserved region of the Six domain almost at the same position wherein Six genes of the Six3/6 type (including Optix) display a negatively charged Aspartate. The data presented here strongly suggest that the introduction of a D residue, rather than the loss of a V, is in fact the critical change conferring dominant activity to the SO^D mutant allele as shown by the observation that $SO^{G>D}$ has SO^D-like activity. Moreover, a D99G substitution in Optix alters its function in vivo confirming the critical importance of the amino acid sequence in this region of the Six domain.

In conclusion, this work supports the notion that SO and Optix play unique roles in the early stages of eye development that cannot be substituted for by each other and strongly suggests that association with specific partners is an important mechanisms underlying their functional specificity. In the context of evolution, the emergence of novel protein–protein interactions as a result of single base-pair changes exemplifies a possible mechanism for the emergence of novel protein functions and the resulting morphological variation.

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References

Bessa, J., Gebelein, B., Pichaud, F., Casares, F., Mann, R.S., 2002. Combinatorial control of *Drosophila* eye development by eyeless, homothorax, and teashirt. Genes Dev. 16, 2415–2427.

Bonini, N.M., Leiserson, W.M., Benzer, S., 1993. The eyes absent gene: genetic control of cell survival and differentiation in the developing *Drosophila* eye. Cell 72, 379–395.

Bonini, N.M., Bui, Q.T., Gray-Board, G.L., Warrick, J.M., 1997. The *Drosophila* eyes absent gene directs ectopic eye formation in a pathway conserved between flies and vertebrates. Development 124, 4819–4826.

Brand, A.H., Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401–415.

Brown, N.L., Sattler, C.A., Markey, D.R., Carroll, S.B., 1991. Hairy gene function in the *Drosophila* eye: normal expression is dispensable but ectopic expression alters cell fates. Development 113, 1245–1256.

Chen, R., Amoui, M., Zhang, Z., Mardon, G., 1997. Dachshund and eyes absent proteins form a complex and function synergistically to induce ectopic eye development in *Drosophila*. Cell 91, 893–903.

Chen, R., Halder, G., Zhang, Z., Mardon, G., 1999. Signaling by the TGF-beta homolog decapentaplegic functions reiteratively within the network of genes controlling retinal cell fate determination in Drosophila. Development 126 (5), 935–943 (Feb.).

Cheyette, B.N., Green, P.J., Martin, K., Garren, H., Hartenstein, V., Zipursky, S.L., 1994. The *Drosophila* sine oculis locus encodes a homeodomain-containing protein required for the development of the entire visual system. Neuron 12, 977–996.

Freeman, M., 1996. Reiterative use of the EGF receptor triggers differentiation of all cell types in the *Drosophila* eye. Cell 87, 651–660.

Greenwood, S., Struhl, G., 1999. Progression of the morphogenetic furrow in the *Drosophila* eye: the roles of Hedgehog. Decapentaplegic and the Raf pathway. Development 126, 5795–5808.

Halder, G., Callaerts, P., Gehring, W.J., 1995. Induction of ectopic eyes by targeted expression of the eyeless gene in *Drosophila*. Science 267, 1788–1792.

Halder, G., Callaerts, P., Flister, S., Walldorf, U., Kloter, U., Gehring, W.J., 1998. Eyeless initiates the expression of both sine oculis and eyes absent during *Drosophila* compound eye development. Development 125, 2181–2191.

Hazelett, D.J., Bourouis, M., Walldorf, U., Treisman, J.E., 1998. Decapentaplegic and wingless are regulated by eyes absent and eyegone and interact to direct the pattern of retinal differentiation in the eye disc. Development 125, 3741–3751.

Heitzler, P., Coulson, D., Saenz-Robles, M.T., Ashburner, M., Roote, J., Simpson, P., Gubb, D., 1993. Genetic and cytogenetic analysis of the 43A-E region containing the segment polarity gene costa and the cellular polarity genes prickle and spiny-legs in *Drosophila melanogaster*. Genetics 135, 105–115.

Hu, Birong, 1997. A genetic and molecular analysis of Drosophila melanogaster visual system development. Ph.d. thesis, University of California, Los Angeles, UMI publisher.

Jarman, A.P., Grell, E.H., Ackerman, L., Jan, L.Y., Jan, Y.N., 1994. Atonal is the proneural gene for *Drosophila* photoreceptors. Nature 369, 398–400.

Kawakami, K., Ohto, H., Ikeda, K., Roeder, R.G., 1996. Structure, function and expression of a murine homeobox protein AREC3, a homologue of *Drosophila* sine oculis gene product, and implication in development. Nucleic Acids Res. 24, 303–310.

Kenyon, K.L, Ranade, S.S., Curtiss, J., Mlodzik, M., Pignoni, F., 2003. Coordinating proliferation and tissue specification to promote regional identity in the Drosophila head. Dev. Cell. 5 (3), 403–414 (Sep.).

Kenyon, K.L., Li, D.J., Clouser, C., Tran, S., Pignoni, F., 2005. Fly SIX-type homeodomain proteins Sine oculis and Optix partner with different cofactors during eye development. Dev. Dyn. (electronic publication ahead of print).

Kumar, J.P., Jamal, T., Doetsch, A., Turner, F.R., Duffy, J.B., 2004. CREB binding protein functions during successive stages of eye development in Drosophila. Genetics 168 (2), 877–893 (Oct.).

Mardon, G., Solomon, N.M., Rubin, G.M., 1994. Dachshund encodes a nuclear protein required for normal eye and leg development in *Drosophila*. Development 120, 3473–3486.

Niimi, T., Seimiya, M., Kloter, U., Flister, S., Gehring, W.J., 1999. Direct regulatory interaction of the eyeless protein with an eye-specific enhancer in the sine oculis gene during eye induction in *Drosophila*. Development 126, 2253–2260.

Pappu, K.S., Mardon, G., 2004. Genetic control of retinal specification and determination in *Drosophila*. Int. J. Dev. Biol. 48, 913–924.

Pascal, E., Tjian, R., 1991. Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism. Genes Dev. 5, 1646–1656.

Pignoni, F., Hu, B., Zavitz, K.H., Xiao, J., Garrity, P.A., Zipursky, S.L., 1997. The eye-specification proteins SO and Eya form a complex and regulate multiple steps in *Drosophila* eye development. Cell 91, 881–891.

Quiring, R., Walldorf, U., Kloter, U., Gehring, W.J., 1994. Homology of the eyeless gene of *Drosophila* to the Small eye gene in mice and Aniridia in humans. Science 265, 785–789.

Robinow, S., White, K., 1991. Characterization and spatial distribution of the ELAV protein during *Drosophila melanogaster* development. J. Neurobiol. 22, 443–461.

Roederer, K., Loralyn, C., Anderson, J., Kumar, J., 2005. Novel dominant negative mutation within the six domain of the conserved eye specification gene sine oculis inhibits eye development in *Drosophila*. Dev. Dyn. 232, 753–766.

Seo, H.C., Curtiss, J., Mlodzik, M., Fjose, A., 1999. Six class homeobox genes in *Drosophila* belong to three distinct families and are involved in head development. Mech. Dev. 83, 127–139.

Seimiya, M., Gehring, W.G., 2000. The *Drosophila* homeobox gene optix is capable of inducing ectopic eyes by an eyeless-independent mechanism. Development 127, 1879–1886.

Serikaku, M.A., O'Tousa, J.E., 1994. Sine oculis is a homeobox gene required for *Drosophila* visual system development. Genetics 138, 1137–1150.

Silver, S.J., Rebay, I., 2005. Signaling circuitries in development: insights from the retinal determination gene network. Development 132, 3–13.

Silver, S.J., Davies, E.L., Doyon, L., Rebay, I., 2003. Functional dissection of eyes absent reveals new modes of regulation within the retinal determination gene network. Mol. Cell. Biol. 23, 5989–5999.

Spitz, F., Demignon, J., Porteu, A., Kahn, A., Concordet, J.P., Daegelen, D., Maire, P., 1998. Expression of myogenin during embryogenesis is controlled by Six/sine oculis homeoproteins through a conserved MEF3 binding site. Proc. Natl. Acad. Sci. 95, 14220–14225.

Staehling-Hampton, K., Jackson, P.D., Clark, M.J., Brand, A.H., Hoffmann, F.M., 1994. Specificity of bone morphogenetic protein-related factors: cell fate and gene expression changes in *Drosophila* embryos induced by decapentaplegic but not 60A. Cell Growth Differ. 5, 585–593.

Sun, Y., Jan, L.Y., Jan, Y.N., 1998. Transcriptional regulation of atonal during development of the *Drosophila* peripheral nervous system. Development 125, 3731–3740.

Treisman, J., Gonczy, P., Vashishtha, M., Harris, E., Desplan, C., 1989. A single amino acid can determine the DNA binding specificity of homeodomain proteins. Cell 59, 553–562.

Zhu, C.C., Dyer, M.A., Uchikawa, M., Kondoh, H., Lagutin, O.V., Oliver, G., 2002. Six3-mediated auto repression and eye development requires its interaction with members of the Groucho-related family of co-repressors. Development 129, 2835–2849.