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Conservation and non-conservation of genetic pathways in eye specification

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ABSTRACT In this review we highlight two genetic pathways important for eye morphogenesis that are partially conserved between flies and vertebrates. Initially we focus on the *ey* paradigm and establish which aspects of this genetic hierarchy are conserved in vertebrates. We discuss experiments that evaluate the non-linear relationship amongst the genes of the hierarchy with a concentration on vertebrate functional genetics. We specifically consider the *Six* genes and their relationship to *sine oculis*, as tremendous amounts of new data have emerged on this topic. Finally, we highlight similarities between Shh/Hh directed morphogenesis mediated by basic Helix-Loop-Helix factors in vertebrate retinal cell specification and in specification of fly photoreceptors.

KEY WORDS: *bHLH gene, eyeless, lens, neural retina, Pax6*

Introduction

Developmental and evolutionary biologists have identified numerous protein families that maintain high sequence conservation across metazoan phyla. Analysis of these gene families reveals a striking conservation of both gene function and of the relationships among gene families in the patterning of analogous structures in evolutionarily distant organisms. In this review we will focus on the genetic hierarchies that control morphogenesis of the vertebrate eye, and we will compare them to the genetic pathways that control patterning of the compound eye of the fruit fly, *Drosophila*. In particular, we will review the *Drosophila eyeless* paradigm that is involved in patterning the fly eye disc and we will assess the conservation of this pathway in the vertebrate lens and retina. Finally, we will review the hedgehog (*hh*) dependent regulation of bHLH transcription factors that specify the *Drosophila* R8 photoreceptor and we will discuss the similarity to the specification of retinal cell fate by *Sonic hedgehog* (*Shh*) and bHLH transcription factors.

Definition of the eye fields

The fly eye field

In *Drosophila*, the eye-antennal disc invaginates from the embryonic ectoderm and for most of three larval stages these epithelial cells proliferate without differentiating. At the end of the third instar larval period, however, a transition from a monolayer of ectoderm to a highly organized compound eye begins with the formation of the morphogenetic furrow (MF) at the posterior edge of the eye imaginal disc (see Fig. 1). Subsequently, a wave of differentiation sweeps across the disc as the MF moves from posterior to anterior. In the anterior compartment the cells are unpatterned and proliferate asynchronously. Just prior to entering the MF, cells become synchronized in the G1 phase of the cell cycle. In the wake of the MF, differentiation of photoreceptor cells begins with the specification of R8, which is necessary for all subsequent cells to be specified and recruited. R8 quickly recruits R2, R3, R4, and R5 to form a pre-cluster. The remaining unspecified cells undergo a second mitotic division prior to specification of the final 14 precursor cells, including four cells that secrete the lens and crystalline cone and six pigment cells that optically isolate each ommatidium. The 19th founder cell divides twice to form the 4-cell mechanosensory bristle (reviewed in Baker, 2001; Hsiung and Moses, 2002). Thus, the transformation of the fly eye imaginal disc from a sheet of proliferative epithelial cells to a highly organized array of approximately 800 ommatidia provides a powerful and genetically tractable system to understand the genetic hierarchies that control patterning and morphogenesis.

Abbreviations used in this paper: AEL, anterior epithelial layer; Ato, atonal; bHLH, basic helix-loop-helix transcriptional regulator; dac, dachshund; Dpp, decapentaplegic; E, embryonic day; ey, eyeless; eyg, eyegone; eya eyes absent; GCL, ganglion cell layer; Hh, hedgehog; INL, inner nuclear layer; LP, lens placode; MF, morphogenetic furrow; NR, neural retina; OC, optic cup; ON, optic nerve; ONL, outer nuclear layer; OS, optic stalk; OV, optic vesicle; P, postnatal day; PN, proneural; PPN, pre-proneural; RGC, retinal ganglion cell; RPE, retinal pigmented epithelium; Shh, Sonic hedgehog; so, sine oculis; toy, twin of eyeless.

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The vertebrate eye field

In vertebrates, the eye field develops mainly from two separate but interactive tissues, the anterior neurectoderm and the head surface ectoderm. The retinal anlage is specified at the end of gastrulation in the anterior neurectoderm. This eye field splits into two symmetric retinal primordia that evaginate from the forebrain as optic vesicles (OV; Fig. 2). Each OV closely approaches the overlying surface ectoderm of the head. The close apposition between the vesicle and the head ectoderm results in the induction of the lens placode (LP), a thickened layer of ectoderm composed of a pseudo-stratified columnar epithelium. The LP and the OV remain closely apposed as development proceeds. Invagination of the OV results in a bi-layered structure that is patterned along a proximal-distal axis into optic stalk (OS), retinal pigmented epithelium (RPE), and neural retina (NR). Invagination of the LP results in the formation of the lens vesicle, which pinches off from the surface ectoderm. Cells in the posterior half of the lens vesicle elongate through the vesicle and differentiate into primary fiber cells. The anterior epithelial layer (AEL) in the lens remains proliferative and cells produced in the AEL migrate laterally to the equatorial region of the lens where they differentiate into secondary fiber cells (reviewed in Ogino and Yasuda, 2000; Ashery-Padan and Gruss, 2001).

Differentiation in the mouse NR begins at the OS, extends to the central retina, and spreads as a wave to the peripheral retina (McCabe *et al.*, 1999). Retinal cell fate determination in the mouse occurs over a broad period of time (E12 to P21) and involves the cessation of mitosis (birth), commitment to one of seven major cell fates, migration from the ventricular portion of the retina to the appropriate cell layer in the laminate retina, and differentiation (Cepko *et al.*, 1996). The first neurons born in the vertebrate eye are always retinal ganglion cells (RGCs), while the birth order of the other retinal cell types varies among species (Cepko *et al.*, 1996). In mice, the birth order for mature retinal cells begins with RGCs and cone photoreceptors, followed by amacrine and horizontal cells, and lastly, rod photoreceptors, bipolar cells, and Müller glia are specified. There is tremendous overlap in the timing of specification owing to the acquisition of properties such as competence and bias which are not tightly defined temporally (reviewed in Cepko *et al*., 1996; Marquardt and Gruss, 2002). In this review, we will focus on genetic aspects of the *Drosophila* eye morphogenetic process that are conserved, at least in part, in vertebrate lens and retinal development.

TABLE I

***DROSOPHILA* EYE SPECIFICATION GENES AND THEIR VERTEBRATE COUNTERPARTS**

Drosophila	Vertebrate	Eye Phenotype (loss of function)	References
Ey	*Pax6*	small eyes, anophthalmia, Aniridia	Hill *et al*., 1991; Glaser *et al*., 1994
Eya	*Eya1-3*	*Eya1:* open eyelids *EYA1:* anterior segment anomalies	Xu *et al*., 1997; Azuma *et al*., 2000
So	*Six1/2*	none	Laclef *et al*., 2003
Optix	*Six3/6*	holoprosencephaly, anophthalmia	Gallardo *et al*., 1999; Wallis *et al*., 1999; Pasquier *et al*., 2000; Li *et al*., 2002;
D-six4	*Six4/5*	*Six5:* adult onset cataracts	Sarkar *et al*., 2000; Klesert *et al*., 2000; Winchester *et al*., 1999
Dac	*Dach1*	none	Davis *et al*., 2001
Hh	*Shh*	holoprosencephaly, cyclopia	Chiang *et al*., 1996
Atonal	*Math5 (Xath5, ath5)*	> 80% loss of RGCs	Brown *et al*., 2001; Wang *et al*., 2001
hairy	*Hes1*	premature retinal neurogenesis resulting in a retina with very few of each major type of neuron	Tomita *et al*., 1996

The eyeless paradigm in *Drosophila*

Studies on the paired domain containing transcription factor encoded by the *eyeless* (*ey*) gene have been central to our understanding of eye morphogenesis in *Drosophila*. *Ey* was coined the "master regulator" of *Drosophila* eye development since removal of *ey* from the eye disc abolishes eye formation (Quiring *et al*., 1994), and ectopic *ey* expression initiates ectopic eye formation (Halder *et al*., 1995). We now know that *ey* is one of several genes (*ey*, *twin of eyeless* (*toy*); *sine oculis* (*so*); *eyes absent* (*eya*); and *dachshund* (*dac*); see Fig. 1 and Table I) that form a nonlinear network of regulatory interactions essential for fly eye morphogenesis. This pathway has been reviewed extensively elsewhere (Desplan, 1997; Gehring and Ikeo, 1999; Wawersik and Maas, 2000**)**. For the purposes of this review, we will refer to this genetic network as the *ey* paradigm.

Conservation and non-conservation of the *eyeless* paradigm

Since the elucidation of the *ey* paradigm in the *Drosophila* eye and the identification of highly related genes in vertebrates, the extent to which the paradigm has been conserved during vertebrate eye morphogenesis has been of considerable interest (Table I). However, comparison of the corresponding genetic networks between *Drosophila* and vertebrates has been complicated by the existence of multiple members of the respective gene families. *Ey* was originally placed at the top of the genetic hierarchy required for *Drosophila* eye specification. Subsequently, two *Drosophila ey*-related genes have been found, *twin of eyeless (toy)* and *eyegone (eyg),* see Czerny *et al*., 1999 and Jang *et al*., 2003. Both of these genes are required for eye formation and function in unique capacities. *Toy* acts upstream of *ey*, directly inducing *ey* expression in the eye disc (Hauck *et al*., 1999; Czerny *et al*., 1999), and is dependent upon *ey* for its function (Czerny *et al*., 1999). *Toy* is not regulated by *ey, eya*, *so*, or *dac* (Czerny *et al*., 1999). *Eyg*, on the other hand, acts in a pathway independent of *ey* (Jang *et al*., 2003), and plays an entirely separate role during eye development (Dominguez *et al*., 2004). *Eyg* promotes growth of the eye disc and acts downstream of *Notch* (Dominguez *et al*., 2004).

In *Drosophila*, two additional *so* family members, *optix* and *D-six4*, have also been found (Kawakami *et al*., 2000; Table I). Like *eyg*, *optix* is essential for eye development but is not involved in the same signaling network as *so* (Seimiya and Gehring, 2000). *D-six4*, on the other hand, is not expressed in the fly eye (Kawakami *et al*., 2000). In vertebrates, the gene families are for the most part larger, and it is therefore difficult to define orthologues. There exists one *ey/toy/eyg* homologue (*Pax6*), six *so/optix/D-six4* homologues (*Six1-6*) (Kawakami *et al*., 2000), four *eya* homologues (*Eya1-4*) (Xu *et al*., 1997; Borsani *et al*., 1999), and two *dac* homologues (*Dach1* and *Dach2*) (Hammond *et al*., 1998; Caubit *et al*., 1999; Davis *et al*., 1999; Heanue *et al*., 1999).

Defining vertebrate orthologues

Pax6 is more closely related to *toy* and *ey* than to *eyg* (Gehring and Ikeo, 1999**),** and may have taken on the functional role of both *ey* and *toy* in vertebrate eye specification (Plaza *et al.*, 1993). Eyg, on the other hand, shares both sequence and functional homology and with the Pax6 isoform Pax6(5a) (Dominguez *et al.*, 2004). The central role that *Pax6* plays in vertebrate eye formation and its remarkable similarity to *ey* has been reviewed extensively (Gehring and Ikeo, 1999; Wawersik and Maas, 2000; Ashery-Padan and Gruss, 2001; Hansen, 2001; van Heyningen and Williamson, 2002; Simpson and Price, 2002), and will be only briefly reviewed here.

The *Six* genes fall into three gene families: *Six1/Six2/so*, *Six3/Six6/optix*, and *Six4/Six5/D-six4*. This classification is based upon molecular phylogeny, chromosomal arrangement, DNA binding specificity, and the ability to interact with Eya proteins (Kawakami *et al.*, 2000). Surprisingly, one of the *so* orthologues, *Six1*, is not expressed during vertebrate eye morphogenesis, and homozygous deletion of *Six1* has no affect on eye development (Oliver *et al.*, 1995; Laclef *et al.*, 2003). *Six2* is expressed in the inner and outer nuclear layers (INL and ONL) and the ganglion cell layer (GCL) of the adult mouse retina (Kawakami *et al.*, 1996), but ectopic expression of *Six2* in developing medaka fish has no affect on eye morphogenesis (Loosli *et al.*, 1999). Thus, it is unlikely that *Six1* or *Six2* is an essential part of a signaling network in vertebrate eye morphogenesis.

Both *optix* orthologues, *Six3* and *Six6*, are expressed throughout eye morphogenesis (Jean *et al.*, 1999), and homozygous mutation of *Six6* in mice deletes the optic chiasm and ON and causes retinal hypoplasia (Li *et al.*, 2002). Deletion of *Six3* expression in medaka fish with a morpholino completely deletes all eye and forebrain structures (Carl *et al.*, 2002). These results indicate that *Six3* and *Six6* are required for vertebrate eye formation, and we will therefore discuss the likelihood that one or both of these genes plays a role comparable to that of *so* in the *Drosophila ey* paradigm.

The remaining two vertebrate *Six* genes, *Six4* and *Six5*, are orthologous to *D-six4,* which is not expressed in the fly eye (Kawakami *et al.*, 2000). *Six4* is expressed in the lens placode of *Xenopus* embryos (Ghanbari *et al.*, 2001). *Six4*, however, is not expressed in the developing eyes of mouse embryos, and homozygous deletion of *Six4* has no effect on eye morphology (Ozaki *et al.*, 2001). *Six4* is also not expressed in the developing eyes of zebrafish embryos (Kobayashi *et al.*, 2000). Thus, like *Six1* and *Six2*, *Six4* does not play a major role in the specification of the vertebrate eye. Although *Six5* is only weakly expressed in the developing mouse lens (Heath *et al.*, 1997; Sarkar *et al.*, 2000) and in the adult NR (Kawakami *et al.*, 1996), mice with either heterozygous or homozygous deletion of *Six5* develop cataracts (Sarkar *et al.*, 2000; Klesert *et al.*, 2000). Similarly, disruption of the *DMPK/SIX5* locus in humans causes myotonic dystrophy, with symptoms including adult onset cataracts (Winchester *et al.*, 1999). Since both mice and humans deficient in *Six5* develop cataracts postnatally, *Six5* likely plays a role in the maintenance of adult lenses. Since there is less evidence supporting a critical role for *Six5* during embryonic eye specification we will focus on *Six3* and *Six6* in our consideration of a vertebrate equivalent of *so*.

Eya1, *Eya2*, and *Eya3* are also expressed during eye development (Xu, *et al.*, 1997; Purcell, 2002), while *Eya4* is expressed in the embryonic craniofacial region, but not in the developing eye (Borsani *et al.*, 1999). Thus, we will focus on the comparison of *eya* to *Eya1, 2*, and *3*, the vertebrate *Eya* genes that are expressed during vertebrate eye development. Lastly, *Dach1* is expressed in the retina in a pattern overlapping with, albeit delayed from, *Pax6* (Hammond *et al.*, 1998; Caubit *et al.*, 1999; Heanue *et al.*, 2002). *Dach2*, on the other hand, is not expressed in the eye (Heanue *et al.*, 1999), and thus, we will compare and contrast *dac* with *Dach1*.

The activity of Pax6 and eyeless is conserved

Similar to *ey* (Quiring *et al.*, 1994), *Pax6* expression is found in the eye as soon as the eye field can be identified. This is true, both in the retinal anlage specified in the anterior neurectoderm and in the presumptive head ectoderm that becomes the lens placode (Walther and Gruss, 1991). As seen with *ey* mutations in the fly, disruption of *Pax6* severely affects vertebrate eye formation.

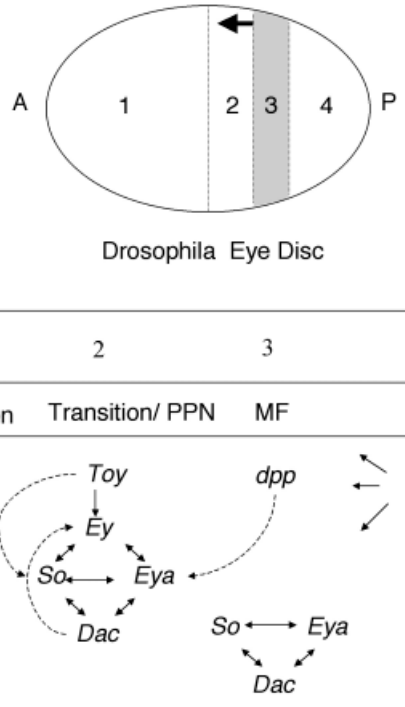

Fig. 1. Schematic representation of *Drosophila* eye development. *Differentiation of the* Drosophila *eye is controlled by a complex series of signaling events that produce precise compartmentalization of transcription factor activity. The MF, marked by hatched lines, is a wave of differentiation that moves from posterior (P) towards anterior (A) across the eye field during the third instar larvae. Compartments of the eye disc are divided by a dotted line. Compartment 1 represents the majority of cells anterior to the MF. The pre-proneural (PPN) region, represented by compartment 2, is just anterior to the MF. Compartment 3 represents the MF. The arrow indicates the direction of furrow progression. In the compartment 4, posterior to the MF, photoreceptor differentiation and ommatidia assembly occur. Below both the major cellular events and the expression domains of* ey *paradigm genes are indicated.* Ey *and* toy *are only expressed anterior to the MF. In the PPN region,* ey *and* toy *induce expression of* so, *while* ey *and* dpp *induce expression of* eya. *Together* ey, so, *and* eya *activate* dac. *The nonlinear regulatory relationship amongst these genes is illustrated and is herein referred to as the* eyeless *paradigm. Just posterior to the MF furrow* so, eya, *and* dac *continue to be expressed in the absence of* eye.

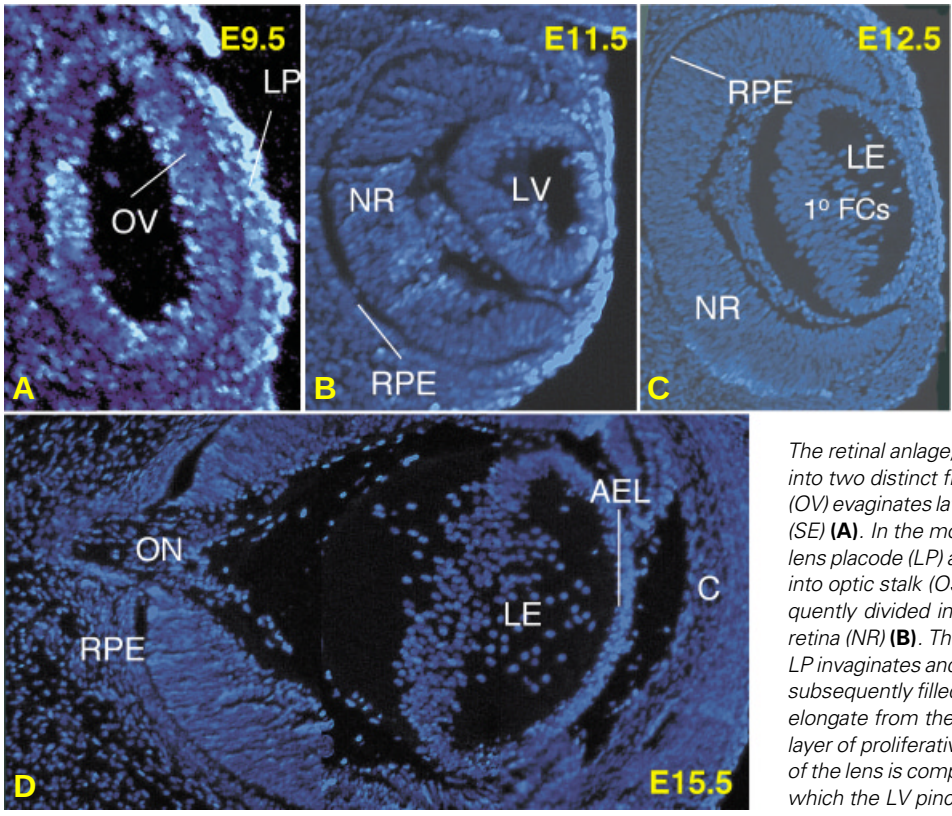

Fig. 2. Vertebrate eye formation. *Four key stages of embryonic mouse eye development are shown. (A E9.5; B E11.5; C E12.5; and D E15.5). Each panel shows a representative DAPI stained section through the eye of a paraffin embedded embryo. The vertebrate eye is formed from two separate tissues the neurectoderm and the head surface ectoderm. The retinal anlage, specified in the anterior neurectoderm, is divided into two distinct fields (not shown). From each field an optic vesicle (OV) evaginates laterally and opposes the overlying surface ectoderm (SE)* **(A)**. *In the mouse, the surface ectoderm is induced to form the lens placode (LP) at roughly E9. The OV is patterned proximal-distally into optic stalk (OS) and optic cup (OC, not shown), which is subsequently divided into retinal pigmented epithelium (RPE) and neural retina (NR)* **(B)**. *The OS matures to form the optic nerve (ON, D). The LP invaginates and forms a hollow lens vesicle (LV; panel B), which is subsequently filled with differentiating primary fiber cells (1º FC) that elongate from the posterior* **(C)**. *In the mature lens (LE), an anterior layer of proliferative epithelial cells (APE) remains and the remainder of the lens is composed of fiber cells* **(D)**. *The surface ectoderm from which the LV pinches off from gives rise to the cornea (C in D).*

Homozygous or compound heterozygous mutation of the *Pax6* gene results in anophthalmia in both humans (Glaser *et al.*, 1994) and *Pax6*^{Sey} mice (Hill *et al.*, 1991).

Similarly, in the zebrafish mutant *cyclops*, which lacks *Shh* expression, the *Pax6* expression domain is expanded and retina specification occurs at the expense of the optic nerve (Macdonald *et al.*, 1995). Ectopic expression of *Shh*, on the other hand, restricts *Pax6* expression, and nearly abolishes the retinal field (Macdonald *et al.*, 1995). Thus, alterations in *Shh* alter *Pax6* expression. This is reminiscent of the fly, where down-regulation of *ey* in the MF coincides with cells receiving Hh signals (Halder *et al.*, 1998).

Ectopic *Pax6* expression in *Xenopus* embryos can produce various eye related phenotypes including the induction of well-organized, ectopic eyes in the head anterior to the hindbrain-spinal cord junction (Chow *et al.*, 1999). This observation is reminiscent of the ectopic eye forming activity of *ey* in *Drosophila* imaginal discs (Halder *et al.*, 1995). Collectively, these experiments show that specification of the eye in vertebrates is tightly linked to *Pax6* expression. Clearly, *Pax6* in the vertebrate eye has retained some striking functional similarities with *ey*.

Do non-orthologous genes in the vertebrate eye fulfill the role of so*?*

The *ey* paradigm is used during vertebrate organogenesis in tissues unrelated to the eye. In two such examples, a gene family member has been substituted into the hierarchy. In the developing kidney, homozygous deletion of *Eya1*, *Pax2*, or *Six1* disrupts early kidney morphogenesis (Torres *et al.*, 1996; Xu *et al.*, 1999; Xu *et al.*, 2003). In addition, compound heterozygous mutants of *Eya1* and *Six1* have hypoplastic kidneys (Xu *et al.*, 2003, Li *et al.*, 2003) demonstrating an interaction between these genes. While all of the *ey* paradigm interactions from the *Drosophila* eye are not conserved in the vertebrate kidney, enough parallels exist to establish the importance of the *Pax2/Eya1/Six1* genetic hierarchy for organogenesis (Torres *et al.*, 1996; Xu *et al.*, 1999; Xu *et al.*, 2003). *Pax2*, however, is substituted for *Pax6*. In the developing somites, *Pax3*, *Six1*, *Eya2*, and *Dach2* synergize to promote myogenesis (Heanue *et al.*, 1999). In this example, *Pax6* is replaced by the

TABLE II

QUALITATIVE AND FUNCTIONAL COMPARISON OF *SIX3* AND *SIX6* TO *SO* AND *OPTIX*

Characteristic	Context	*So*	*Optix*
Pattern of Expression	N/A	-	+
Eye Inducing Capacity	N/A	-	+
Null Mutations	N/A	NI	NI
Interaction with Eya proteins	N/A	-	+
Relationship with *Pax6 / Ey*			
	Mouse Lens (*Six3*)	+	-
	Mouse NR	-	+
	Fish NR	+	+
	Frog NR (*Six3*)	+	-
	Mouse OS (*Six6*)	-	+
	Frog OS (*Six3*)	+	-
	Fish RA (*Six3*)	+	+
	Frog RA	+	-

In the vertebrate eye it is unclear if *Six3* and *Six6* function more like *optix* or like *so*. We assessed their role in vertebrate eye formation and have classified them as behaving either like *optix* or *so*. In some instances *Six3* and *Six6* have characteristics of both *Drosophila* genes. These instances are indicated by a (+) for each *so* and *optix*. Abbreviations; N/A, not applicable; NI, not informative; (+) similar; (-) not similar; NR, neural retina; OS, optic stalk; RA, retinal anlage

Pax3 gene. Thus, there are clear examples in which the *Drosophila ey* paradigm has been utilized in vertebrate organogenesis, but the hierarchy has been modified by substituting tissue-appropriate family members.

Is it possible that a non-orthologous *Six* gene, *Six3* or *Six6*, replaces the function of *so* in the vertebrate eye? As noted earlier, two *Six* genes are broadly expressed in the vertebrate eye during morphogenesis, *Six3* and *Six6* (Jean *et al.*, 1999). These genes, however are orthologous to *optix* (Kawakami *et al.*, 2000), which is expressed during and is important for eye development in *Drosophila* (Seimiya and Gehring, 2000), and not to *so*. To determine if *Six3* or *Six6* might replace *so* in the lens or NR, we would like to know whether these *Six* genes behave more like *so* or more like *optix* during vertebrate eye development. For both genes we will assess their qualitative and functional similarity to *so* and to *optix* by considering their temporal and spatial expression patterns, their ability to induce ectopic eyes, their homozygous null phenotypes, their regulatory relationships with *Pax6*, and their ability to interact with Eya proteins (Table II).

Patterns of expression

Optix and *so* have different patterns of expression in *Drosophila* eye development (Seimiya and Gehring, 2000). *Optix* has an expression pattern similar to *ey* in the eye primordium and anterior to the MF in the differentiating disc, while *so* has an expression pattern comparable to *eya*, and is found in cells adjacent to and including the MF (Seimiya and Gehring, 2000). *Six3* has an expression pattern nearly identical to that of *Pax6,* as both genes are found in the retinal anlage, the LP, and throughout the developing lens vesicle and OC (Walther and Gruss, 1991; Oliver *et al.*, 1995). *Six6* expression overlaps that of *Pax6* in derivatives of the retinal anlage (optic stalk and neural retina) but is absent from the actual anlage (Toy *et al.*, 1998; Toy and Sundin, 1999; Jean *et al.*, 1999; Bernier *et al.*, 2000). *Six6* is completely absent from the head surface ectoderm and its derivatives (Oliver *et al.*, 1995; Toy *et al.*, 1998; Toy and Sundin, 1999; Jean *et al.*, 1999). *Six3* and *Six6* are expressed earlier than and more broadly than *Eya1*, *Eya2* or *Eya3* (Xu *et al.*, 1997). Thus, *Six3* and *Six6* are expressed in patterns similar to that of *Pax6*, and therefore in this regard more closely resemble *optix* than *so*.

Ectopic eye inducing capacity

In the fly, ectopic expression of *optix* alone in the antennal disc induces eyes, whereas ectopic *so* expression does not (Seimiya and Gehring, 2000; Pignoni *et al.*, 1997). Similar to *optix*, ectopic expression of *Six3* in medaka fish induces ectopic retinal primordia in competent locations within the brain, and at a much lower frequency ectopic lenses in the head ectoderm near the otic vesicle (Loosli *et al.*, 1999). In *Xenopus* ectopic expression of either *Six3* or *Six6* converts anterior neural plate to retina (Bernier *et al.*, 2000.) In these experiments low concentrations of *Six3* of *Six6* expand the size of the retina, while high concentrations of either gene transform the midbrain to retina and delete the normal eye (Bernier *et al.*, 2000). Thus, *Six3* and *Six6* resemble *optix* in their ability to induce an eye-specific developmental program in non-ocular tissue.

Homozygous null phenotypes

So is required for all aspects of visual system development in *Drosophila* (Cheyette *et al.*, 1994; Serikaku and O'Tousa *et al.*, 1994), while an *optix* fly mutant has not been described. *Six6* null mice exhibit retinal hypoplasia and often lack both an optic nerve and an optic chiasm (Li *et al.*, 2002). In addition, heterozygous mutation of *SIX6* in humans correlates with anophthalmia (Gallardo *et al.*, 1999). Mutation of *SIX3* in humans causes holoprosencephaly, with phenotypes ranging from cyclopia to hypotelorism (Wallis *et al.*, 1999; Pasquier *et al.*, 2000). Morpholino inhibition of *Six3* expression in medaka fish deletes both forebrain and eye tissue (Carl *et al.*, 2002). The absence of an *optix* fly mutant makes it unclear whether *Six3* and *Six6* mutation in vertebrates are more reminiscent of *so* or *optix*, although given the severity of each of the reported null phenotypes it is unlikely that this feature would provide a definitive distinction.

Regulatory relationship to ey/Pax6

In the fly, *optix* expression is truly independent of *ey*, as eyes ectopically induced by *optix* do not express *ey*, and *optix* can induce eyes in *ey* deficient flies (Seimiya and Gehring, 2000). In contrast, *so* expression is dependent upon *ey* (Pignoni *et al.*, 1997; Halder *et al.*, 1998; Niimi *et al.*, 1999; Michaut *et al.*, 2003). Moreover *so* acts in conjunction with *eya* to induce *ey* expression in ectopic eyes (Pignoni *et al.*, 1997; Bonini *et al.*, 1997).

In vertebrates, the relationship of *Six3* or *Six6* to *Pax6* depends upon both the species in question and the particular compartment

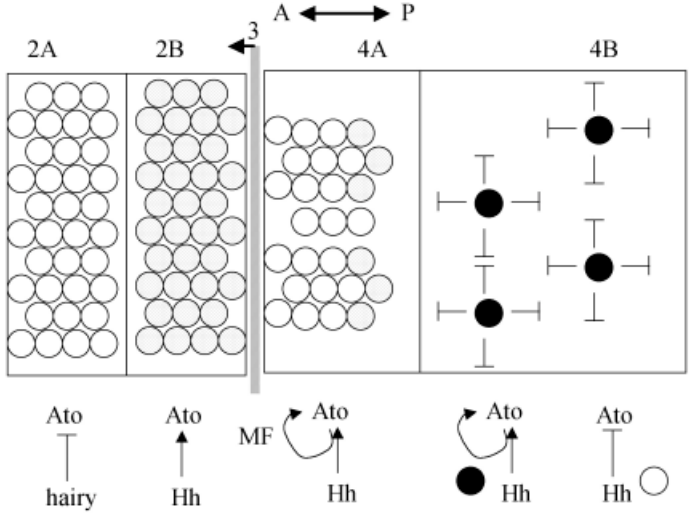

Fig. 3. Specification of the R8 founder cell in the *Drosophila* retina. *Shown is a schematic representation of R8 cell specification in the Drosophila eye disc. The same compartmental nomenclature is used, although compartments 2 and 4 are expanded into A and B sub compartments to provide more detail. Anterior (A) is to the left and posterior (P) is to the right. The morphogenetic furrow (MF; striped line at 3) progresses anteriorly. Most cells anterior to the MF, in compartment 2A (open circles), do not express* ato *because it is repressed by* hairy. *In cells just anterior to the furrow, compartment 2B, Hh induces* ato *expression.* Ato *positive cells (dotted circles) are progressively restricted in number, in compartment 4B, until there is one founder cell (filled circles) around which each ommatidia is assembled. The restriction process occurs in four major stages: 1) induction of* ato *(2B); 2) restriction of* ato *to an intermediate group of 10-20 cells (not shown); 3) restriction of* ato *to an equivalence group of 2-3 cells (4A); and 4) restriction of* ato *to a single founder cell, R8 (4B). During R8 differentiation,* hh *is expressed in cells that do not express* ato, *and it activates* ato *in the R8 founder cell and contributes to* ato *repression in other cells.*

of developing eye (Table II). In mice, *Six6* is expressed in the optic stalk and in the OV remnant in homozygous *Pax6*^{Sey} mice at E9.5 (Jean *et al.*, 1999), demonstrating that *Six6* expression is independent of *Pax6*. Furthermore, *Six6* null mice have normal *Pax6* expression in both the lens and retina (Li *et al.*, 2002). Since the expression of *Six6* and *Pax6* are independent in mice, their relationship is reminiscent of that between *ey* and *optix*. *Six3* and *Pax6*, however, regulate each other's expression in mouse surface ectoderm derivatives (Goudreau *et al.*, 2002). In *Pax6*^{LacZ} mice, expression of *Six3* is unchanged in the retina, but is reduced in the AEL of the lens (Goudreau *et al.*, 2002). Ectopic expression of either *Pax6* or *Six3* in lens fiber cells induces the expression of the other gene (Goudreau *et al.*, 2002). Thus, while *Six3* expression is independent of *Pax6* in the retina, the relationship between *Six3* and *Pax6* in the mouse lens is interdependent, and therefore, resembles the *ey-so* relationship.

In *Xenopus*, ectopic *Pax6* expression expands *Six3* expression in the OV at the midline (presumptive optic stalk) and reduces *Six3* expression distally (presumptive NR; Chow *et al.*, 1999). In addition, ectopic expression of either *Six3* or *Six6* induces ectopic retinas coincident with the expansion of *Pax6* expression (Bernier *et al.*, 2000). Likewise, in medaka fish, ectopic expression of either *Six3* or *Six6* expands *Pax6* expression and induces ectopic retinas (Loosli *et al.*, 1999). In each of the aforementioned examples misexpression is achieved by injection of high concentrations of RNA into blastomeres during early stages of development. Thus, expression of the injected gene is not necessarily at a physiological concentration and neither the time nor the place of expression is regulated. Therefore, such overexpression experiments define relationships that may occur in normal development but do not prove that they do occur. The relationship between *Pax6* and *Six3* predicted by overexpression experiments is, however, supported by complementary reduced expression data. Down-regulation of *Six3* expression by morpholino interferes with *Pax6* expression, whereas interference with *Pax6* expression does not interfere with *Six3* (Carl *et al.*, 2002). Thus, in both *Xenopus* and medaka fish *Pax6* and *Six3/Six6* are at least partly inter-dependent and, therefore, resemble *ey* and *so*.

Synergism with eya

Lastly, in *Drosophila*, *so* acts synergistically with *eya*, both by direct protein-protein interaction and in the cooperative induction of ectopic eyes (Pignoni *et al.*, 1997). Optix, however, does not form a protein-protein complex with Eya (Ohto *et al.*, 1999), and co-expression with *eya* does not effect the frequency of ectopic eye induction by *optix* (Seimiya and Gehring, 2000). Six3 also does not interact strongly with Eya proteins in biochemical assays *in vitro* (Ohto *et al.*, 1999; Purcell, 2002). Six6, however, acts as a co-repressor with Dach proteins both *in vitro* and in chromatin immunoprecipitations, suggesting that this protein-protein interaction occurs *in vivo* (Li *et al.*, 2002). No direct interaction between either So or Optix and Dac proteins have been reported in the fly. This Six-Dach interaction may, therefore, represent a feature unique to vertebrates. However, due to their inability to interact strongly with Eya proteins, Six3 and 6 may be more similar to Optix than to So.

Thus, based on the aforementioned criteria (summarized in Table II), *Six3* and *Six6* in the eye do not behave exactly like *so*. They have some functional characteristics of both *optix* and *so*. Overall, however, they have more in common with *optix* than they have with *so*.

Eyes absent *related genes are not critical for vertebrate eye formation*

As stated above, Eya1 and Six3 do not interact strongly at the protein level (Ohto *et al.*, 1999; Purcell, 2002). What then becomes of the *eya* component of the *ey* paradigm in vertebrates? Are other aspects of *eya* function conserved?

Disruption of *eya* activity in the fly prevents eye formation (Bonini *et al.,* 1997), and the collective expression of *Eya1*, *Eya2*, and *Eya3* encompass most tissues of the developing mouse eye. *Eya1* is expressed in the lens, OS, and NR (Xu *et al.*, 1997). *Eya2* is absent from the lens, but is expressed in the NR in a pattern complementary to *Eya1* (Xu *et al.*, 1997). In the retina, *Eya1* is in the ONL and the peripheral retina, while *Eya2* is in the posterior region and the INL (Xu *et al.*, 1997). *Eya3* is present in the OV and the periocular mesenchyme, but is absent from the lens (Xu *et al.*, 1997).

These expression patterns suggest that *Eya* genes may play a role in vertebrate eye morphogenesis and that each *Eya* gene may have taken on a different component of *Drosophila eya* activity. Homozygous mutation of *Eya1*, however, results only in a mild, extrinsic eye phenotype, open eyelids at birth (Xu *et al.*, 1999), and mice with compound homozygous mutations in both *Eya1* and *Eya2* also retain morphologically normal eyes (P.Y. Xu and R.L. Maas, unpublished; Purcell, 2002). The null mutation of *Eya3* in mouse has not yet been reported. Thus, in the mouse, *Eya1* and *Eya2* are not required for eye morphogenesis and, therefore, cannot play a role comparable to the requisite role of *eya* in *Drosophila*. Three human cases, however, have been identified in which heterozygous mutation of *EYA1* correlates with anterior segment defects (Azuma *et al.*, 2000). Additional insight into the mechanism for this human defect is needed, however, since many other mutations in *EYA1* have been reported that have no effect on eye development (Vervoot *et al.*, 2002). Hopefully these mutations will ultimately provide insight into the function of *Eya* genes in the vertebrate eye.

Drosophila eya is downstream of *ey* and critical for its function (Pignoni *et al.*, 1997; Bonini *et al.*, 1997). Indeed, both the frequency and size of ectopic eye induction are enhanced when *eya* and *ey* are co-expressed (Bonini *et al.*, 1997). In mice, neither *Eya1* nor *Eya2* expression is changed in the early eyes of *Pax6*^{LacZ} heterozygous mice (Goudreau *et al.*, 2002). Unfortunately, expression of vertebrate *Eya* genes begins too late to reliably assess expression patterns in homozygous *Pax6*^{Sey} mice since the tissues in which these genes are expressed fail to form (Purcell, 2002). Clearly, however, if *Eya1* and *Eya2* genes can be disrupted with no effect on eye morphogenesis (Xu *et al.*, 1999; Purcell, 2002), they cannot be essential downstream mediators of *Pax6* function.

At this time, there is very little evidence to support a homologous relationship between vertebrate *Eya* genes and fly *eya*. Perhaps the best evidence that these genes retain some functional homology comes from the demonstration that the mouse *Eya1, Eya2,* and *Eya3* genes can each rescue fly eye formation in *eya* mutants (Bonini *et al.*, 1997; Bui *et al.*, 2000). We know, however, that *Eya1* and *Eya2* have some capacity to behave like *Drosophila eya* with regards to the *ey* paradigm during organogenesis in other vertebrate tissues (Heanue *et al.*, 1999; Xu *et al.*, 1999; Xu *et al.*, 2003). Nonetheless, the ability to replace *eya* in the *Drosophila* eye does not indicate that vertebrate *Eya1* and *Eya2* have a specific role in vertebrate eye development.

Dach1 *is not essential for vertebrate eye formation*

In the fly, the pattern of *dac* expression coincides with that of *eya* (Chen *et al.*, 1997). In the vertebrate eye, *Dach1* is expressed in the lens and the periphery of the retina (Caubit *et al.*, 1999; Purcell, 2002), similar to the expression of *Eya1* (Xu *et al.*, 1997). Thus vertebrate *Dach1* is similar to *dac* in its ocular co-localization with *Eya1*. *Dach1* expression also overlaps with that of *Pax6*, but is significantly delayed in its time of appearance (Hammond *et al.*, 1998; Caubit *et al.*, 1999; Davis *et al.*, 1999; Heanue *et al.*, 2002). However, while loss of *dac* expression disrupts *Drosophila* eye formation (Shen and Mardon, 1997; Chen *et al.*, 1997), homozygous mutation of *Dach1* in mice has no affect on eye morphogenesis (Davis *et al.*, 2001). Thus, while *dac* is essential in the fly, *Dach1* is not essential for vertebrate eye morphogenesis.

In *Drosophila*, *dac* acts downstream of *ey*, *eya* and *so* (Shen and Mardon, 1997; Chen *et al.*, 1997; Michaut *et al.*, 2003). In mouse, expression of *Dach1* in the OC is not dependent upon *Pax6* as its expression is maintained in the presumptive NR of homozygous *Pax6*^{Sey} mice (Heanue *et al.*, 2002). Expression of *Dach1* is, however, disrupted in the lens ectoderm of homozygous *Pax6*^{Sey} mice (Purcell, 2002). This loss of *Dach1* expression is not the result of global loss of gene expression in a quiescent tissue because other genes are still expressed (Purcell, 2002). In the fly, *dac* expression is involved in the maintenance of *ey*, *eya*, and *so* expression (Chen *et al.*, 1997). In *Dach1* mutant mice, neither *Pax6* nor *Six3* expression is altered in the developing eyes (Purcell, 2002). Thus, although *Dach1* is downstream of *Pax6* in the lens ectoderm, most aspects of the *ey-dac* relationship are missing in the vertebrate eye.

As observed for the rescue of *Drosophila eya* mutants with *Eya1, 2* and *3*, *Dach2* expression in *dac* mutant flies rescues the eye phenotype (Heanue *et al.*, 1999). This is not surprising since *Dach2* and its relationship to *Pax3, Six1, and Eya2* during somitogenesis is comparable to the relationship in the fly *ey* paradigm (Heanue *et al.*, 1999). Thus, the ability of vertebrate *Dach2* to rescue the *Drosophila dac* eye phenotype reflects the conservation of the *ey* paradigm in vertebrate organogenesis rather than a specific conservation of *Dach* function in the vertebrate eye.

The potential conservation of the *ey* paradigm in vertebrate oculogenesis has received tremendous attention. In the preceding sections, we have compared each genetic component of the *ey* paradigm to equivalent vertebrate genetics. While homologues of all of the genes from the fly *ey* paradigm are expressed during development of the vertebrate eye, the function of each of these genes has not been strictly preserved. The most notable example of non-conservation is the failure of mutations in *Eya1* and *Eya2* to produce an embryonic eye phenotype. It is intriguing to note, however, that the vertebrate genes are capable of many of the interactions present in the *Drosophila* eye, as evident from the vertebrate genes either rescuing *Drosophila* mutants or inducing ectopic eyes in the fly. This suggests that the orthologous vertebrate genes have maintained their molecular function but that the components have, to some extent, become uncoupled. In addition it is important to note that some aspects of the *ey* paradigm are well conserved. In particular, *Pax6* is highly reminiscent of *ey*, while *Six3* and *Six6* have some characteristics of *so*. Thus, despite the lack of strict conservation of the *ey* paradigm, it is significant that several critical eye regulator genes have been preserved between the morphologically divergent fly and vertebrate eye.

Parallel genetic hierarchies control retinal differentiation in the fly and vertebrates

Interestingly, another parallel genetic pathway between *Drosophila* and vertebrates has also emerged between R8 photoreceptor differentiation and vertebrate RGC specification. Once again this conservation is found in analogous structures that are morphologically quite different. Below, we briefly highlight the genetic similarities between the retinal developmental pathways in *Drosophila* and vertebrates. In both systems, the transition from a naive, progenitor state to a proneural state is controlled in part by hedgehog signaling, and by the antagonistic relationship between a proneural transcriptional activator and a proneural repressor of the basic helix-loop-helix (bHLH) class.

In the *Drosophila* eye disc, the MF spatially and temporally precedes a wave of differentiation (Figs. 1,3). Initiation and progression of the MF depend in part upon signaling by Hh, which is secreted from differentiating photoreceptors posterior to the MF (Dominguez and Hafen, 1997; reviewed in Treisman and Heberlein, 1998). Hh initiates photoreceptor differentiation through two distinct signals, one long-range and one short-range (Greenwood and Struhl, 1999; Kango-Singh *et al.*, 2003). Decapentaplegic, Dpp, mediates the long-range signal within the MF (Greenwood and Struhl, 1999; Fig. 3) and facilitates the shift from a naive cell to a pre-proneural (PPN) cell. The shift to a PPN state is marked by the upregulation of the bHLH transcription factor *hairy* (Greenwood and Struhl, 1999; Fig. 3). *Hairy* is a proneural repressor and marks the PPN state (Greenwood and Struhl, 1999). In the PPN compartment, cells exit the cell cycle and prepare for neuronal differentiation (Greenwood and Struhl, 1999).

The mediator of the second, short-range signal downstream of Hh is unknown but uses the Raf pathway (Greenwood and Struhl, 1999). The result of this short-range signal is the expression of Atonal, a bHLH transcription factor that induces a proneural state (Jarman *et al.*, 1994). *Hairy* and *atonal* share a sharp expression boundary at the border between the PPN and the proneural (PN) compartments (Fig. 3; Greenwood and Struhl, 1999). Cells that do not pass through the PPN (*hairy*+) to PN (*atonal*+) transition do not differentiate into R8 photoreceptors (Greenwood and Struhl, 1999). Once beyond the MF, the expression of *atonal* becomes gradually restricted from all of the cells in the MF to one per cluster, the R8 founder cell (reviewed in Treisman and Heberlein, 1998; Frankfort and Mardon, 2002). This restriction is partially dependent on Hh signaling (Dominguez and Hafen, 1997; Greenwood and Struhl, 1999).

A wave of Shh signaling also marks retinal differentiation in vertebrates

In zebrafish, *Shh* is expressed in the retinal GCL, ventral and nasal to the optic disc (Neumann and Nuesslein-Volhard, 2000). This zone of *Shh* gradually spreads across the retina as a wave that temporally matches the specification of RGCs (Neumann and Nuesslein-Volhard, 2000). Disruption of *Shh* expression as it expands across the retina blocks both the wave of RGC differentiation, as well as the continued expression of *Shh* (Neumann and Nuesslein-Volhard, 2000). Similar to the secretion of Hh by differentiating photoreceptors posterior to the MF in the fly, Shh is normally secreted by RGCs *in vivo*, and this drives the wave of differentiation across the retina (Neumann and Nuesslein-Volhard,

2000). During the wave of RGC specification, the number of differentiating RGCs can be increased or decreased by altering Shh levels (Zhang and Yang, 2001). Low concentrations of Shh induce an increase in the number of differentiating RGCs, while high concentrations of Shh inhibit RGC differentiation and reduce their numbers (Zhang and Yang, 2001). This is reminiscent of photoreceptor differentiation in *Drosophila* where Hh induces the expression of the R8 neuronal precursor marker, *atonal*, in all cells at the MF, but interferes with expression of *atonal* in cells deselected as the R8 neuronal precursor, posterior to the MF (Fig. 3). Alteration of the concentration of Hh in *Drosophila* alters the number of R8 neuronal precursors (Dominguez and Hafen, 1997). Thus, a wave of expression of both Hh and Shh drive differentiation, and their concentrations are critical for specification of appropriate numbers of either R8 founders or RGCs, respectively.

In mice, complete abolition of *Shh* expression produces a single retinal anlage that fails to divide into symmetric retinal primordia, and therefore results in the formation of a single, centrally located OC (Chiang, *et al.*, 1996). Due to the timing and severity of the phenotype resulting from mutation of *Shh* in mice, assessing the role of Shh in retinal specification is complicated. However, the single OC in *Shh* mutant mice is severely dysmorphic: the two-layered structure is inappropriately patterned along the proximal-distal axis (Chiang *et al.*, 1996). Specifically, NR is lost at the expense of RPE, suggesting that *Shh* may play an important role in NR specification. Additionally, *Shh* expression has been observed in the NR of mice at the time of RGC specification (Jensen and Wallace, 1997). Definitive evidence of a role for *Shh* in mouse retinal specification awaits the generation of a conditional knock out.

A family of bHLH genes specifies neuronal identity

The mouse RGC marker, *Math5*, is the orthologue of the fly R8 progenitor cell marker, *atonal* (Brown *et al.*, 1998). Expression of *Math5* precedes that of other bHLH PN genes (*Ngn2*, *NeuroD*, and *Mash1*) in mice (Brown *et al.*, 1998). *Math5* is expressed initially in the mouse retina at E11.5 in the central OC, and correlates with the appearance of early neurons (Brown *et al.*, 2001; Wang *et al.*, 2001). *Math5* expression expands throughout the retina in a wave and peaks at E13.5 (Brown *et al.*, 2001;Wang *et al.*, 2001). During RGC birth at E15.5, *Math5* is expressed in the periphery of the retina where neuronal specification occurs (Brown *et al.*, 2001; Wang *et al.*, 2001). Similarly, the zebrafish and *Xenopus atonal* orthologues, *ath5* and *Xath5* respectively, predict the pattern of neuronal differentiation with their expression (Kaneker *et al.*, 1997; Masai *et al.*, 2000). Thus, the dynamic expression patterns of the vertebrate proneural genes *Math5/ath5/Xath5* resemble the expression of *atonal* that moves across the fly eye disc in front of the MF as photoreceptor differentiation proceeds.

Mice bearing a homozygous deletion of *Math5* lose up to 80% of cells expressing RGC markers. This leaves an excess of progenitor cells in the proliferative layer of the retina (Brown *et al.*, 2001; Wang *et al.*, 2001). The presence of these excess retinal precursor cells after the first wave of neurogenesis leads to the specification of large numbers of amacrine cells (Brown *et al.*, 2001; Wang *et al.*, 2001). In zebrafish, mutation of *ath5* (*lak*) abolishes the first wave of differentiation in the retina, which produces RGCs (Kay *et al.*, 2001). Likewise, cells in the Drosophila eye disc that do not transition through and *ato*+ state, cannot differentiate into photoreceptors (Greenwood and Struhl, 1999).

In the developing vertebrate retina, the neuronal repressor *Hes1*, a homologue of the fly repressor *hairy,* is expressed in the ventricular zone and is absent from the GCL (Tomita *et al.*, 1996). *Hes1* positive cells remain in the proliferative layer and do not differentiate into neurons (Tomita *et al.*, 1996). In *Hes1* deficient mice, the waves of differentiation that produce the different neurons of the retina are greatly accelerated (Tomita *et al.*, 1996), and neurogenesis proceeds at the expense of proliferation in RPCs. Loss of both proneural repressor proteins (Hairy and Extramacrochaete) in *Drosophila* also results in premature differentiation of photoreceptors (Brown *et al.,* 1995). In *Hes1-/-* mice, fewer progenitor cells are available for specification at each stage so the resulting retinas have very few neurons (Tomita *et al.,* 1996). Based on these data, *Hes1* is believed to repress neurogenesis in proliferative cells, thereby preventing premature differentiation. Consistent with this idea, overexpression of *Hes1* in postnatal rat retinal progenitors increases the number of Müller glia at the expense of neurons (Furukawa *et al.*, 2000).

Hes1 and *Math1* have an antagonistic relationship in vertebrate retinal neurogenesis. *Hes1* is expressed in progenitor cells and represses premature differentiation, while *Math5* is expressed in early neural precursors and promotes RGC differentiation. Additionally, in heterozygous *Pax6*^{Sey} mice, fewer *Math5* expressing cells are present, while the domain of *Hes1* expression is expanded (Brown *et al.*, 1998). In homozygous *Pax6*^{Sey} mice, *Math5* expression is abolished (Brown *et al.*, 1998). Since the loss of *Math5* expression correlates with an expansion of *Hes1*, these data provide additional evidence for the antagonistic relationship between these genes in vertebrates. This antagonistic relationship is highly reminiscent of that observed between *hairy* and *atonal* in the fly.

Summary

In conclusion, we have reviewed the *ey* paradigm as characterized in *Drosophila*, and we have evaluated its potential conservation in vertebrates. The evidence to date does not support the idea that the entire *ey* genetic hierarchy is conserved in the vertebrate eye. On the other hand, some genetic parallels do exist, as *Pax6* activity is highly reminiscent of *ey*, and *Six3* and *Six6* have some characteristics of *so*. Nonetheless, it is the overall epistatic relationship amongst the vertebrate homologues of *ey*, *so*, *eya*, and *dac* that appears to be specifically absent in the vertebrate eye.

We have also briefly reviewed some aspects of neuronal specification in the vertebrate retina, as the control of both cell proliferation and RGC identity by bHLH transcription factors is highly reminiscent of R8 photoreceptor differentiation in the fly. Thus in both cases, specific features of developmental regulatory cassettes have been retained and re-deployed in vertebrate ocular organogenesis.

As vertebrate geneticists, how then do we best utilize the wealth of genetic information that is available to us? The aim is to continue to utilize the wealth of knowledge emerging from studies in *Drosophila* to guide our inquiries into mechanisms of vertebrate development. In the event that a genetic cassette is not maintained, this too is instructive, as it indicates that the particular role that the genetic hierarchy evolved to accomplish is either not relevant, or not sufficient, to meet the complexity of the vertebrate system. It is, however, increasingly clear that genetic pathways orchestrating *Drosophila* eye formation have been adapted in vertebrates for

multiple organogenic processes, and that they have been partially maintained in the eye, despite the significant divergence of vertebrate and invertebrate eyes.

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