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Novel Dominant-Negative Mutation Within the Six Domain of the Conserved Eye Specification Gene *sine oculis* Inhibits Eye Development in *Drosophila*

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Abstract

The development of the compound eye of *Drosophila* is controlled, in part, by the concerted actions of several nuclear proteins that form an intricate regulatory system. One member of this network is *sine oculis* (*so*), the founding member of the Six gene family. Mutations within *so* affect the entire visual system, including the compound eye. The vertebrate homologs Six3 and Six6 also appear to play crucial roles in retinal formation. Mutations in Six3 inhibit retinal formation in chickens and fish, whereas those in Six6 are the underlying cause of bilateral anophthalmia in humans. Together, these phenotypes suggest a conserved role for the Six genes in eye development. In this report, we describe the effects of a dominant-negative mutation of *sine oculis* on the development of the compound eye of *Drosophila*. The mutation resides within the Six domain and may have implications for eye development and disease.

Keywords

sine oculis; eye specification; retina; *Drosophila*

INTRODUCTION

The stereotyped development and simple cellular architecture of the *Drosophila melanogaster* compound eye makes the insect retina an excellent model system for studying the molecules and mechanisms that control the earliest steps in retinal development. Overt development of the compound eye begins at mid-embryo-genesis when a small group of cells invaginates from the surface ectoderm and develops into a monolayer epithelium called the eye imaginal disc (Cohen, 1993). As the embryo hatches and continues developing through the first two larval stages, the eye primordium proceeds to proliferate without undergoing differentiation. At the beginning of the third and last larval instar, pattern formation initiates at the posterior margin of the eye field and progresses toward the anterior border. The leading edge of the wave can be visualized by an indentation within the epithelium and is called the morphogenetic furrow (Ready et al., 1976). As this mobile patterning front sweeps across the eye imaginal disc, the once undifferentiated cells are organized into periodic clusters that will eventually give rise to the future ommatidia or unit eyes (Wolff and Ready, 1991). A stereotyped pattern of cellular recruitment takes place within each unit eye until all 8 photoreceptors and 12 accessory cells are specified and positioned correctly (Tomlinson and Ready, 1987a,b). This process involves choreographed morphogenetic movements and precise

regulation of gene expression (Heberlein and Moses, 1995; Kumar and Moses, 1997). Ultimately, the approximately 800 identical ommatidia are organized and packed into a hexagonal array so precise that it has been referred to as a neurocrystalline lattice (Ready et al., 1976; Ready, 1989; Wolff and Ready, 1993).

Despite many distinctions in morphology and different embryological origins, the eyes of mammals and insects such as *Drosophila* are specified by a conserved system of signal transduction cascades and transcription factors (Treisman, 1999; Wawersik and Maas, 2000; Hanson, 2001; Kumar, 2001; Zuber et al., 2003). The ensemble of transcription factors that specify the retina has been best studied in the fruit fly, and to date, eight nuclear proteins have been shown to regulate the earliest events in retinal determination (Bonini and Choi, 1995; Heberlein and Treisman, 2000; Kumar and Moses, 2001c). These proteins are collectively referred to as the “eye specification cascade” and include the Pax6 genes *eyeless* (*ey*) and *twin of eyeless* (*toy*), the Pax genes *eye-gone* (*eyg*) and *twin of eyegone* (*toe*), the Six family members *sine oculis* (*so*) and *optix*, and the founding members of the Eya and Dach gene families *dachshund* (*dac*) and *eyes absent* (*eya*; Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994; Quiring et al., 1994; Serikaku and O'Tousa, 1994; Jun et al., 1998; Czerny et al., 1999; Seimiya and Gehring, 2000; Aldaz et al., 2003). In cases where loss-of-function mutations exist, retinal development is completely inhibited, suggesting that the activity of each member is necessary for eye development to proceed. In addition, ectopic expression of each individual member (with the notable exception of *sine oculis*) has the ability to redirect other tissues into an eye fate (Halder et al., 1995; Bonini et al., 1997; Pignoni et al., 1997; Shen and Mardon, 1997; Czerny et al., 1999; Seimiya and Gehring, 2000; Jang et al., 2003).

These factors do not function in a simple linear or biochemical manner but rather are entwined in a tortuous labyrinth of regulatory interactions (Kumar, 2001; Kumar and Moses, 2001c). The genetic, molecular, and biochemical data collected so far suggest that the eight known eye specification genes function together, certainly within the same tissue but presumably within the same cells, to specify a retinal cell fate. In addition, molecular epistasis experiments have shown that the expression of each member of the eye specification cascade is dependent upon the activity of at least one other member of the pathway. However, the evidence also indicates that *twin of eyeless* and *eyeless* lie atop this hierarchical structure and together regulate a myriad of downstream genes (Gehring, 1996; Czerny et al., 1999; Gehring and Ikeo, 1999). One direct target is *sine oculis* which encodes a homeodomain protein and plays a crucial role in the development of the entire visual system, including the embryonic optic lobe, the larval photoreceptor organ known as Bolwig's organ and the adult ocelli and compound eyes (Halder et al., 1998; Niimi et al., 1999; Punzo et al., 2002).

The two *Drosophila* Six family members have been placed into this grouping by the dual presence of a homeobox and the Six protein-interaction domain. Multiple homologs of *sine oculis* and *optix* have been recovered in a wide variety of organisms, including the frog, nematode, chicken, zebrafish, mouse, and human (Kawakami et al., 2000; Hanson, 2001; Zuber et al., 2003). Currently, the Six superfamily is subdivided into the Six1 subclass, the Six3 subclass, and the Six4 subclass (Seo et al., 1999; Kawakami et al., 2000). Despite some early debates, *sine oculis* and *optix* have now been placed in the Six1 and Six 3 subclasses, respectively (Seimiya and Gehring, 2000). These assignments are based on sequence homologies, intron–exon positions and biochemical interactions with Eya family members.

Of interest, whereas both *Drosophila* members play roles in retinal determination, only the vertebrate members of the Six3 and Six4 subclass appear to function during early eye specification. For instance, Six3 and Six6 (also a member of the Six3 subclass) and Six4 are the only Six genes to be expressed within the developing mammalian retina (Oliver et al., 1995; Kawakami et al., 1996; Niiya et al., 1998; Jean et al., 1999; Leppert et al., 1999; Loosli

et al., 1999). In addition, ectopic expression of mouse Six3 is capable of directing tissues into a retinal fate (Oliver et al., 1996; Lagutin et al., 2001). And while mutations have been recovered in several mammalian *sine oculis* homologs, only mutations within human Six6, which are the underlying cause of bilateral anophthalmia, results in early eye specification defects (Gallardo et al., 1999; Tomarev, 1999; Gallardo et al., 2004). The roles for members of the Six3 subclass in eye development are apparently conserved as Six3/Six6 orthologs are also expressed within the retinas of chicken, frogs, and zebrafish (Bovolenta et al., 1996; Seo et al., 1998; Kawakami et al., 2000; Zhou et al., 2000; Ghanbari et al., 2001). It should be noted, however, that other Six genes function during later stages of retinal development as mice that are deficient for Six5 manifest ocular cataracts among other abnormalities (Klesert et al., 2000; Sarkar et al., 2000).

The Six gene family also plays pivotal roles in a wider range of developmental processes than just retinal development. Of particular interest is the role that this gene family plays in head morphogenesis due to the demonstration that mutations within human Six3 lead to holoprosencephaly (Wallis and Muenke, 1999, 2000; Wallis et al., 1999; Pasquier et al., 2000). Both *sine oculis* and *optix* genes are expressed within the developing embryonic head as are all four Six genes in the nematode *Caenorhabditis elegans* (Dozier et al., 2001; Kumar and Moses, 2001b). Other Six gene family members are involved in patterning muscle tissue, the otic vesicle, the mammalian kidney, cranial placodes, branchial arches, and somites (Boucher et al., 2000; Laclef et al., 2003a,b; Xu et al., 2003; Zheng et al., 2003; Ozaki et al., 2004; Ruf et al., 2004). Together, these expression patterns and phenotypes suggest a crucial role for the Six genes in early patterning decisions.

Mutations within *sine oculis* were first recovered in 1939, but the gene was not formally named until 1946. The original allele (*so¹*) was characterized by an absence of the ocelli, which are three simple eyes located at the vertex of the head, and a dramatic reduction in the size of the compound eyes (Cheyette et al., 1994; Serikaku and O'Tousa, 1994). It was subsequently shown that a deletion of an eye-specific enhancer element is the underlying cause of the eyeless phenotype of *so¹* mutants (Halder et al., 1998; Niimi et al., 1999; Punzo et al., 2002). Null alleles of *sine oculis* (*so³*) have also been identified and are known to result in embryonic lethality. Examination of null mutant embryos identified additional abnormalities in the development of the optic lobes and Bolwig's organ (Cheyette et al., 1994; Serikaku and O'Tousa, 1994). Additionally, a dominant allele of *sine oculis* (*so^D*) was isolated. Although heterozygous *so^D/+* flies are eyeless, the external appearance of the fly head is significantly different from that of adults homozygous for the eye-specific *so¹* loss-of-function allele. We have shown previously that the underlying lesion in *so^D* is a novel valine to aspartic acid substitution within a highly conserved portion of the Six domain (Kumar et al., 2004). As all other known molecular lesions within Six family members are loss-of-function mutations, the *so^D* allele is unique and could be particularly relevant for human eye development and disease, especially because dominant-negative versions of Pax6 and Gli2 are known to adversely affect retinal development in zebrafish and mammals. Also, while traditionally holoprosencephaly has been associated with mutations within the homeodomain of Six3, a newly discovered mutation within the Six domain also leads to the disease, making an understanding of the Six domain all the more important (Pasquier et al., 2000).

In this report, we characterize the retinal phenotypes of the *so^D* dominant-negative mutation and contrast it with that of the *so¹* eye-specific allele. In particular, we demonstrate that the inhibitory effects of *so^D* upon the visual system of *Drosophila* occur through a mechanism that is distinct from loss-of-function alleles. *so^D* does not appear to affect eye specification itself or the establishment of the dorsoventral (D/V) compartment boundaries. The primary defect appears to be a block in the initiation of the morphogenetic furrow at the posterior margin of the eye field. We searched for genes that interact with *sine oculis* by isolating mutants that

modified the no-eye phenotype of the *so^D* dominant-negative mutant. The results from our genetic screen indicate that *sine oculis* lies genetically upstream of the Hedgehog (Hh), Decapentaplegic (Dpp), and Wingless (Wg) signaling cascades, all of which are known to be involved in the regulation of the morphogenetic furrow.

RESULTS

Eye Development Is Restored in *so^D* Retinas by Addition of *sine oculis*

Loss-of-function mutations within the *Drosophila* Six family member *sine oculis* lead to a complete block in retinal development (Cheyette et al., 1994; Serikaku and O'Tousa, 1994; Fig. 1A,B,F,G). These mutants are of particular interest, because mutations within the mammalian homologs of *sine oculis* also inhibit human eye formation (Gallardo et al., 1999, 2004). Of interest, a dominant mutation within *sine oculis* (*so^D*) exists in which eye formation is also halted (Fig. 1C,H). However, the adult phenotype of *so^D* is surprisingly different from that of traditional loss-of-function mutants. The adult heads of *so^D* flies contain a nonpigmented retina-like structure, whereas flies harboring the *so^L* loss-of-function contain head cuticle within the regions where the eye would normally be located. Using a variety of molecular markers, we attempted to determine the identity of the retina-like structure within the *so^D* adult head. Our analysis indicates that these cells within the *so^D* adult are neither typical retinal cells (i.e., photoreceptors, cone, and pigment cells) nor are they of the same fate as the surrounding head regions (data not shown). Thus, these cells might represent an interesting intermediate cell type in which specification initiates but terminal differentiation is not completed.

We have shown previously that the molecular lesion responsible for the no-eye phenotype resides within the Six domain, a highly conserved portion of the SO protein that interacts with the EYA protein (Kumar et al., 2004). The formation of this heterodimeric protein complex (SO-EYA) is a key step in the progression of eye specification (Pignoni et al., 1997). We used the UAS/GAL4 expression system to introduce additional copies of each of the eye specification genes (twin of eyeless, eyeless, eye gone, twin of eyegone, eyes absent, dachshund, and *sine oculis*) into *so^D* retinas by means of the *eyeless* and *dpp^{blk}* enhancer elements. The *eyeless* enhancer directs expression ahead of the advancing morphogenetic furrow, whereas the *dpp^{blk}* enhancer is restricted to the posterior-lateral margins of the eye field (Blackman et al., 1991; Hauck et al., 1999). With either enhancer, the addition of *sine oculis* appears to be the only condition that resulted in a rescue of the no-eye phenotype (Fig. 1D,E,I,J). This result suggests that *sine oculis* may have a role in the initiation of the morphogenetic furrow in addition to its role in eye specification because expression at the eye field margins is sufficient to restore eye development. Furthermore, the inability of other eye specification genes to rescue the no-eye phenotype suggests that the effect of *so^D* is independent of the other retinal determination genes.

Eye Specification Is Not Affected in *so^D* Retinas

Prior reports have described the expression patterns of several eye specification genes within *so^L* retinas and have suggested that eye development is inhibited, in part, by a decrease in the transcription of these genes (Halder et al., 1998; Curtiss and Mlodzik, 2000). To determine whether the molecular mechanism of eye inhibition in *so^D* retinas is similar or different from that of the *so^L* loss-of-function mutant, we looked at the expression patterns of several retinal determination genes, including *eyeless*, *sine oculis*, *eyes absent*, and *dachshund* within *so^D* eyes. A wealth of genetic and molecular evidence places *eyeless* atop the eye specification cascade. In normal retinas, EY protein is restricted to regions anterior to the morphogenetic furrow where it directly regulates *sine oculis* (Fig. 2A). Like *so^L* loss-of-function mutants, *so^D* eye discs have normal levels of EY protein. As a consequence of the furrow not initiating

at the posterior margin, EY protein is found in all cells of the retinal epithelium (Fig. 2A,E). These results indicate that *eyeless* expression is independent of *sine oculis*.

We next looked at the expression pattern of *sine oculis* itself using a so-lacZ reporter construct. In wild-type retinas, so-lacZ is detected in all cells ahead and behind the morphogenetic furrow (Fig. 2B). In *so^l* mutant eyes, *sine oculis* expression is completely lost within the retina due to the deletion of an eye-specific enhancer (Niimi et al., 1999). However, in *so^D* eye discs, *sine oculis* transcription appears to be at near normal levels (Fig. 2F). This finding is a clear suggestion that different developmental mechanisms are responsible for the no-eye phenotypes in the two mutants of *sine oculis*.

A further distinction concerns the expression of *eyes absent* in *so^l* and *so^D* retinas. The conventional wisdom is that SO and EYA proteins are obligate binding partners that regulate the transcription of target genes. This theory is based on (1) the in vitro binding of SO to EYA, (2) the synergistic activity of these proteins in producing ectopic eyes, and (3) the near identical expression profile of both genes in the developing wild-type eyes (Fig. 2B,C). Interestingly, in *so^l* loss-of-function mutants, EYA protein levels are dramatically reduced, suggesting that EYA protein is not only a binding partner but that the *eyes absent* gene itself is a transcriptional target of *sine oculis* (Halder et al., 1998). But in *so^D* mutant retinas EYA protein levels appear unaffected, further suggesting that the loss of retinal tissue is not due to a reduction in the levels of eye specification protein levels. It is known from studies on Six family proteins in both flies and vertebrates that Six proteins can bind to members of the EYA and GROCHO families (Pignoni et al., 1997; Zhu et al., 2002; Lopez-Rios et al., 2003). An SO-EYA complex is usually associated with the promoting of transcription, whereas an SO-GRO complex is thought to inhibit transcription of target genes. One possible explanation for how SO-D protein inhibits eye development is that, although the ability to regulate the transcription of *eyes absent* is unaffected, the mutant protein may now have a stronger affinity for binding to GRO repressor proteins. Thus, such a situation would allow for normal transcriptional levels of *eyes absent*, while still inhibiting eye development.

The eye specification gene *dachshund* lies downstream of *sine oculis* and *eyes absent*. During normal development DAC protein is present in all cells ahead of the morphogenetic furrow and in subsets of cells within the developing unit eyes (Mardon et al., 1994). Expression of *dachshund* gradually expires after approximately 10 columns of ommatidia have developed (Fig. 2D). In *so^l* mutant discs DAC protein levels are dramatically reduced, suggesting that *sine oculis* regulates *dachshund* (J. Kumar, unpublished results). However, in *so^D* retinas, DAC protein appears to be at or near normal levels (Fig. 2H).

We conclude that the no-eye phenotype of *so^D* is not due to an alteration in the levels of eye specification gene expression, because all of the genes we examined were expressed at near normal levels in *so^D* mutant retinas. This finding is unique among all other known mutations in *sine oculis*. In addition, we generated homozygous (*so^D/so^D*) retinal mosaic clones and observed that the expression of the eye specification genes is still normal within the clone during development and within the adult the region of the clone was transformed into head cuticle (data not shown). This finding further suggests that the no-eye phenotype is not due to a loss of the eye specification genes.

D/V Patterning Is Not Affected in *so^D* Retinas

Because the primary defect in *so^D* retinas does not appear to involve the specification of the retina, we then looked to see if the developing eye field is correctly partitioned into dorsal and ventral compartments, which is a key step in eye formation. Failure to correctly split the eye into dorsal and ventral halves can lead to a block in eye development as is the case in mutants of *fringe* (*fng*), *mirror* (*mirr*) and *Lobe* (*L*; McNeill et al., 1997; Cho and Choi, 1998;

Cavodeassi et al., 1999; Chern and Choi, 2002). During normal development all cells within the eye imaginal disc are initially of the ventral fate (Singh and Choi, 2003). Subsequently, the Iroquois complex, which includes *mirror*, establishes the dorsal compartment (Cavodeassi et al., 1999). At the equator, the dorsal compartment expression of *mirror* is abutted by the expression of *fringe* in the ventral compartment (Yang et al., 1999). Localized activity of the Notch signaling pathway maintains the boundary between compartments (Dominguez and de Celis, 1998). We used a *mirr-lacZ* reporter, which is expressed exclusively in the dorsal half of the retina and faithfully reflects the endogenous expression of *mirror*, to assay D/V patterning in *so^D* mutant retinas. Despite blocked eye development, *so^D* retinas still maintain *mirr-lacZ* expression solely within the dorsal compartment, suggesting that the retina is correctly partitioned (Fig. 3A,B). Other markers for ventral fate such as *fringe* were also normally expressed (data not shown), further suggesting that the no-eye phenotype is not due to a loss in D/V patterning.

Expression of *hedgehog*, *decapentaplegic*, and *wingless* in *so^D* Retinas

A third common cause for a block in eye formation is the failure in the initiation of pattern formation. The morphogenetic furrow initiates at the posterior margin of the eye imaginal disc and proceeds toward the anterior edge (Ready et al., 1976). Several signaling pathways, including the *Epidermal Growth Factor Receptor* (*Egfr*), *Notch* (*N*), *hedgehog* (*hh*), *decapentaplegic* (*dpp*), and *wingless* (*wg*), are known to influence the progression of the furrow (Heberlein et al., 1993; Ma et al., 1993; Heberlein and Moses, 1995; Heberlein et al., 1995; Ma and Moses, 1995; Treisman and Rubin, 1995; Dominguez and Hafen, 1997; Borod and Heberlein, 1998; Greenwood and Struhl, 1999; Kumar and Moses, 2001a). For example, mutations in either *hedgehog* or *decapentaplegic* can block the initiation of the furrow, whereas the loss-of-function *wingless* alleles show precocious retinal development. The expression of each of these genes was monitored using *lacZ* reporter constructs that recapitulate endogenous expression patterns. In normal development the *dpp-lacZ* product is first localized to the posterior edge of the eye field (Heberlein et al., 1993). As the furrow proceeds toward the anterior edge, cells within and ahead of the furrow will express the reporter (Fig. 4A). The propagation of the morphogenetic furrow across the eye disc depends upon sufficient levels of HH protein begin made by developing photoreceptors posterior to the furrow (Fig. 4B; Ma et al., 1993). Cells within and ahead of the furrow will receive the signal and begin to differentiate into photoreceptor cells. In contrast, *wg-lacZ* expression is seen at low levels just ahead of the furrow at the dorsal-lateral margin (Fig. 4C). The role of *wingless* appears to be to prevent pattern formation from initiating at the dorsal margin (Ma and Moses, 1995; Treisman and Rubin, 1995).

In *so^D* retinas, both *dpp-lacZ* and *hh-lacZ* expression is restricted to the posterior margin, suggesting that the morphogenetic furrow fails to initiate, resulting in a block in pattern formation (Fig. 4D,E). Because the reporters appear to be expressed at fairly high levels the block in pattern formation is not due to direct down-regulation of *decapentaplegic* and *hedgehog* transcription by *sine oculis*. It is more likely that the amino acid change within the Six domain alters the ability of SO to interact correctly with its binding partner or could possibly recruit novel partners that inhibit eye development by interfering with Hh and Dpp signaling. We also examined the expression of the *wg-lacZ* reporter and find that, although the expression pattern of *wingless* is not significantly altered, the levels of *wingless* transcription appear to be higher in *so^D* discs (Fig. 4F). The lack of effect on eye specification and D/V patterning suggests that the primary cause of the no-eye phenotype in *so^D* retinas is a block in the initiation of the morphogenetic furrow at the posterior margin of the presumptive eye field. Furthermore, our results suggest that *sine oculis* regulates Hedgehog and Decapentaplegic signaling.

Identification of Potential Targets and Binding Partners of *sine oculis*

To confirm and extend the molecular epistasis data described above, we conducted a genetic screen (see Experimental Procedures section) to isolate genes that potentially interact with the *so^D* mutant allele by screening for suppression of the no-eye phenotype (Fig. 5). By using the *so^D* dominant-negative allele, we expected to recover loss-of-function mutations in either downstream target genes or in loci that encode for binding partners. In addition, we expected that all of the interacting genes would encode for negative regulators of eye development. We determined that there were 14 regions of the *Drosophila* genome harboring genes that potentially interacted with *sine oculis* (Fig. 5; Table 1). After testing all available mutations that are uncovered by these deletions, we identified seven genes as potential candidates for interacting with *sine oculis* (Fig. 5; Table 1).

so^D eye imaginal discs are characterized by a complete absence of photoreceptor development (Fig. 6E). However, because eye specification gene expression and D/V patterning appear to be normal, the eye disc itself does not degenerate, and in the adult, a clear nonpigmented and nondifferentiated field is present (Fig. 6A). This finding is different than *so¹* loss-of-function mutants in which the eye field is replaced with head cuticle. Both adult and disc phenotypes are 100% penetrant; thus, any deviation from this can be reliably classified as suppression.

The level of suppression by the deletion chromosomes can be classified into three broad categories. These classes are based on the relative number of ommatidia that are formed and the organization of the retina. In all cases, the morphogenetic furrow will initiate from the posterior margin and will express the appropriate genes such as *decapentaplegic* (data not shown). Class 1 suppressors are the mildest and have a very slight roughening of the clear cuticle field and contain several short mechanosensory bristles (Fig. 6B). The entire retinal field can be seen as pigmented under the light microscope (data not shown), suggesting that retinal development has been partially restored. Developing imaginal discs show several columns of photoreceptor clusters at the posterior edge of the eye field, with each cluster containing two to three cells (Fig. 6F). Class 2 suppressors are moderate in degree and show a much more developed, albeit unorganized, adult retina with several partially formed ommatidia being visible (Fig. 6C). Within the developing retina several more rows of ommatidia are present. Additionally, many clusters have the full complement of 8 photoreceptor neurons (Fig. 6G). Finally, the class 3 suppressors are the strongest and show 30–50 completely developed ommatidia along with mechanosensory bristles (Fig. 6D,H). This level of suppression is equivalent to expressing a single exogenous copy of a wild-type *sine oculis* gene (Fig. 1D,E). It is interesting to note that suppression is not complete even with class 3 suppressors that have a normal looking furrow. A likely explanation for this is that the mutant SO-D protein may be present at high enough levels to block furrow progression even though the furrow is able to initiate.

sine oculis Lies Upstream of Several Negative Regulators of Eye Development

From our genetic screen, we expected to recover downstream target genes of *sine oculis* that are also negative regulators of eye development. Of the seven genes that we were able to identify, five (*patched*, *wingless*, *teashirt*, *homothorax*, and *groucho*) play roles in regulating eye development by either delimiting the eye field itself or by inhibiting the initiation of the morphogenetic furrow or both (Table 1). We tested several alleles of each gene, and in all cases, the results were consistent.

wingless inhibits eye development (1) by negatively regulating the expression of eye specification genes at the boundary between the eye and head structures and (2) by blocking inappropriate initiation of morphogenetic furrows from the dorsal and ventral margins of the eye field (Ma and Moses, 1995; Treisman and Rubin, 1995; Heberlein et al., 1998; Baonza and

Freeman, 2002). Loss of *wingless* within the eye field leads to an up-regulation of several eye specification genes and the initiation of ectopic morphogenetic furrows from the dorsal and ventral margins.

The inhibition of retinal development by *wingless* ahead of the morphogenetic furrow is mediated by the *teashirt* (*tsh*) and *homothorax* (*hth*) genes, which encode for zinc-finger transcription factor and a homeodomain-containing protein, respectively. Together with EY, TSH and HTH form a multiprotein complex that represses the expression of several eye specification genes, including *eyes absent* and *dachshund* (Pichaud and Casares, 2000; Bessa et al., 2002; Singh et al., 2002, 2004). The repression of eye development is restricted to zones of the eye in which all three genes are expressed (Bessa et al., 2002). In regions where only *eyeless* and *teashirt* are expressed, eye development is promoted. This conclusion is supported by the formation of ectopic eyes as a response to the overexpression of *teashirt* in some domains of the eye-antennal imaginal disc (Pan and Rubin, 1998).

During normal development *hedgehog*, like *wingless*, appears to play roles in the specification of the eye and the initiation of the morphogenetic furrow. Loss of *hedgehog* expression blocks the initiation of the morphogenetic furrow, whereas the formation of ectopic eyes is highly dependent upon *hedgehog* expression (Heberlein et al., 1993, 1995; Ma et al., 1993; Dominguez and Hafen, 1997; Borod and Heberlein, 1998; Greenwood and Struhl, 1999; Curtiss and Mlodzik, 2000). In contrast to *hedgehog*, *patched* negatively regulates eye development (Ma and Moses, 1995). PTC is a transmembrane protein that blocks the activity of the Hedgehog signaling pathway in the absence of the HH protein (Hooper and Scott, 1989; Hahn et al., 1999). Removal of PTC ahead of the morphogenetic furrow leads to an up-regulation of eye specification gene transcription and the generation of ectopic furrows in the anterior regions of the retina (Ma and Moses, 1995).

Of the five known negative regulators of eye development that we identified, *groucho* is the only gene that may encode a potential binding partner for SO. In both *Drosophila* and mammalian systems GRO proteins function as corepressors of transcription (Fisher and Caudy, 1998; Chen and Courey, 2000; Li, 2000). Furthermore, in vertebrate eye development, both Six3 and Six6 (*sine oculis* homologs) are known to form heterodimeric complexes with GRO proteins (Kobayashi et al., 2001; Zhu et al., 2002; Lopez-Rios et al., 2003). Due to the high levels of conservation among molecules and mechanisms between retinal systems, it is likely that SO and GRO also interact during fly eye specification. One possibility is that an SO-GRO complex functions to down-regulate genes such as *wingless*, *patched*, *teashirt*, and *homothorax*.

***patched* and *wingless* Function Together to Regulate Eye Development**

In our screen, we identified both *patched* and *wingless* as weak (class 1) dose-dependent suppressors of *so^D* (Fig. 7A,B,D,E). Loss-of-function of several downstream components of *wingless* signaling such as *sloppy paired* (*slp*) and *frizzled* (*fz*) also suppresses the *so^D* retinal phenotype (data not shown). We were interested in determining if the repressive activities of *patched* and *wingless* are coordinated in normal development. We simultaneously removed both *patched* and *wingless* in a *so^D* mutant background and assayed the degree of suppression. If each gene works independently, then removing both genes would at best be expected to yield an intermediate (class 2) suppression phenotype (see Fig. 6C,G). However, a relatively strong (class 3) suppression phenotype (as assayed by the well-formed ommatidia in the adult eye) was observed, indicating a synergistic effect of simultaneous reduction of *patched* and *wingless* (Fig. 7C,F, compare with Fig. 6D,H). We also tried to rule out a simple additive effect by combining the other class 1 suppressors (*hth*, *tsh*, *gro*, *lid*, *ifc*) with either *patched* or *wingless* and did not observe the well-formed ommatidia of the adult. We only observed a phenotype similar to that shown for class 2 suppressors in Figure 6C (data not shown). This

finding suggests that one role for *sine oculis* in normal development might be to repress the coordinated activities of *patched* and *wingless*.

Expression of *hedgehog* Is Sufficient to Completely Suppress *so^D*

Our results suggest that, in normal development, *sine oculis* represses the activity of several negative regulators, including *patched* and *wingless*, consequently allowing both eye specification and furrow initiation to proceed normally. We were interested in determining if an increase in Hedgehog or TGF β signaling would be sufficient to overcome the no-eye phenotype. We used a dpp-GAL4 driver to express either *hedgehog* or the two known TGF β genes *decapentaplegic* and *glass bottom boat* (*gbb*) along the posterior margin of *so^D* eye discs. Amazingly, expression of *hedgehog* was sufficient to restore the *so^D* phenotype to wild-type (Fig. 8A,B). In contrast, expression of either TGF β ligand provided only a nominal degree of rescue (Fig. 8C,D). We also attempted to rescue the no-eye phenotype of *so^D* by expressing components of the Egr and Notch signaling cascades (which are known also to regulate movement of the morphogenetic furrow), but none of these conditions resulted in substantial rescue (data not shown). These results are in concordance with our genetic screen data that suggest that one of the major roles played by *sine oculis* is in the initiation of the morphogenetic furrow through the positive regulation of the Hedgehog signaling pathway. It should be noted that the expression levels of hh-lacZ are normal in the *so^D* eye disc. A potential explanation is that the mutant SO-D protein and the Hedgehog signaling pathway compete for a common target with Hedgehog signaling promoting its transcription, whereas the SO-D protein acts as an inhibitor. The addition of exogenous HH protein might be expected to then tip the balance and promote eye development.

Unmapped Suppressor Mutants

We are currently attempting to identify the remaining unmapped interacting genes by conducting additional genetic screens. In all cases, there is substantial restoration of photoreceptor development; thus, these are likely to represent additional interacting genes (Fig. 9). We also predict that the identified genes will encode for negative regulators of eye development.

DISCUSSION

In this report, we have described eye development in flies harboring a dominant-negative mutation within *sine oculis*, a member of the Six gene superfamily and a component of the *Drosophila* eye specification network. Six family members positively influence retinal development in several systems, including the mammalian retina (Cheyette et al., 1994; Serikaku and O'Tousa, 1994; Oliver et al., 1995, 1996; Seo et al., 1998; Jean et al., 1999; Leppert et al., 1999; Loosli et al., 1999; Lagutin et al., 2001; Zhu et al., 2002). Loss-of-function mutations within the human *sine oculis* homolog Six6 are the underlying cause of bilateral anophthalmia (Gallardo et al., 1999, 2004). Mutations within another human homolog Six3 lead to holoprosencephaly, suggesting that Six genes have broad developmental roles (Wallis et al., 1999; Pasquier et al., 2000). In *Drosophila*, mutations within *sine oculis* lead to defects in the development of the optic lobes, visual primordium, Bolwig's organ in addition to inhibiting retinal formation (Cheyette et al., 1994; Serikaku and O'Tousa, 1994).

The *so^D* allele harbors a unique amino acid change (V200D) within the Six protein interaction domain that confers dominant-negative activity (Kumar et al., 2004). As molecular lesions are now known to cause holoprosencephaly in humans, an understanding of the Six domain is all the more relevant (Pasquier et al., 2000). Dominant-negative mutations in vertebrate *Pax6* and *Gli2* are known to negatively regulate eye development in mammals and zebrafish (Singh et al., 1998; Duncan et al., 2000; Kondoh et al., 2000). It is likely that understanding the

mechanisms by which *so^D* inhibits retinal development may have implications for human retinal disorders caused by lesions in Six family members.

We had initially expected the no-eye phenotype of *so^D* to result from inhibition of eye specification or D/V patterning, two processes that are crucial to eye formation. However, our results indicate that neither eye specification nor D/V patterning is adversely affected in *so^D*. Rather, the primary defect appears to be a block in the inhibition of the morphogenetic furrow at the posterior margin of the presumptive eye field. In normal development *hedgehog*, *decapentaplegic*, and *wingless* control the initiation and progress of the furrow across the eye disc (Heberlein et al., 1993, 1995; Ma et al., 1993; Ma and Moses, 1995; Treisman and Rubin, 1995; Dominguez and Hafen, 1997; Borod and Heberlein, 1998; Greenwood and Struhl, 1999; Curtiss and Mlodzik, 2000). Mutations within these genes either block movement of the furrow, as is the case for *hedgehog* and *decapentaplegic*, or promote precocious eye development in the case of *wingless*. In *so^D* mutants, the expression of these genes is not abolished as might be expected. Most notably *hedgehog* and *decapentaplegic* expression is restricted to the posterior of the eye disc as a result of the failure of the morphogenetic furrow to initiate.

In addition, we conducted a genetic screen for genes that interact with *sine oculis*. The screen was designed to look for suppressors of the *so^D* noeye phenotype. Due to the dominant-negative activity of *so^D*, we expected (and identified) genes that are likely to be downstream targets of *sine oculis* that are negative regulators of eye development. Of the seven identified complementation groups, five are known negative regulators (*wingless*, *patched*, *teashirt*, *homothorax*, and *groucho*).

wingless and *patched* have been shown to affect both eye specification and morphogenetic furrow initiation (Ma and Moses, 1995; Treisman and Rubin, 1995; Baonza and Freeman, 2002). A simple idea is that during wild-type eye development, *sine oculis* inhibits both *wingless* and *patched* within and posterior to the furrow, thereby allowing retinal development to occur as the furrow moves forward (Fig. 10). Alternatively, because Wingless signaling is antagonized by the Hedgehog and Decapentaplegic pathways in many developmental contexts, it is also possible that the effects on *wingless* may also be mediated indirectly by the effects of *sine oculis* on both *patched* and *hedgehog*.

Just ahead of the furrow the expression of *teashirt* and *homothorax* overlaps with that of *eyeless*. It has been suggested that an EY-TSH-HTH complex ahead of the furrow also blocks precocious eye development, in part, by down-regulating the expression of several retinal determination genes (Bessa et al., 2002). Another potential role of *sine oculis* might be to repress the expression of *teashirt* and *homothorax* within the furrow, again allowing the furrow to proceed across the presumptive eye field (Fig. 10).

How is this repression accomplished given that SO protein can bind to DNA but appears to be insufficient to regulate transcription of target genes? The answer may lie in the observation that, in our genetic screen, we uncovered mutations in *groucho*, a known transcriptional corepressor (Fisher and Caudy, 1998; Chen and Courey, 2000). Interestingly, several mammalian GRO proteins are known to form heterodimeric complexes with Six proteins and repress eye development (Li, 2000; Kobayashi et al., 2001; Lopez-Rios et al., 2003). It is a reasonable possibility that a GRO-SO complex down-regulates the expression of *wingless*, *patched*, *teashirt*, and *homo-thorax* (Fig. 10).

The data presented here suggest that *sine oculis* is a focal point for regulating retinal development and is likely to alleviate the repressive effects of at least three inhibitory pathways. We are currently attempting to identify seven other potential targets of *sine oculis*. Each of these is predicted also to be a negative regulator of eye development. It will be interesting to

see if these genes are part of extant inhibitory pathways or if they represent novel mechanisms for delimiting the development of the retina both temporally and spatially. It will be of interest also to determine whether the interplay between *sine oculis* and these inhibitory mechanisms is conserved in the mammalian retina.

EXPERIMENTAL PROCEDURES

Fly Stocks

The following fly stocks were used in this study: *so*¹, *so*^D/CyO, *UAS-dpp*, *UAS-lacZ*, *UAS-GFP*, and Deficiency Kits for chromosomes X,2,3,4 (Bloomington Stock Center); *so*³/CyO (gift of Larry Zipursky); *ey-GAL4*, *UAS-toy*, *UAS-ey*, and *UAS-optix* (gifts of Walter Gehring); *dpp-GAL4* (gift of Janice Fischer); *UAS-eya* (gift of Nancy Bonini); *UAS-so* and *UAS-dac* (gifts of Graeme Mardon); *UAS-eyg* and *UAS-toe* (gifts of Claude Desplan); *UAS-hh* and *hh-lacZ* (gifts of Kevin Moses); *UAS-gbb* (gift of Kristi Wharton); *UAS-ci* (gift of Robert Holgrem); *mirr-lacZ* (gift of Henry Sun). All crosses were conducted at 25°C.

Genetic Screen

We crossed the 235 stocks that constituted the Bloomington Deficiency Kit, which provides nearly 90% coverage of the genome, to *so*^D/CyO flies and assayed the ability of each deficiency to modify the no-eye phenotype of *so*^D/+ heterozygotes. Several deficiencies scored positive in our assay by suppressing the *so*^D/+ retinal phenotype. We mapped the suppressing activities by crossing all available single gene mutations that were uncovered by the seven deficiencies to *so*^D/+ and scored the ability of each single gene mutation to mimic that of the overlying deficiency. We identified seven complementation groups that suppressed (this report) the *so*^D/+ retinal phenotype. We tested all available mutations that were uncovered by these deletions but were unable to refine the maps for an additional seven regions of the genome. There are two possible explanations for these results. First, because mutations do not exist for all predicted genes within these deletions we may not have been able to test the correct mutations. Second, the suppressing phenotype may be due to some other mutation within the deficiency strain. These regions are listed in Table 1 but are not considered further here.

Antibodies, Immunohistochemistry, and Microscopy

The following antibodies were used in this study: mouse anti-22C10 (1:10, gift of Seymour Benzer), mouse anti-ELAV (1:100, gift of Gerald Rubin), mouse anti-EYA (1:5, gift of Seymour Benzer), mouse anti-DAC (1:5, gift of Gerald Rubin), rabbit anti-β-galactosidase (1:100, Cortex Biochemical). The following secondary antibodies were obtained from Jackson Laboratories: goat anti-mouse fluorescein isothiocyanate (1:100) and goat anti-mouse horseradish peroxidase (1:100). F-actin was visualized using phalloidin-tetrarhodamine isothiocyanate (1:500; Molecular Probes). Dissection and treatment of imaginal discs and preparation of adult eyes for scanning electron microscopy were as previously described in (Kumar et al., 1998). Adult eyes were sectioned for light microscopy as described in Kumar et al. (2003).

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Fig. 1.

A dominant-negative mutation of *sine oculis* deletes the eye. **A–E:** Scanning electron photomicrographs of adult eyes. **F–J:** Confocal images of developing eye imaginal discs. Red, F-actin; green, Elav. A,F: Wild-type. B,G: *so^l*, the arrow notes that the compound eye is replaced with head cuticle. C,H: *so^D*, the arrow indicates that an undifferentiated field remains. D,I: *so^D/+; ey-GAL4/UAS-so*, the arrow indicates developing photoreceptor cells. E,J: *so^D/+; dpp-GAL4/UAS-so*. Anterior is to the right. Arrow indicates developing photoreceptor cells.

Fig. 2. Expression of eye specification genes in *so^D/+* retina. A–H: Confocal images of developing eye imaginal discs. **A–D:** Wild-type (wt). **E–H:** *so^D/+*. Note that the levels of each eye specification gene are at near normal levels. However, initiation of the morphogenetic furrow is blocked. Visualized molecules are listed above each column. Arrows in C,D,G,H indicate the position of the ocelli. Anterior is to the right.

Fig. 3. Normal dorsoventral patterning in *so^D/+* retinas. Confocal images of developing eye imaginal discs. **A:** Wild-type (wt). **B:** *so^D/+*. In both wild-type and *so^D/+* discs, the mirr-lacZ reporter is expressed in the dorsal compartment, suggesting that dorsoventral patterning is normal in *so^D/+* retinas. Anterior is to the right.

Fig. 4.

Dpp, Hh, and Wg expression in *so^D/+* retinas. A–F: Confocal images of developing eye imaginal discs. A–C: Wild-type (wt). D–F: *so^D/+*. **A:** The dpp-lacZ reporter leaves the posterior edge and moves with the morphogenetic furrow in wild-type eye discs. **B:** The hh-lacZ reporter is expressed in all developing photoreceptor cells posterior to the furrow in wild-type. **C:** The wg-lacZ reporter is expressed at the anterior–dorsal margin ahead of the morphogenetic furrow (arrow) and within the antennal disc (arrowhead). **D:** In *so^D/+* discs, the dpp-lacZ reporter is restricted to the posterior margin (arrow). **E:** In *so^D/+* discs, the hh-lacZ reporter is also relegated to the posterior margin (arrow). **F:** The pattern of wg-lacZ expression is not significantly altered in *so^D/+* discs (arrow and arrowhead). However, the levels of expression are slightly elevated. Anterior is to the right.

Fig. 5.

A genetic screen for modifiers of the *so^{D/+}* no-eye phenotype. The initial screen involved crossing the “deficiency kit” of 235 deletion stocks to *so[D]/+* mutants and scoring for the level of suppression in the F1 generation. Next, single-gene mutants that are uncovered by the overlying deficiency were tested for their ability to suppress *so^{D/+}* to the same degree as the deletion stock.

Fig. 6. Retinal phenotypes of $Su(so^D)$ suppressors. **A–D:** Scanning electron photomicrographs of adult eyes. **E–H:** Confocal images of developing eye imaginal discs. Red = F-actin, green = ELAV. **A,E:** $so^D/+$. Note that $so^D/+$ retinas are devoid of ommatidia. **B,F:** $so^D/class\ 1$ suppressor. Note the slight roughening of the adult field (arrow in B) and the rows of ommatidia (arrowhead in F). **C,G:** $so^D/class\ 2$. Note the moderate roughening of the adult eye field and the partial formation of ommatidia (arrow in C). Also note the increasing number of photoreceptor clusters (arrowhead in G). **D,H:** $so^D/class\ 3$. **D,H:** Between 30 and 100 fully formed ommatidia are formed (D), and a normal morphogenetic furrow is seen in developing eye imaginal discs (H). Anterior is to the right.

Fig. 7.

so lies upstream of both Hedgehog and Wingless signaling. **A–C:** Scanning electron photomicrographs of adult eyes. **D–F:** Confocal images of developing eye imaginal discs. **A,D:** *so^D/ptc⁷*; note the slight roughening of the eye field (arrow in A) and the few ommatidia in developing eye discs. This finding is characteristic of class 1 suppressors. **B,E:** *so^D/wg^{1G22}*; note the class 1 level of suppression. **C,F:** *so^D/ptc⁷ wg^{1G22}*; note that the simultaneous removal of both *patched* and *wingless* results in a class 3 level of suppression. Several well-formed ommatidia are seen in the adult eye and organized rows of developing photoreceptor clusters are visible in eye imaginal discs. Anterior is to the right.

Fig. 8.

Expression of *hedgehog* completely rescues the no-eye phenotype of *so^D/+*. A–D: Scanning electron photomicrographs of adult eyes. **A:** *so^D/+*. **B:** *so^D/+; dpp-GAL4/UAS-hh*; note the complete restoration of eye development. **D:** *so^D/+; dpp-GAL4/UAS-gbb*; note the slight rescue level similar to class 1 suppression levels. **C:** *so^D/+; dpp-GAL4/UAS-dpp*, similar to *gbb* expression of *dpp*, results in only minor levels of suppression. Anterior is to the right.

Fig. 9.

Suppressors of *so^D/+* partially restore photoreceptor development. **A–H:** Confocal photomicrographs of developing eye imaginal discs. **A,B:** Class 1 suppressors. **C–E:** Class 2 suppressors. **F,G:** Class 3 suppressors. **H:** *so^D/+*. Names of deletion stocks are listed in each panel. Red, F-actin; green, Elav. Anterior is to the right.

Fig. 10.

Models for *sine oculis* function. Our genetic screen isolated mutants in *wingless*, *patched*, *teashirt*, *homothorax*, and *groucho*. **A:** A model in which *sine oculis* negatively regulates the expression of all five genes. **B:** A model in which SO and GRO proteins form a repressor complex and down-regulates the expression of the other four genes. Anterior is to the right.

TABLE 1List of Mutants and Deficiency Stocks that Suppress the *so*[D]/+ no-eye Phenotype

Mutant/Deficiency	Cytological Region	Class
<i>ptc</i>	—	1
<i>wg</i>	—	1
<i>hth</i>	—	1
<i>tsh</i>	—	1
<i>gro</i>	—	1
<i>lid</i>	—	1
<i>ifc</i>	—	1
Df(2L)net-PM47C	21A7–21B7	3
ln(2LR)DTD16[L]DTD42[R]	23C1–23E6	1
Df(2L)BSC5	26B1–26D1	1
Df(2R)H3E1	44D1–44F12	2
Df(2R)03072	51A5–51C1	3
Df(2R)X58–12	58D1–59A1	2
Df(3L)kto2	76B1–76D5	2