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The Molecular Circuitry Governing Retinal Determination

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Abstract

The developing eye of the fruit fly, *Drosophila melanogaster*, has become a premier model system for studying the genetic and molecular mechanisms that govern tissue determination. Over the last fifteen years a regulatory circuit consisting of the members of the Pax, Six, Eya and Dach gene families has been identified and shown to govern the specification of a wide range of tissues including the retina of both insects and mammals. These genes are not organized in a simple developmental pathway or cascade in which there is a unidirectional flow of information. Rather, there are multiple feedback loops built into the system rendering its appearance and functionality more in line with the workings of a network. In this review I will attempt to describe the genetic, molecular and biochemical interactions that govern the specification of the *Drosophila* compound eye. In particular, the primary focus will be on the interactions that have been experimentally verified at the molecular and biochemical levels. During the course of this description I will also attempt to place each discovery in its own historical context. While a number of signaling pathways play significant roles in early eye development this review will focus on the network of nuclear factors that promote retinal determination.

Keywords

Drosophila; retinal determination; eye specification; molecular circuit

Introduction

Over the past three decades the compound eye of the fruit fly *Drosophila melanogaster* has become a premier model system for studying a wide range of developmental decisions including tissue determination and patterning, cell fate specification, compartment boundary establishment and maintenance, cell rotation and polarity as well as cell proliferation and death. A landmark paper by Ready and co-workers on patterning in the fly retina was published in *Developmental Biology* in 1976 and served as starting point for a series of detailed studies on the cellular mechanisms underlying pattern formation within the eye (Lebovitz and Ready, 1985; Cagan and Ready, 1989a,b; Tomlinson and Ready, 1987a,b, 1988; Wolff and Ready, 1991a,b; Longley and Ready, 1995). Collectively, these studies have served as the baseline for hundreds of studies on mutants that affect the development, structure and physiology of the retina.

During the mid to early 1990s several laboratories developed a renewed interest in a set of mutations that eliminated the compound eyes in adult flies. Many of these mutations had

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languished in obscurity for decades while researchers focused their attentions on the more “exciting” processes of cell fate determination and signal transduction. But the astonishing demonstration by Georg Halder and coworkers identifying a selector gene for the eye, one whose orders could at times supercede the instructions that had already been set in place within non-retinal tissues (see below), sparked a renewed interest in these mutants and the underlying genes (Figure 1, Halder et al., 1995).

According to the current state of the field, the early fate of the *Drosophila* eye is determined by the activities of approximately ten nuclear proteins and five signal transduction cascades. Each nuclear factor and signaling pathway is represented in vertebrates with several clinical retinal disorders being associated with mutations in the human orthologs of these genes (Figure 2; reviewed in Hanson, 2001). In flies these nuclear proteins are comprised of the Pax6 homologs *eyeless* (*ey*) and *twin of eyeless* (*toy*), the Six family members *sine oculis* (*so*) and *optix*, a tyrosine phosphatase *eyes absent* (*eya*) and a distant relative of the Ski/Sno family of proto-oncogenes, *dachshund* (*dac*; Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994; Serikaku et al., 1994; Halder et al., 1995; Cznery et al., 1999; Seimiya and Gehring, 2000). In support are the activities of *teashirt* (*tsh*), a transcription factor with multiple zinc finger DNA binding domains and *homothorax* (*hth*), a homeobox containing transcriptional co-activator (Pai et al., 1997; Pan and Rubin, 1998). As this review will highlight, these genes are not organized in a simple developmental pathway or cascade in which there is a unidirectional flow of information. Rather, there are multiple feedback loops built into the system rendering its appearance and functionality more in line with the workings of a network (Figure 3). Integrated into this system at multiple points are the activities of several bona fide signaling cascades including the Notch, EGF Receptor, Hedgehog, TGF β and Wnt pathways. Certain pathways appear to promote eye development, some are inhibitory while others appear to switch between promoting and inhibiting eye development depending upon the temporal and spatial setting (Treisman and Rubin, 1995; Chen et al., 1999; Kurata et al., 2000; Hsaio et al., 2001; Kumar et al 2001; Baonza and Freeman, 2002). This review will focus strictly on the network of nuclear proteins that make up the “traditional” retinal determination or eye specification network.

Pax6: the first master of the eye

May Hoge first identified the *eyeless* (*ey*) locus in 1915 when she recovered a set of mutants that mapped genetically to the fourth chromosome and whose compound eyes were completely eliminated (Hoge, 1915). This seminal discovery began what has been a long but exciting adventure into the mechanisms that govern eye development not just in flies but also in a sweeping range of organisms that includes mice, primates and humans. The crowning achievements in this story were made in the 1990s and are reported in a pair of papers from Walter Gehring's group in Switzerland. In the first manuscript, the *eyeless* gene was shown to encode a transcription factor that contained both a paired box and a homeobox. More exciting was the observation that *ey* shared extensive homology with the mouse and human *Pax6* genes (Quiring et al., 1994). Disruptions in these three genes are the underlying cause of the eyeless phenotype in flies, the Small eye (Sey) phenotype in mice and the human disorder Aniridia (Hill et al., 1991, 1992; Ton et al., 1991; Hanson et al., 1993; Quiring et al., 1994). Following on the heels of this finding was the explosive discovery that forced expression of fly *eyeless* and mammalian *Pax6* in non-retinal tissues is capable of coaxing such tissues into adopting an eye fate (Halder et al., 1995). The impact of this paper has been remarkable: it strengthened the argument that studies of the *Drosophila* retina could have major developmental and clinical implications on studies of the vertebrate eye, a tissue with a very different structure. Furthermore, it sparked a profound rethinking of the evolutionary origins of the eye (Halder et al., 1995b; Callerts et al., 1997; Gehring and Ikeo, 1999). Together, phenotypes of loss-of-function mutants and the effects of forced expression appeared to place *ey* at or near the top of

a yet to be identified regulatory cascade. These characteristics became the benchmarks by which all subsequent genes are measured. Only those that fulfill these criteria (and several followed) are given the coveted title along with *eyeless* of being a “master control gene” for eye development (Figure 1). But for the moment *Ey/Pax6* was the first and only master of the eye.

Subsequent analysis identified a second Pax6 homolog in *Drosophila* called *twin of eyeless* (*toy*; Cznery et al., 1999). This was unexpected as both basal insects such as the flour beetle, *Tribolium*, and all vertebrate species, including mice and humans, have only a single *Pax6* gene. The discovery of *toy* begged the question of why flies require two *Pax6* genes when virtually all other organisms get along fine with a single gene. Do these genes play redundant functions? Do they split the chores of vertebrate *Pax6* and activate different sets of target genes?

While both genes are expressed in nearly identical patterns within undifferentiated cells of third instar eye imaginal discs, several observations indicate that there is a one-way flow of information between the two Pax6 genes. A comparison of transcriptional profiles revealed that *toy* expression is initiated throughout the visual primordium, including the eye primordia, prior to that of *ey* (Cznery et al., 1999). And later it was determined that expression of *ey* within the eye disc is dependent upon functional Toy (Krohnhamn et al., 2002). Genetic epistatic experiments demonstrated that forced expression by *toy* is sufficient to activate *ey* transcription but not vice versa (Cznery et al., 1999). Located within an intron of *ey* is an eye specific enhancer that contains several Pax6 binding sites that can be recognized by Toy (Cznery et al., 1999; Hauck et al., 1999). In contrast, functional Pax6 sites have not been identified within the *toy* transcriptional unit. These data suggest that (1) *toy* transcription is activated independently of any Pax6 inputs (2) Toy protein binds to Pax6 sites located within an enhancer element of *ey* thereby directing its expression; and (3) that a feedback loop in which Ey regulates *toy* transcription does not exist. The first step of the regulatory network has been set. Interestingly, a comprehensive genome wide search for *ey* binding sites identified several putative sites within the *ey* transcription unit itself suggesting that Ey may regulate and amplify its own expression (Ostrin et al., 2006). An earlier effort involving a microarray analysis of gene expression during ectopic eye development also implicated Ey in its own auto regulation (Michaut et al., 2003). However, it is not clear if this auto regulation occurs during normal eye development, as a transcriptional reporter containing the *ey* eye specific enhancer can be activated even in *ey* mutants (Halder et al., 1998).

While it appeared that the two fly Pax6 genes do not act redundantly in the retina, the results raised the question of what were the individual roles of Toy and Ey in eye development. Is the role of *toy* just to activate *ey* in the retinal primordia or does *toy* go on further to activate other downstream genes? The latter model appears to be more accurate with at least one verified example of a target being bound and activated by both Pax6 genes. Initial efforts towards identifying Pax6 transcriptional targets focused on *sine oculis*, a gene required for the development of the entire visual system (Cheyette et al., 1994; Serikaku et al., 1994). Activation of *so* transcription in the eye imaginal disc is directly regulated by both Pax6 genes through binding of Ey and Toy to different sites within an eye specific enhancer (Halder et al., 1998; Nimi et al., 1999; Punzo et al., 2002). This result clearly established a direct link between Pax6 and *So*, a member of the Six family of homeobox transcription factors. Satisfyingly, *so* has also been independently identified as a target of Ey in a genome-wide bioinformatic screen (Ostrin et al., 2006).

This same screen by Ostrin and co-workers identified another nineteen genes of which three (*eyeless*, *eyes absent* and *optix*) are known retinal determination genes. The remaining genes were novel with respect to potential roles in eye development. One identified target, *optix*, is considered an evolutionary cousin of *so* as both genes arose from the duplication of an ancestral

Six gene (see below). An enhancer element within *optix* containing Ey binding sites was identified and subsequently verified experimentally by its ability to direct expression of a reporter in the normal *optix* expression pattern and by EMSA for Ey binding (Ostrin et al., 2006). However, mutations in the *optix* eye specific enhancer do not yet exist thus the phenotypic consequence of uncoupling Ey from regulating *optix* during eye specification remains undetermined.

Mutations within an eye specific enhancer of *eya* were shown to be the underlying cause of the no-eye phenotype of certain loss-of-function mutants (Bui et al., 2000; Zimmerman et al., 2000). Prior work had suggested that Ey can induce expression of *eyes absent* and it had always been expected that this would be the result of direct binding of Ey to an eye specific enhancer (Halder et al., 1998). The recovery of *eya* in two genome wide genomic/bioinformatic screens for Ey targets appeared to corroborate this prediction (Michaut et al., 2003; Ostrin et al., 2006). However, it is interesting that the predicted Ey binding sites, while being experimentally verified by EMSAs, neither map to the previously identified eye specific enhancer element nor drive expression of a reporter in imaginal discs (Ostrin et al., 2006).

In summary, the first known eye specification gene to be activated in the developing eye primordia is the Pax6 gene *toy*. Toy sits atop the cascade and directly induces the expression of the other Pax6 gene *ey*, presumably among other targets. Together both Pax6 genes bind to and regulate the expression of the downstream network member *so*. These steps in the cascade have been verified by (1) expression pattern analysis during normal development, in loss-of-function mutants and during the forced induction of ectopic eye assays; (2) bioinformatic analysis of specific enhancer elements as well as genome wide interrogations; (3) *in vivo* molecular epistatic assays with enhancers fused to molecular reporters; and (4) electrophoretic mobility shift assays (EMSA). Several, but not all of these methods, have also supported roles for Ey in the direct regulation of *optix* and *eyes absent*.

Let's play king of the hill: the Six, Dach and Eya Genes

In addition to *ey*, loss-of-function mutations in a number of genes including *sine oculis*, *eyes absent*, *dachshund* and *eyegone* are known to display a similar no-eye phenotype (Milani, 1941; Ives, 1942; Sved, 1986; Mardon et al., 1994). Despite this dramatic phenotype, several of the mutants were forgotten and neglected for decades. It was not until the phenomenal revelation that *ey* is a selector gene for the eye that these genes experienced a significant resurgence in interest and excitement. Renewed curiosity led to the demonstration that, like *ey*, forced expression of these genes in non-retinal tissues is also sufficient to redirect tissue fates and support eye formation (Shen and Mardon; Bonini et al., 1998; Jang et al., 2003; Weasner et al., 2007). Ey/Pax6 no longer appeared to be the sole ruler of the retina and the race began to see who was the true master of the eye.

The Six Gene Family

sine oculis is the founding member of the Sine oculis box (Six) family of homeobox containing transcription factors. *so* along with *optix* and *DSix4* arose through a series of duplication events and comprise the three family members in *Drosophila* (Seo et al., 1999). These duplications appear to be quite ancient as both *Tribolium* and *Oncopeltus* have all three genes. The evolutionary history of this family includes an additional duplication of each member as vertebrate systems have a total of six family members with Six1/2, Six3/6 and Six4/5 being the homologs of *sine oculis*, *optix* and *DSix4* respectively (Seo et al., 1999; Seimiya et al., 2000). Each protein contains a homeobox DNA binding domain and the Six domain through which protein-protein interactions are mediated. Of the three genes *DSix4* does not appear to be expressed or function during retinal development.

The original characterizations of *so* loss-of-function mutations indicated that *so* is required for the specification of the entire visual system. In particular, the compound eyes fail to correctly proliferate, are plagued by massive cell death and are incapable of supporting photoreceptor development (Cheyette et al., 1994; Serikaku et al., 1994). For many years ectopic eyes were not recovered in forced expression experiments. However, it was considered a member of the eye specification network because of its effect on the eye in loss of function mutants, its direct link to *ey* and through the exciting observation that So formed a biochemical complex with Eya, a transcriptional co-activator (Cheyette et al., 1994; Serikaku et al., 1994; Nimi et al., 1999; Pignoni et al., 1997). The formation of the So-Eya complex was postulated to be required for the activation of downstream target genes. Consistent with this model, co-expression of the two genes had a synergistic effect and ectopic eyes were recovered at frequencies that exceeded their individual rates (Pignoni et al., 1997). More recently however, *so* has been shown capable of inducing eye formation on its own although the range of tissues and cell types that can be transformed into retina by *so* is far more limited than that of *ey* (Weasner et al., 2007). One potential explanation is that several Six proteins appear to be a weak transcriptional co-activators (Yu et al., 2004). It might be the case that in just the right cell types and temporal expression patterns So is sufficient to promote eye formation without the strong co-activator, Eya.

One of the major pieces of molecular and biochemical evidence suggesting that the eye specification cascade functions more like a network than a linear pathway came from the identification of So binding sites within the *ey* eye specific enhancer (Pauli et al., 2005). The clear implication of this result is that a critical feedback loop between two members of the network exists. Earlier reports had discounted such an interaction based on the continued demonstrable levels of Ey in discs mutant for *so* (Halder et al., 1998). The activation of *ey* in *so* mutants can now be seen in a different light, in which *ey* continues to be activated, at least in part, by the activity of Toy at the *ey* eye specific enhancer. The *ey* $\longleftrightarrow$ *so* interaction remains to this day the only example of an experimentally verified molecular feedback loop within the retinal determination network. It should be noted that there is a mountain of genetic, expression and epistatic evidence to suggest that a significant number of retinal determination genes participate in positive and/or negative feedback loops (Pignoni et al., 1997; Shen and Mardon, 1997; Bonini et al., 1998; Halder et al., 1998; Pan and Rubin, 1998).

So binding sites have also been identified within *so* itself, implicating it in an auto regulatory feedback loop (Pauli et al., 2005). It is not obvious why such auto regulation would be required anterior to the morphogenetic furrow as *ey* is co-expressed with *so*. However, as *toy* and *ey* expression ceases at the furrow the *so* auto regulatory loop may be required to maintain high *so* levels in developing photoreceptor cells posterior to the advancing furrow. Additionally, *so* binding sites were identified within the segment polarity gene *hedgehog* (*hh*; Pauli et al., 2005). In addition to its role in correctly establishing segmental boundaries in the embryo, the *hh* signaling pathway is known to play key roles in the establishment of compartment boundaries within imaginal discs (Ingham et al., 1991; Tabata and Kornberg, 1994; Basler and Struhl, 1994). One key role for *hh* is in the initiation and progression of the morphogenetic furrow, the mobile compartment boundary in the eye separating anterior undifferentiated cells from posterior terminally differentiated photoreceptor cells (Ma et al., 1993; Heberlein et al., 1993). Flies harboring the *bar-3* mutation have small kidney shaped eyes, hence the name. The underlying cause for this phenotype is the deletion of an eye specific enhancer of *hh*. Deletion of this enhancer removes two So binding sites as well as three sites for the binding of the Ets transcription factor Pointed (Pnt), through which instructions from the EGF Receptor pathway are transmitted (Pauli et al., 2005; Rogers et al., 2005). Hh protein is produced in cells posterior to the furrow. Cells within and ahead of the furrow are competent to receive this signal and undergo changes in cell morphology and gene expression in response (reviewed in Heberlein and Moses, 1995). Thus, it may be that So binds to the *hh* eye specific enhancer in cells along

the posterior margin of the eye field where the furrow initiates and in developing photoreceptors (this time with Pnt) to promote the continued progression of the furrow across the retinal epithelium.

A consensus-binding site for *optix* mammalian homologs Six3/6 has been identified (Zhu et al., 2002(Jemc and Rebay, 2007b)). The binding site is similar to that which has been identified for So and its vertebrate homologs Six1/2. However, a genome wide search for *optix* target genes in *Drosophila* has not been published therefore the extent to which So and Optix share common targets in flies is unknown. However, it is likely that their activities will be considerably different. So and its homologs bind strongly to the Eya family of transcriptional co-activators. In contrast, Optix and its homologs show the strongest interactions with members of the Groucho (Gro) family of co-repressors (Kobayashi et al., 2001; Zhu et al., 2002; Lopez-Rios et al., 2003; Kenyon et al., 2005). As *ey* appears to directly activate the expression of both Six genes ahead of the furrow it will be of the utmost importance to identify and compare both target gene lists.

Eya: A family of transcriptional activators and tyrosine phosphatases

The *eyes absent* gene is the founding (and only) member of the Eya family of transcriptional co-activators in *Drosophila* (Bonini et al., 1993). In contrast, this family is represented in vertebrates by four homologs, Eya1-4 (reviewed in Hanson et al., 2001; Jemc and Rebay, 2007). The rarity of finding viable recessive mutations that eliminate the compound eyes in adult flies made several *eya* mutants valuable for studying early decisions in eye formation. And as interest in mutants with this unusual phenotype grew, *eya* became a prime candidate for membership in the retinal determination network. However, this connection was not necessarily obvious as the first major paper on the role that *eya* played in the retina appeared several years ahead of the demonstration that *ey* could induce ectopic eye formation and focused on the role that *eya* played in cell survival and death in the retina (Bonini et al., 1993). But, as other genes with similar mutant phenotypes were shown to induce ectopic eye formation, it became clear that these factors were likely to work together in a single pathway (Halder et al., 1995; Shen and Mardon, 1997). And as one would expect forced expression of *eya* was sufficient to induce retinal development thus placing *eya* squarely within the retinal determination network (Bonini et al., 1997; Pignoni et al., 1997; Jemc and Rebay, 2007a).

eya, unlike all other RD network members, is not predicted to encode a transcription factor. Instead, it functions as a transcriptional co-activator through interactions with several DNA binding proteins including So and Dachshund (Dac; Pignoni et al., 1997; Chen et al., 1997). These interactions are mediated through a highly conserved 271 amino acid motif called the Eya domain (ED). A second less conserved domain (ED2) harbors the activation domain (Silver et al., 2003). The best-studied interaction involves the formation of the So-Eya complex, which is thought to be an integral step in eye specification as both genes have identical expression patterns in the eye. Additionally, co-expression of both factors synergizes to produce ectopic eyes at a frequency higher than that seen with either individual gene (Pignoni et al., 1997). The Eya-Dac complex may also play a significant role in the eye as Dac is predicted to be a DNA binding protein and co-expression of both genes also synergistically increases the rate of ectopic eye formation (Chen et al., 1997). Interestingly, in the eye Dac function does not absolutely require the domain that mediates interactions with Eya (Tavsanli et al., 2004). Coupled to this unexpected finding is the demonstration that several Six family members bind to members of the Dach family (Li et al., 2002). Thus raising the possibility that So may act as a bridge for interactions between Eya and Dac within the developing eye.

What are the transcriptional targets of Eya? Ideally one would like to discuss this in terms of So-Eya and Dac-Eya activation complexes. But as direct targets for Dac in the genome have

yet to be identified the most extensive knowledge is from studies on So and the So-Eya complex. With respect to genes involved in eye specification So binds to sites within the eye specific enhancers of *ey*, *so* itself and *hh*. Therefore the expectation is that Eya contributes significantly to the activation of these three genes through its physical interactions with So. A recent study that combined a microarray analysis of the effects of forced expression of *eya* and *in silico* predictions of So binding sites identified additional putative targets of the So-Eya complex (Jemc and Rebay, 2007b). One potential target is *string*, a gene that has been implicated in the cell cycle and pattern formation in the retina (Thomas et al., 1994; Jemc and Rebay, 2007b). Loss of *so* or *eya* in the eye disc, either in clones or in whole discs, has dramatic effects on cell proliferation and tissue growth (Bonini et al., 1993; Pignoni et al., 1997). These results indicate that the retinal determination cascade, particularly *so* and *eya*, interact with the cell cycle machinery to regulate the balance between tissue growth and specification.

In addition to its role as a transcriptional co-activator, Eya has been shown to possess tyrosine phosphatase activity and is a member of the haloacid dehalogenase (HAD) superfamily (Li et al., 2003; Rayapureddi et al., 2003; Tootle et al., 2003). This activity has been mapped to the conserved C-terminal Eya domain. To date, three biologically relevant substrates have been identified *in vitro*: Eya itself, RNA Polymerase II and MAPK (Li et al., 2003; Tootle et al., 2003; Rayapureddi et al., 2005). These substrates have yet to be verified *in vivo* but their identification serves as a starting point for furthering our understanding of the mechanisms that underlie the roles that Eya plays in regulating its targets. Although So and Dac have not been identified *in vitro* or *in vivo* as targets of dephosphorylation by Eya, it is tantalizing to think of models in which Eya, through its phosphatase activity, regulates the formation, stability and function of the So-Eya and Dac-Eya complexes during eye development.

A much predicted and sought after target of Eya transcription (and that of So as well) is *dachshund*. A fair amount of circumstantial evidence has long suggested that *dac* is a direct target of the So-Eya complex. For instance, all three genes are co-expressed in identical patterns in anterior regions of the eye (Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994; Serikaku et al., 1994). Additionally *dac* transcription is largely dependent upon the activity *so* and *eya* (Pappu et al., 2005; Anderson et al., 2006). A dissection of *dac* regulatory sequences has identified and experimentally verified enhancers that direct expression in the eye (Pappu et al., 2005). One of these enhancers contains a set of functional So binding sites (Pappu et al., 2005). Interestingly and somewhat disturbingly, several genome-wide *in silico* screens have failed to identify *dac* as a potential direct target of So binding (Pauli et al., 2005; Jemc and Rebay, 2007b). Thus there is a note of caution attached to the results of *in silico* screens for binding sites. Much is dependent upon individual parameters that are used in the searches, thus the failure to identify targets in such screens is not necessarily an indication that a predicted interaction does not take place. The activity of a second eye specific enhancer appears to be dependent upon *ey* but not of *so* or *eya*. This interaction appears to be indirect as the enhancer is responsive to Ey but does not contain Pax6 binding sites (Pappu et al., 2005). It raises the exciting possibility that an unidentified intermediate step lies between these two network members.

Dac: The last stop on the road to eye specification?

As the name implies, mutations in *dachshund*, were first identified based on their severe effects on leg development although the original designation for the gene was l(2)36Ae (Ashburner et al., 1990). These mutant flies also had rough eyes providing the first hint that l(2)36Ae played a role in retinal development. Several additional alleles were isolated in screens within the eye for dominant suppressors of a hypermorphic allele of the EGF Receptor. These newly named *dac* mutants also had severely deformed legs and eyes that were dramatically reduced in size which, in some cases were completely absent (Mardon et al., 1994). This initial report focused

on the role that *dac* plays in the initiation and progression of the morphogenetic furrow rather than any potential function in eye specification. However, the tantalizing co-expression of *dac* with *ey*, *so* and *eya* ahead of the morphogenetic furrow as well as the eyeless phenotype of viable pharate adult mutants suggested that bigger things were destined for *dac*. Indeed, a few years later *dac* was shown to also induce ectopic eye formation, placing it within the elite group of retinal selector genes (Shen and Mardon, 1997).

Members of the Dach family share limited homology with the Ski/Sno co-repressors and contain two highly conserved domains, DD1 and DD2 (Hammond et al., 1998). The DD1 domain contains a winged helix domain that is capable of contacting DNA, a transactivation domain and a binding site for members of the Six family of DNA binding proteins (Chen et al., 1997; Kim et al., 2002; Li et al., 2002). Additionally, the DD2 domain interacts physically with Eya proteins (Chen et al., 1997). These results immediately suggest several interesting mechanisms, each of which has very different transcriptional outputs. *Dac* could modulate transcription of target genes on its own, in separate heterodimeric complexes with *So* and *Eya*, or within the context of a trimeric complex that includes all three proteins. These predictions are not mutually exclusive. Thus, all three *Dac* dependent factors could function within a single tissue or even a single cell for that matter. A caveat to these results is that a structure/functional analysis of the *Dac* protein indicated that one of the conserved domains, DD2, which mediates interactions with *Eya*, is not required for normal *Dac* function in the eye (Chen et al., 1997; Tavsanlı et al., 2004). This suggests that, at least within the eye, a heterodimeric *Eya-Dac* complex does not exist. It still leaves open the possibility for *Dac* to either function on its own or within the context of the trimeric *Eya-So-Dach* complex.

The impact that *Dac* has on the eye specification regulatory circuit is currently difficult to ascertain as consensus binding sites and direct transcriptional targets are yet to be identified for this member of the retinal determination network. The role of Dach proteins in mammalian eye development is also somewhat of a mystery as mutations in the individual two mouse Dach genes fail to show any gross retinal abnormalities (Davis et al., 2001, 2006). Genetic/molecular epistatic experiments have indicated that forced expression of *dac* is sufficient to activate the expression of *ey* and *eya* (Shen and Mardon, 1997). Once a consensus binding site for *Dac* is identified, the eye specific enhancers of both genes (which have already been identified) would be prime candidates for bioinformatics and EMSA analysis. As *dac* has also been implicated in the initiation of the morphogenetic furrow, a genome wide analysis for direct downstream targets is likely to clarify the link between the eye specification cascade and the pathways that regulate pattern formation in the retina.

Teashirt: Going back and forth in the eye

It is actually quite rare to isolate a homozygous loss-of-function mutant that lacks the adult compound eyes. In fact mutations in only five genes with that phenotype have been identified so far (*ey*, *eyg*, *eya*, *so* and *dac*). This is likely due to the fact that nearly all of the genes involved in eye development also play significant roles in the development of other non-retinal tissues including those in the embryo. Null mutations for any of these genes leads to embryonic lethality. The viable no-eyed mutants (of the above mentioned genes) actually contain mutations/deletions of eye specific enhancers, a hard feat to repeat over and over again in the hundreds of genes that are thought or estimated to regulate the development of the eye (Thaker and Kankel, 1992).

Several lines of evidence (not presented here) suggest that the cast of characters that are likely to function within the retinal determination network is significantly larger than those discussed within this article. So how can those additional genes be identified? One innovative method for identifying new retinal determination genes was employed by D.J. Pan and Gerald Rubin.

They inserted the *dpp* disc enhancer at random positions within the genome in an effort to over express genes in all imaginal tissues. In a subset of these insertions the gene driven by the *dpp* enhancer would be expected to promote eye development in non-retinal tissues. Five insertions, all driving expression of *teashirt* (*tsh*), a gene previously known to function in the establishment of the trunk segments in the embryo, were shown to induce ectopic eye formation on the antenna (Fasano et al., 1991; Pan and Rubin, 1998).

Unlike the other retinal determination genes whose roles can be simply and unambiguously described as promoting eye formation, the role of *tsh* in eye development is complicated and at times contradictory in nature. This was first noticed during a careful survey of the expression patterns of the eye specification genes (Bessa et al., 2002). The authors demonstrated that in more anterior regions of the retina *tsh* could work with other members of the eye specification network, *ey* and *homothorax* (*hth*) to repress the expression of other network members, *so*, *eya* and *dac*. In contrast, within adjacent regions that abut the morphogenetic furrow it appears that *tsh* switches its activity and actually cooperates to promote the expression of the same genes that it was repressing earlier. One significant difference between the two adjacent domains is the loss of *hth* expression in the zone that borders the furrow. This difference is all the more interesting as Hth can form independent biochemical complexes with both Ey and Tsh suggesting that the interaction with Hth serves as the switch between the promoting and suppressing functions of *tsh* during eye development (Bessa et al., 2002; Singh et al., 2002).

tsh also functions disparately within the dorsal and ventral compartments of the early eye field (Singh et al., 2002, 2004). As is the case in the most anterior regions of the third larval eye disc, *tsh* represses eye formation in the early ventral eye primordium. This is achieved in part by the induction of *hth* by *tsh* (Singh et al., 2002). It has not been experimentally demonstrated, but it is predicted that following the induction of *hth*, a Tsh-Hth complex forms and represses eye specification gene expression in a manner similar to that of the anterior most portion of the eye. In contrast to its role in the ventral eye, *tsh* appears to promote eye development in the dorsal compartment of the retinal primordium (Singh et al., 2002). The transcriptional targets of *tsh* in the dorsal half of the retina are yet unidentified so a molecular mechanism for eye promotion in this area of the presumptive eye field is not known. One potential target is *ey* as *tsh* induces its expression during the formation of ectopic eyes (Pan and Rubin, 1998).

The ability of *tsh* to promote or suppress eye development depending upon the circumstances is likely tied to the early history of the eye primordium. All cells within the very early retina initially adopt a ventral fate. Early in the first instar dorsal identity is imprinted onto a subset of cells (Singh and Choi, 2003). Unsurprisingly, the regulatory circuits and signaling cascades that subdivide the retina into ventral and dorsal compartments cooperate with *tsh* to promote eye formation in the dorsal eye and suppress retinal development in the ventral half (Singh et al., 2004).

The effect that removal of *tsh* had on normal eye development has been harder to examine as removal of *tsh* in retinal mosaic clones yielded flies with no visible phenotypes, prompting the prediction that the requirement for *tsh* may be masked by at least one additional gene (Pan and Rubin, 1998). This prophecy may be in the process of being fulfilled by a second gene called *tiptop* (*tio*), which along with *tsh* arose through the duplication of an ancestral *tsh/tio* gene (Laugier et al., 2005). This second gene is expressed in an identical pattern to *tsh* in the developing eye and might be another eye specification gene. Its role in retinal determination is currently somewhat murky, as loss-of-function mutants have no effect on eye development (Laugier et al., 2005). Despite the absence of a phenotype, which could be masked by the activity of Tsh, the induction of ectopic eyes by forced expression of *tio* is an encouraging sign that it does play a role in early patterning (R. Datta and J.P. Kumar, unpublished results). More

molecular tools such as RNAi may have to be employed to elucidate the role that *tio* plays in eye specification.

Homothorax: A suppressor of eye development

As discussed above, *hth*, which is a homolog of mammalian Meis1, functions to suppress eye specification at the most anterior regions of the eye disc and within the ventral compartment of the early retinal primordium (Pai et al., 1998). Another function of *hth* is to prohibit the initiation of the morphogenetic furrow (Pichaud and Casares, 2000). The induction of *hth* is mediated by two signals: one from *tsh* and the other from the *wg* signaling pathway (Pan and Rubin, 1998; Pichaud and Casares, 2000). These signals had been shown previously to repress both eye specification and morphogenetic furrow initiation (Triesman and Rubin, 1995; Bessa et al., 2002; Baonza and Freeman, 2002; Singh et al., 2002). We now know that these inputs pass, in part, through *hth* but it is still unclear if the regulatory interactions are direct. Likewise, the consensus binding sites for Hth and its transcriptional targets are unknown. Thus it remains an open question as to how *hth* interacts with other members of the retinal determination network. Hth is known to function as a transcriptional activator (Inbal et al., 2001) suggesting that the suppression of eye development could be mediated by the activation of an intermediate transcriptional repressor. As consensus binding sites for both Hth and Exd have been determined (Ebner et al., 2005) *in silico* genome wide searches for targets and experimental verification will be critical to solving this piece of the retinal determination puzzle.

Pax6(5a): To grow or not to grow, that is the question for the eye

Within the retinal determination network lies a pair of genes called *eyegone* (*eyg*) and *twin of eyegone* (*toe*). These genes arose from a duplication of an ancestral gene and as the name implies, flies harboring mutations within *eyg* lack compound eyes (Ives, 1942). Like several of the other retinal determination genes, *eyg* mutants were essentially placed on a shelf and forgotten until the late 1990s when efforts to identify and clone retinal determination genes had reached a frenzied pace. It was around this time that *eyg* was shown to code for a unique Pax gene that contains a Prd class homeodomain with a serine at position 50 but has only one of the two functional DNA binding motifs (RED) that make up the Prd domain (Jones et al., 1998; Jun et al., 1998). This makes *Eyg* structurally similar to Pax6(5a), an isoform of vertebrate Pax6, which arises from an alternate splicing event (Epstein et al., 1994). Forced expression of *eyg* was sufficient to induce ectopic eye formation although the location of the newly formed retinas was limited to the ventral side of the head adjacent to the normal compound eye (Jang et al., 2003). *eyg* appeared to meet the two requirements needed for being placed within the privileged group of retinal selector genes.

The initial loss and gain of function phenotypes strongly suggested that *eyg* functions as a bona fide retinal determination gene. However, upon further examination it became clear that *eyg* played a greater role in cell proliferation than in retinal determination. Among the evidence that supports this contention is the demonstration that *eyg* is expressed along the equator of the eye (Chao et al., 2004). The equator, which serves as a dividing line for dorsal-ventral patterning, is also the region for high Notch activation levels (Cho and Choi, 1998). Notch has been shown to regulate cell proliferation and tissue growth in the early eye primordium (Cho and Choi, 1998; Dominguez and de Celis, 1998). Second, forced expression of *eyg* can stimulate cell proliferation in wild type retinas and can rescue proliferation defects in Notch mutants (Jang et al., 2003; Chao et al., 2004; Dominguez et al., 2004). And finally, *eyg* expression is lost in discs or clones of cells that have reduced Notch pathway activity (Chao et al., 2004; Dominguez et al., 2004). Third, *eyg* is capable of inducing ectopic morphogenetic furrows in the eye disc but cannot promote retinal development in non-retinal tissues (Chao et al., 2004; Dominguez et al., 2004).

The ability to promote tissue growth in the retina appears to set *Eyg* apart from the canonical Pax6 gene *Ey*, whose major role in the eye is in early tissue specification (Dominguez et al., 2004). These Pax genes also differ in the way they influence the transcription of their respective target gene sets. Unlike *Ey*, which functions as a transcriptional activator, both *Eyg* and *Toe* serve as dedicated transcriptional repressors (Yao and Sun, 2005, Yao et al., 2008). So how are these two genes integrated into the retinal determination network? Do they work in concert with *Ey* and the other eye specification genes or do they influence a completely independent set of target genes?

Genetic and molecular epistatic interaction studies have suggested that *ey* and *eyg* represent two separate branch points within the overall retinal determination network (Hunt, 1970; Dominguez et al., 2004). However, both genes are targets of the Notch signaling pathway. As we have discussed Notch mediated signaling activates *eyg* expression, which in turn stimulates cell proliferation (Chao et al., 2004; Dominguez et al., 2004). The Notch pathway also mediates the determination of appendage identity by influencing *ey* and *toy* expression (Kurata et al., 2000; Kumar and Moses, 2001). As to the targets of the fly Pax6(5a) genes, *eyg* has been shown to promote cell proliferation by activating unpaired (*upd*) expression and to function in the initiation of pattern formation by repressing *wingless* (*wg*; Hazelet et al., 1998). As binding sites for either *Eyg* (or *Toe* for that matter) have not been determined it is not known if either of these interactions are direct. Nor is it known if any of the remaining retinal determination genes are targets of either Pax6(5a) gene. Conversely, to date neither *eyg* nor *toe* has been shown in *in silico* studies to harbor consensus-binding sites for any of the other network members. Thus, the relative position of either *eyg* or *toe* to each other or to the other eye specification genes remains an open question. It is clear however that these two Pax6(5a) genes serve as a molecular link between two circuits; one responsible for specifying the fate of the eye and one responsible for regulating cell proliferation.

Concluding Remarks

This review has highlighted various aspects of the regulatory circuitry that governs eye specification in *Drosophila*. In particular I have focused on the regulatory interactions that have been tested at the molecular and biochemical level. In the process of documenting experimentally verified binding sites in eye specific enhancers and the formation of biochemical complexes I hope that it has become clear that despite significant progress, our journey towards a total understanding of how the eye is specified is not yet over. In fact, it is more likely the case that the voyage has in many ways just begun. A myriad of putative genetic interactions remain at that genetic level. Each of these will have to be pursued further if we are to have any hope of understanding the network in its entirety. The work on the fly eye also indicates that there are likely to be many more genes within the RD network. These will be hard to find and, consequently, creative molecular, genetic and bioinformatic screens will have to be designed to identify them. And finally, many members of the retinal determination network function as a unit to specify the fates of many other tissues. How this circuit is modified to produce multiple outcomes, each one quite distinct from the other, may be one of the most interesting questions. The answer may turn out to be one of the most elusive. We can be encouraged, however, by the continual development of better *in silico* algorithms, the increasing number of sequenced genomes and the ever-increasing sophistication level of experimental verification. It is my hope that future authors of similar reviews will be able to trace the molecular and biochemical development of the eye from its embryonic beginning on through the labyrinth of interactions and on to the final steps of eye specification.

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Figure 1.
RD genes are necessary and sufficient to promote eye development

Drosophila Gene(s)	Molecular Function	Domain(s)	Vertebrate Gene(s)
eyeless, twin of eyeless	transcription factor	paired/homeodomain	Pax6
eyegone, twin of eyegone	transcription factor	paired (truncated)/homeodain	Pax6(5a)
eyes absent	co-activator/phosphatase	P/S/T	Eya1-4
dachshund	transcription factor	winged helix turn helix	Dach1-2
sine oculis/optix/DSix4	transcription factor	homedomain	Six1-6
teashirt	transcription factor	zinc finger	Tsh1-4
homothorax	transcription factor	homeodomain	Meis1

Figure 2.
Molecular function of RD genes in flies and vertebrates

Figure 3.
Molecular Circuit Governing Eye Development