

Transcriptome Analysis of the Planarian Eye Identifies *ovo* as a Specific Regulator of Eye Regeneration

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SUMMARY

Among the millions of invertebrate species with visual systems, the genetic basis of eye development and function is well understood only in *Drosophila melanogaster*. We describe an eye transcriptome for the planarian *Schmidtea mediterranea*. Planarian photoreceptors expressed orthologs of genes required for phototransduction and microvillus structure in *Drosophila* and vertebrates, and optic pigment cells expressed solute transporters and melanin synthesis enzymes similar to those active in the vertebrate retinal pigment epithelium. Orthologs of several planarian eye genes, such as *bestrophin-1* and Usher syndrome genes, cause eye defects in mammals when perturbed and were not previously described to have roles in invertebrate eyes. Five previously undescribed planarian eye transcription factors were required for normal eye formation during head regeneration. In particular, a conserved, transcription-factor-encoding *ovo* gene was expressed from the earliest stages of eye regeneration and was required for regeneration of all cell types of the eye.

INTRODUCTION

The vertebrate eye is a structure of striking complexity in form and function. Consequently, understanding the evolution and development of eyes has been a classic challenge. The human eye is also subject to numerous pathologies that are poorly understood, such as heritable retinopathies and age-related degeneration. Genetic studies in model invertebrates have the potential to advance the understanding of eye evolution and development, and of the functions of conserved genes associated with eye disorders.

The relevance of invertebrate models to vertebrate systems depends in part on whether homology exists between most bilaterian eyes, a difficult point to establish based on morphological studies alone. Establishing some degree of common ancestry

between vertebrate and invertebrate (primarily *Drosophila*) visual systems has been a success of comparative molecular genetics. Two findings figure prominently in understanding the relationship between diverse animal eyes. First, two photoreceptor neuron categories, rhabdomeric (microvillar) and ciliary, exist throughout the Bilateria and rely on conserved R-opsin and C-opsin signal transduction pathways, respectively (Arendt, 2003). Second, embryonic development of multiple eye types involves *Pax-6*, *Sine oculis*, *Eyes absent*, and *Otx* gene family members. These observations have led to the suggestion that photoreceptor cell types were already present prior to the existence of the Bilateria, and that the common ancestor of the Bilateria utilized transcription factors for eye development still commonly used in extant eyes (Nilsson, 2009).

To explore many conserved features of eye biology, additional invertebrate model eyes must be established. The morphology, cell type composition, and set of gene activities present in ancestral bilaterian eyes remain largely unknown, and can only be inferred using data from multiple extant eyes belonging to diverse animal phyla. Invertebrate models are not yet established for extensive genetic study of several common aspects of eye biology not characteristic of *Drosophila* eyes, such as the formation of optic cups. Furthermore, many genes associated with eye disease have not yet been identified and studied in invertebrate models. Certain invertebrate species allow for study of regenerative repair of eye damage, an increasingly important therapeutic approach.

The planarian *Schmidtea mediterranea* is an emerging invertebrate model system that is highly amenable to gene function studies with RNAi. Planarians are ideal for the study of eye evolution because they are members of the Lophotrochozoa, the sister grouping of phyla to the Ecdysozoa (which includes *Drosophila* and *Caenorhabditis elegans*), and therefore exist at an important and understudied position in the animal phylogeny (Tessmar-Raible and Arendt, 2003). Planarian eyes are true cerebral eyes, as they connect via axon tracts to the brain (Agata et al., 1998), and express orthologs of *Otx* (Umesono et al., 1999), *Sine oculis* (Pineda et al., 2000), and *Eyes-absent* (Mannini et al., 2004). Planarians have rhabdomeric photoreceptor neurons (PRNs), pigment cells (PCs), and a pigmented optic cup structure - features that are common among cerebral eyes (Figure 1A). Photoreceptive organelles in the planarian eye face the optic cup in an inverse orientation, similar to the orientation

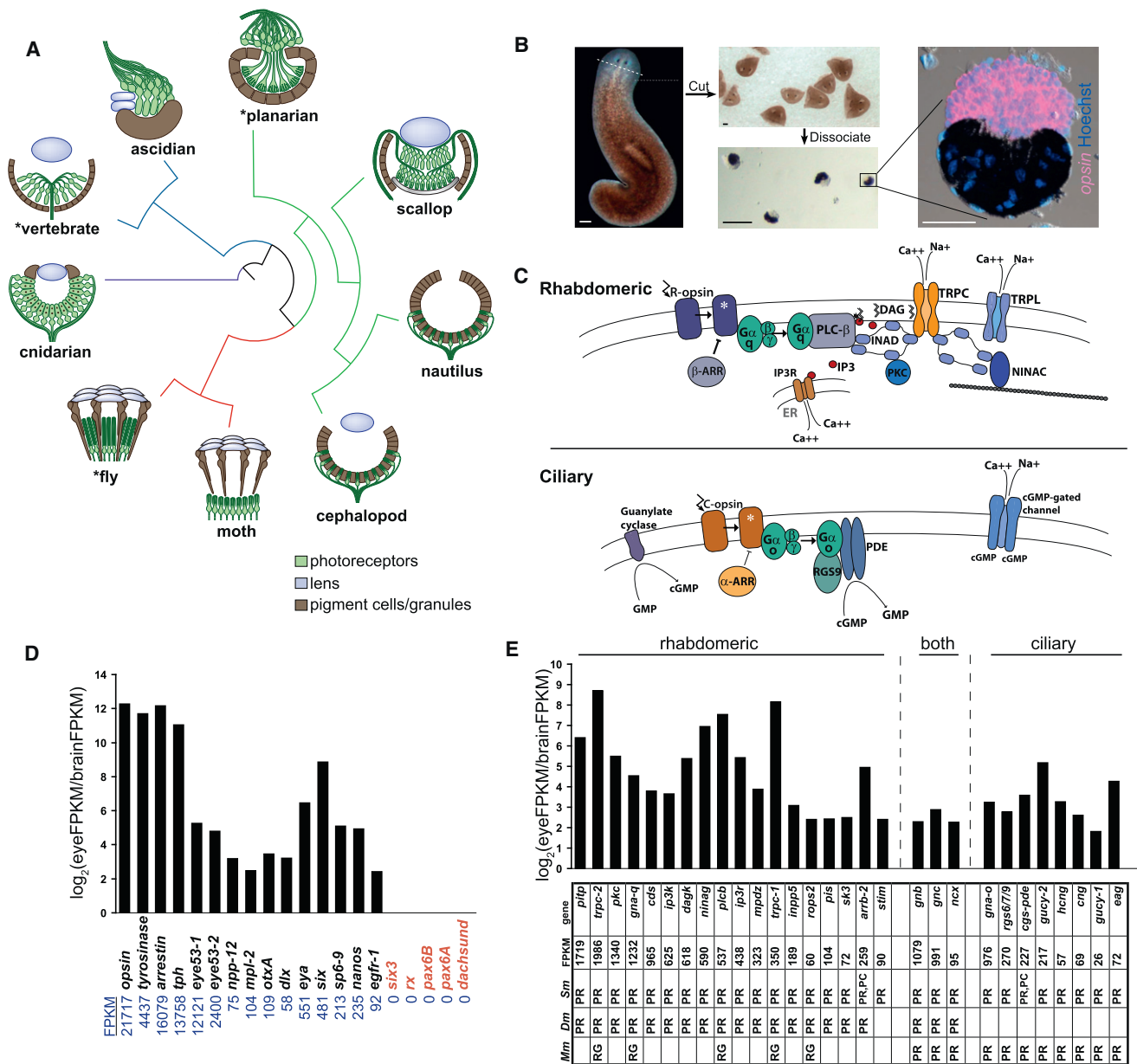


Figure 1. cDNA Sequencing of Purified Planarian Eyes

(A) Schematic of various metazoan eye types. The red branch represents Ecdysozoa; green branch, Lophotrochozoa; blue branch, Deuterostomia; purple branch, Cnidaria. Asterisks indicate that planarians, *Drosophila*, and vertebrate eyes can currently be studied with a completed genome and loss of gene function tools.

(B) Planarian eye purification. *opsin* RNA probe and pigment (melanin) were used to assess purity of purified eyes.

(C) Schematic of signaling downstream of R-opsin and C-opsin.

(D) Plot of relative FPKM values for planarian genes previously described in the literature with expression in the eye (black), or lacking expression in the eye (red). (E) Enriched expression was detected for genes typical of the R-opsin cascade (left set), the C-opsin cascade (right set), or either form of phototransduction (middle set). Table shows FPKM values for genes in the eye sample and summarizes expression data from mouse (*Mm*), *Drosophila* (*Dm*) and planarian (*Sm*); see also Figure 2). PR, photoreceptor neuron; PC, optic pigment cell; RG, retinal ganglion cell. Retinal ganglion cells are vertebrate retinal neurons with some photoreceptive ability, but which rely on an R-opsin cascade-like phototransduction mechanism (Sexton et al., 2012).

Scale bars, 200 μm (B, left and middle); 50 μm (B, right).

of ciliary vertebrate photoreceptors with respect to the retinal pigment epithelium (RPE) (Figure 1A). Finally, planarians possess unique abilities to regenerate entire eyes, and replenish eye

tissue throughout adulthood. These abilities require a population of adult regenerative cells (neoblasts) that includes pluripotent stem cells (Wagner et al., 2011).

Here we use eye purification and RNA-seq to identify most genes active in planarian eyes. These data demonstrate conserved features of the R-opsin signaling cascade, candidate rhabdomeric microvillus-regulating genes, and unexpected similarities between rhabdomeric and ciliary cell types. In addition, a high degree of similarity exists between the types of solute transporters expressed in planarian optic PCs and the vertebrate RPE, suggesting that optic pigment cells have an ancient role as an accessory eye cell type distinct from photoreceptors. We describe several conserved transcription factors expressed in the planarian eye that are functionally required for eye regeneration, including a central role for the conserved gene *ovo* in formation of all eye progenitors.

RESULTS

Planarian Eye Purification and the Eye Transcriptome

To obtain pure eye tissue for gene expression analysis, we developed a dissociation protocol for isolating ~200 morphologically intact planarian eyes in 1–2 hrs (Figure 1B). Greater than 96% of cells in the purified eye preparation were indeed eye cells, as determined using an *opsin* RNA probe for PRNs and melanin as a marker for PCs (Figure 1B). RNA from purified eyes was used to generate a cDNA library for quantitative RNA sequencing (RNA-seq). Animals were fed prior to eye harvesting to stimulate growth and incorporation of new cells in eyes. All genes previously described to be expressed in planarian eyes, and many phototransduction-related genes, displayed at least 4-fold enrichment in our eye reads compared to reads from control tissue (ventral, anterior tissue enriched for neurons), in terms of fragments per kilobase per million fragments mapped (FPKM) values (Figures 1C–1E) (See Experimental Procedures for details). *pax6A*, *pax6B*, *rax*, *six3*, and *dachshund* are orthologs of genes with roles in eye development in other organisms, but have been described to lack detectable expression and function in the planarian eye (Lapan and Reddien, 2011; Mannini et al., 2008; Pineda et al., 2002; Pineda and Saló, 2002); all of these genes displayed FPKM values of zero (Figure 1D), demonstrating specificity of the data and further suggesting that these genes have no role in planarian eyes. We conclude that transcript levels in the RNA-seq data set can be highly predictive of gene expression *in vivo*.

For an *in situ* expression screen we selected unpublished planarian genes with similarity to genes in *Drosophila* and vertebrates and > 4-fold expression enrichment in the eye. Of these approximately 600 genes (Table S1), nearly 200 were tested by *in situ* hybridization (Figure 2 and Table S2) in regenerating heads 7 days after amputation. PRNs and PCs can be identified by position and whether the eye expression domain curves toward (PRNs) or away from (PCs) the midline (Figure 2A). 93% of genes screened by *in situ* had detectable expression in eyes. 60% were expressed in PRNs, 21% were expressed in the pigmented optic cup cells, and 12% were detected in both cell types (Table S2).

Planarian Phototransduction Genes

Rhodopsin signaling components displayed eye-enriched expression in the RNA-seq data set (Figures 1C and 1E) and *in situ* hybridizations revealed expression in photoreceptor

neurons for two *R-opsin* orthologs (Sánchez Alvarado and Newmark, 1999), two β -*arrestin* orthologs (Agata et al., 1998), *G α -q* (*Smed-gna-q*), *PLC- β* (*Smed-plcb*), *INAD* (*Smed-mpdz*), *PKC*, and two Trp channel-encoding genes (*Smed-trpc-1*, *Smed-trpc-2*) (Fain et al., 2010) (Figures 1C, 1E, and 2B). Planarian PRNs also had abundant expression of genes encoding enzymes of the phosphoinositide cycle (Figures 1C, 1E, and 2B), which replenishes PIP2 after its hydrolysis by PLC (Wang and Montell, 2007). These include *retinal degeneration a* (*Smed-dagk*), *retinal degeneration b* (*Smed-pitp*), *dpis* (*Smed-pis*), and *cds* (*Smed-cds*).

Genes with predicted roles in cGMP signaling and intracellular calcium regulation, which are involved in phototransduction in multiple organisms, were also expressed in planarian eyes. Specifically, PRNs expressed orthologs of IP3-receptors (*Smed-ip3r*) and STIM (*Smed-stim*) (Figures 2B and S1), which regulate intracellular calcium levels (Smyth et al., 2010). PRNs also expressed genes encoding the cGMP pathway components guanylate cyclase (*Smed-gucy-1*, *Smed-gucy-2*), cGMP-dependent phosphodiesterase (*Smed-cgs-pde*), RGS7/9 (*Smed-rgs6/7/9*), a cGMP-gated ion channel (*Smed-cng*), and a hyperpolarization-activated cyclic nucleotide-gated channel (*Smed-hcng*) (Figures 1E, 2B, and S1). Many of these components mediate signaling downstream of C-opsin in vertebrates (Arendt, 2003), but we were unable to identify an ortholog of C-opsin in planarians. GO analysis of the RNA-seq data indicated that Rhodopsin signaling, cGMP signaling, and intracellular calcium regulation were all enriched categories in the data set of eye-expressed genes (Table S3).

Expression of Microvillus-Related Genes in Photoreceptor Neurons

Unexpectedly, orthologs of genes involved in the function and development of auditory hair cells displayed enriched expression in planarian eyes and were prominently represented in GO analysis (Figures 2B and 2C; Table S3). Like planarian photoreceptors, hair cells are sensory neurons with abundant apical microvilli. Microvilli are actin-based structures, and we identified enriched PRN expression of genes encoding actin (*Smed-actin-1*), the actin-regulating proteins Ezrin/Radixin/Moesin (*Smed-erm*), Clic (*Smed-clic*), Maguk (*Smed-maguk*), Gelsolin (*Smed-gls*), and the actin-based motor Myosin VI (*Smed-myoVI*). These genes have all been shown to have roles in morphogenesis and function of vertebrate hair cells (Gagnon et al., 2006; Kitajiri et al., 2004; Mburu et al., 2006, 2010; Self et al., 1999). Auditory hair cells of human and mouse also express Usher syndrome genes, which function in morphogenesis of microvillus bundles (Frolenkov et al., 2004). Planarian PRNs expressed orthologs of three Usher syndrome genes, *Myosin VIIA/Ush1B* (*Smed-myoVIIA*), *Sans/Ush1G* (*Smed-sans*), and *Cadherin 23* (*Smed-cdh23*) (Figures 2B and 2C). These genes are also expressed in vertebrate photoreceptors, despite the fact that the latter are ciliary. The function of Usher syndrome proteins in vertebrate photoreceptors is not understood (Williams, 2008), but many localize to the periciliary region and Usher syndrome can result in aberrant photoreceptor cilia morphology (Hunter et al., 1986). Many of the hair cell-related genes described here, including Usher syndrome genes, have not

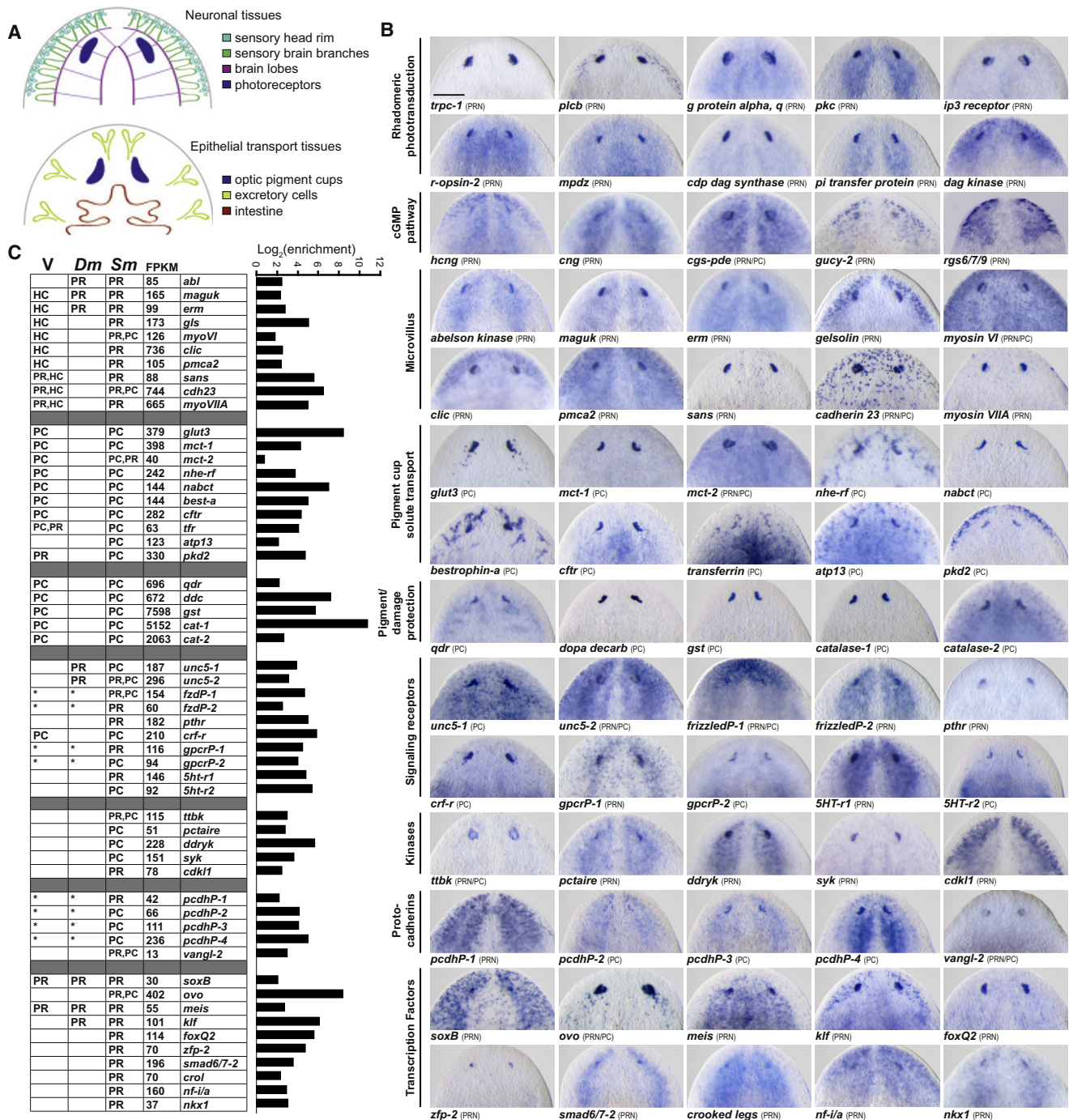


Figure 2. An In Situ Hybridization Screen of Genes with Enriched Expression in Planarian Eyes

(A) Schematic of tissue types in the head that appear in expression patterns. PRN expression domains curve toward the midline, and PC expression domains curve away from the midline.

(B) In situ hybridization of eye-enriched transcripts, grouped by predicted function. Full names and additional orthology information are given in Table S1.

(C) Plot of the log₂ of fold enrichment (FPKM eye sample/FPKM control sample) for genes screened (see also Table S2). Values for rhodomeric phototransduction and cGMP pathway genes are shown in Figure 1. Table shows FPKM values for genes in the eye sample and summarizes expression data from vertebrate (V), *Drosophila* (Dm), and planarian (Sm). PR, photoreceptor neuron; PC, optic pigment cell; HC, inner ear hair cell; asterisk indicates that planarian gene orthology could only be assigned to a broad gene family for which functions have been identified in the eyes of *Drosophila* or vertebrate. Scale bar, 200 μ m.

See Figures S1 and S2 as well.

been described to be expressed in *Drosophila* photoreceptors. Therefore, our analysis describes unexpected similarity between a rhabdomeric photoreceptor and auditory hair cells, identifying genes that are candidates to regulate microvillus-like apical membrane specialization.

Genes of the Pigmented Optic Cup

The vertebrate RPE supports photoreceptor function by providing organic molecules, maintaining extracellular ion concentrations, phagocytosing aging photoreceptor components, and absorbing light energy (Strauss, 2005). Many genes expressed in planarian PCs were orthologs of genes important for the support function of RPE cells. The metabolic rate of the vertebrate retina necessitates high levels of available glucose. Planarian PCs expressed orthologs of *Glut3* (*Smed-glut3*) (Figures 2B and 2C) and MCT transporters (*Smed-mct-1* and *Smed-mct-2*) (Figures 2B and 2C), which transport glucose to the retina (Ban and Rizzolo, 2000) and remove lactic acid waste (Bergersen et al., 1999), respectively. Lactic acid uptake is coupled to proton uptake (Lin et al., 1994; Lin and Miller, 1991) and two genes encoding pH-regulatory proteins, a Na⁺/H⁺ exchanger regulatory cofactor (*Smed-nhe-rf*) and a sodium bicarbonate cotransporter (*Smed-nabct*), were expressed specifically in the planarian PCs (Figures 2B and 2C). High metabolic activity in the retina results in excess water production, which is eliminated using chloride gradients across the RPE (Strauss, 2005). Orthologs of the RPE-expressed chloride channels Bestrophin-1 (*Smed-best-a*) and Cfr (*Smed-cfr*) were strongly expressed in the PCs of planarian eyes (Figures 2B and 2C).

Planarians are similar to vertebrates, but differ from most other invertebrates, in the use of melanin as the primary eye shading pigment (Hase et al., 2006; Strauss, 2005). Among the most abundantly expressed genes in planarian PCs were those encoding orthologs of enzymes required for melanin synthesis in vertebrates, including Tyrosinase (Lapan and Reddien, 2011) (Figure 1D), Aromatic amino acid hydroxylase (Nishimura et al., 2007) (see “*tph*,” Figure 1D); and Dopa decarboxylase (*Smed-ddc*) (Figures 2B and 2C). PCs also expressed orthologs of Quinoid dihydropteridine reductase (*Smed-qdr*), which produces an essential cofactor of tyrosine/phenylalanine hydroxylase (Schallreuter et al., 2008); and Glutathione-S-transferase (*Smed-gst*), which catalyzes addition of glutathione during pheomelanin polymerization (del Marmol et al., 1996) (Figures 2B and 2C).

Peroxide is generated by melanin-synthesizing enzymes and is required for melanin polymerization, but it can also cause cytotoxicity in melanocytic cells (Mastore et al., 2005). The GO category for “Response to hydrogen peroxide” was significantly enriched in the eye RNA-seq data (Table S3). Two orthologs of *catalase*, *Smed-cat-1* and *Smed-cat-2*, were expressed in the planarian PCs. Similar to *tyrosinase*, *Smed-cat-1* was one of the most strongly and specifically expressed genes in the planarian PCs (Figures 2B and 2C and Table S2). Glutathione-S-transferase might also function as an antioxidant enzyme in the eye (Giblin, 2000; Singhal et al., 1999). Therefore, planarian and vertebrate optic PCs express similar melanin-synthesizing enzymes and enzymes that protect from oxidative damage.

Heterogeneity of the PRN and PC Populations

Recently, planarian photoreceptor neuron heterogeneity has been described, with pro-hormones and *smad6/7-2* expressed in distinct domains of the PRN population (Collins et al., 2010; González-Sastre et al., 2012). *Smed-soxB*, *Smed-best-b*, *Smed-pcdhP-1*, and *Smed-pthr* were expressed only in anterior photoreceptors (Figure S2A). *Smed-fzdp-1* was expressed primarily in dorsal and anterior photoreceptors, as well as progenitors near the eye (Figure S2A). *Smed-zfp-2*, *Smed-pctaire*, and *Smed_19866* were expressed only in posterior photoreceptors (Figure S2B). We also identified a gene, *Smed-actin-2*, expressed in a subset of pigment cup cells, indicating that heterogeneity also exists within this population (Figure S2C).

Identification of an Eye-Specific Transcription Factor-Encoding Gene, *Smed-ovo*

Molecular regulators remain to be identified for key steps in planarian eye formation, including progenitor migration, homotypic cell aggregation, mesenchymal-to-epithelial transition, and cupping morphogenesis. In situ screening identified enriched expression in the planarian eye for many genes that are good candidates to encode proteins regulating these processes, including 14 kinases, 5 protocadherins, and 18 signaling receptors (Figures 2B, 2C, and S1).

Transcription factors that control eye development in both *Drosophila* and vertebrates have received close attention for their capacity to elucidate the evolution of eye development. Here, we have identified ten conserved genes predicted to encode transcription factors with enriched expression in planarian eyes (Figures 2B and 2C). The gene with the most prominently enriched expression was a member of the *Ovo* family of zinc finger transcription factor-encoding genes (Figure S3), which includes the *Drosophila* gene *ovo* (*shavenbaby*) and mouse *ovo1-3* genes. Expression of *Smed-ovo* (*ovo*) was detected in both PRNs and PCs, as well as in mesenchymal cells posterior to the eye in day 7 head blastemas (Figures 2B and 3A). After head amputation, *ovo*⁺ cells first became apparent throughout the dorsal anterior region, near the wound, by 2 days post-amputation (Figure 3A). At later regeneration time points *ovo*⁺ eye aggregates formed in the anterior blastema, while dispersed cells posterior to the eye remained visible. This pattern was similar to a recently defined population of regenerative eye progenitors in planarians (Lapan and Reddien, 2011). Accordingly, nearly all *ovo*⁺ cells at day 2 and day 7 of regeneration also displayed expression of *six-1/2* and *eya*, which marks eye progenitors (Figures 3B and 3C).

In regenerating or intact animals, no *ovo* expression was detected outside of eyes or eye progenitors (see below). Furthermore, in the control (ventral anterior tissue) sequencing data, the FPKM value for *ovo* was near zero (Table S2 and Figure S4A). Other previously known eye-expressed transcription factors, *eya*, *six-1/2*, *sp6-9*, and *otxA* also have expression in the brain and/or elsewhere in the body (Lapan and Reddien, 2011; Pineda et al., 2000; Umesono et al., 1999). Therefore, *ovo* is the only known planarian transcription factor with expression exclusively in the eye and eye progenitors. It is one of only three transcription factor-encoding genes,

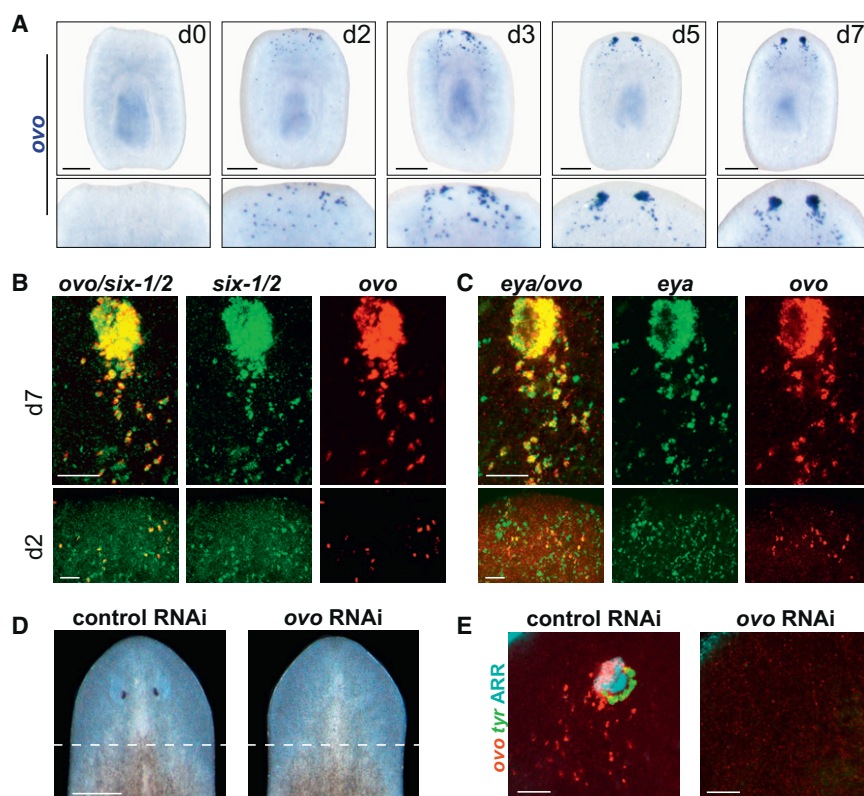


Figure 3. *Smed-ovo* Is Expressed Specifically in the Eye and Eye Progenitors and Is Required for Eye Regeneration

(A) Regeneration time course showing *ovo* expression after amputation of head and tail. Bottom panel is a magnified view of the anterior wound/blastema.

(B) *ovo*⁺ cells express the eye progenitor marker *six-1/2* at early and late time points of regeneration (d2: 96% ± 5% overlap, d7: 100% overlap, $\alpha = 0.05$).

(C) *ovo*⁺ cells also express the eye progenitor marker *eya* (d2: 90% ± 6% overlap, d7: 100% overlap, $\alpha = 0.05$).

(D) RNAi of *ovo* leads to failed eye regeneration (n = 10/10). Dashed lines indicate approximate site of head amputation 12 days prior.

(E) *ovo*(RNAi) animals lack expression of *ovo*, tyrosinase, and Arrestin after head regeneration (n = 10/10).

Scale bars, 200 μ m (A), 50 μ m (B,C, and E), and 1 mm (D).

See Figure S3 as well.

together with *six-1/2* and *eya*, that is expressed in all cells of the eye.

***ovo* Expression Is Required for Eye Regeneration**

RNAi of *ovo* followed by decapitation resulted in animals that failed to regenerate eyes (Figure 3D). These animals also lacked detectable tyrosinase mRNA and Arrestin protein (Figure 3E), and no eye structure could be detected by differential interference contrast microscopy (DIC) (Figure S4B). We studied *ovo*(RNAi) animals using *six-1/2/eya* co-expression to label all eye progenitors, and *otxA/eya* and *sp6-9/eya* co-expression to label PRN and PC progenitors, respectively (Lapan and Reddien, 2011). *ovo* RNAi resulted in failure to generate any eye aggregate (primordium), and loss of most eye progenitors (Figures 4A and 4B). We observed no defects that were unrelated to the eyes, consistent with the highly specific expression of this gene. For example, other cell types of the head, such as *sp6-9*⁺/*eya*⁻ cells of the head rim appeared unaffected (Figure 4A). Therefore, eyes and eye progenitors are strongly and specifically affected in *ovo*(RNAi) animals.

Formation of *ovo*⁺ Progenitors Requires Expression of Other Eye Transcription Factors

In gene regulatory networks involved in *Drosophila* and vertebrate eye development, several transcription factors that regulate eye formation are required for the expression of each other (Silver and Rebay, 2005; Zuber et al., 2003). We tested whether

ovo⁺ progenitor cells are sensitive to expression of other planarian eye transcription factors. RNAi of *six-1/2* and *eya* resulted in animals with greatly reduced or absent *ovo* signal in the eyes and eye progenitor region (Figures 4C and 4D). RNAi of the cell type-specific transcription factors *otxA* and *sp6-9*, required for PRN and PC regeneration respectively, also impacted the presence of *ovo*⁺ progenitors (Figures 4E and 4F). RNAi of *otxA* led to a 34% reduction in *ovo*⁺ cells; this reduction was primarily a result of loss of *ovo*⁺ PRN progenitors (Figures 4E and 4F), because most remaining *ovo*⁺ cells were *sp6-9*⁺. Similarly, RNAi of *sp6-9* led to a 60% reduction in *ovo*⁺ cells, primarily a result of loss of *ovo*⁺ PC progenitors, because most remaining *ovo*⁺ cells were *otxA*⁺ (Figures 4E and 4F). These large decreases indicate that *ovo*-expressing cells, even in early stages of eye progenitor formation, are sensitive to the expression of *six-1/2*, *eya*, *otxA*, and *sp6-9* transcription factors.

***ovo* Is Expressed in a Population of Eye Progenitors during Homeostasis and Is Required for Maintenance of Intact Eyes**

The specificity of *ovo* expression for eyes and eye progenitors allowed us to ask for the first time whether subsets of neoblasts in uninjured animals are specialized for particular differentiation paths. In intact animals undergoing normal adult homeostasis, *ovo* was expressed not only in the eye but also in sparse cells posterior to the eye and anterior to the pharynx (Figure 5A). Adult planarians undergo perpetual tissue turnover (Newmark and Sánchez Alvarado, 2000), but the identity and origin of progenitors that replenish eyes in uninjured animals is unknown. *ovo*⁺ cells posterior to the eye in intact animals expressed *eya* and *six-1/2* (Figure 5B), and those

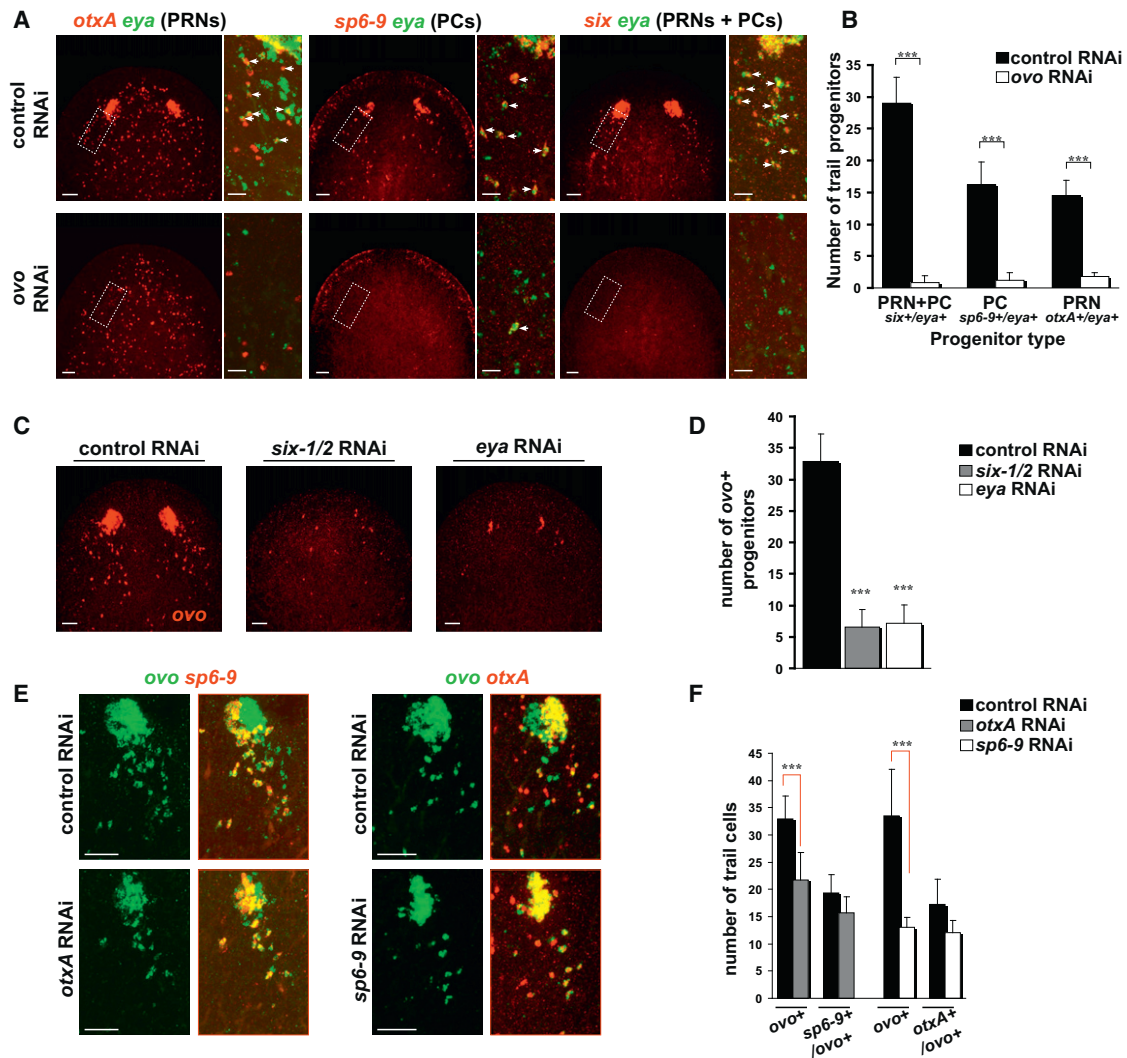


Figure 4. *ovo* Is Required for Formation of Regenerative Eye Progenitors, and *ovo*⁺ Progenitor Presence Requires Other Eye Transcription Factors

(A and B) *ovo RNAi* eliminates regenerative eye progenitors as assessed by markers for PRN (*otxA*⁺/*eya*⁺), PC (*sp6-9*⁺/*eya*⁺), and general eye progenitors (*six-1/2*⁺/*eya*⁺). Arrows indicate double-positive cells.

(C and D) Most regenerative *ovo*⁺ progenitor cells are eliminated by *six-1/2* and *eya RNAi*.

(E and F) *sp6-9 RNAi* eliminates most *ovo*⁺ pigment cell progenitors (*ovo*⁺/*otxA*⁺), and *otxA RNAi* eliminates most *ovo*⁺ photoreceptor neuron progenitors (*ovo*⁺/*sp6-9*⁻).

n = 8 eyes + trails for all quantifications.

Error bars represent SD. ***p < .005. Scale bars, 50 μm (A,C, and E), and 20 μm (A, insets).

See Figure S4 as well.

nearer to the eye also expressed markers for differentiated photoreceptor neurons (*trpc-1*) or PCs (*tyrosinase*) (Figure S5A). Therefore, *ovo*⁺ cells that exist posterior to the eye in intact animals have a gene expression profile seen elsewhere only in cells of the eye.

The localization of the *ovo*⁺ cells posterior to the eye in intact animals is consistent with the location of eye progenitors during regeneration following head amputation (Lapan and Reddien, 2011). Strikingly, these cells were eliminated within 1 day of irradiation (Figure 5A), a treatment known to specifically eliminate

the dividing cells of the adult animal (the neoblasts) (Reddien and Sánchez Alvarado, 2004). Furthermore, many *ovo*⁺ cells in this prepharyngeal population expressed the neoblast-specific markers histone *h2b* (Figure 5C) and *smedwi-1* (Figure S5B) (Reddien et al., 2005). It is unknown whether these *ovo*⁺ neoblasts are self-renewing or constantly produced from a more naive cell. These results suggest that eye progenitors constitutively exist in the neoblast population.

ovo RNAi in intact animals resulted in gradual loss of eyes, with pigment cups undetectable following two months of RNAi

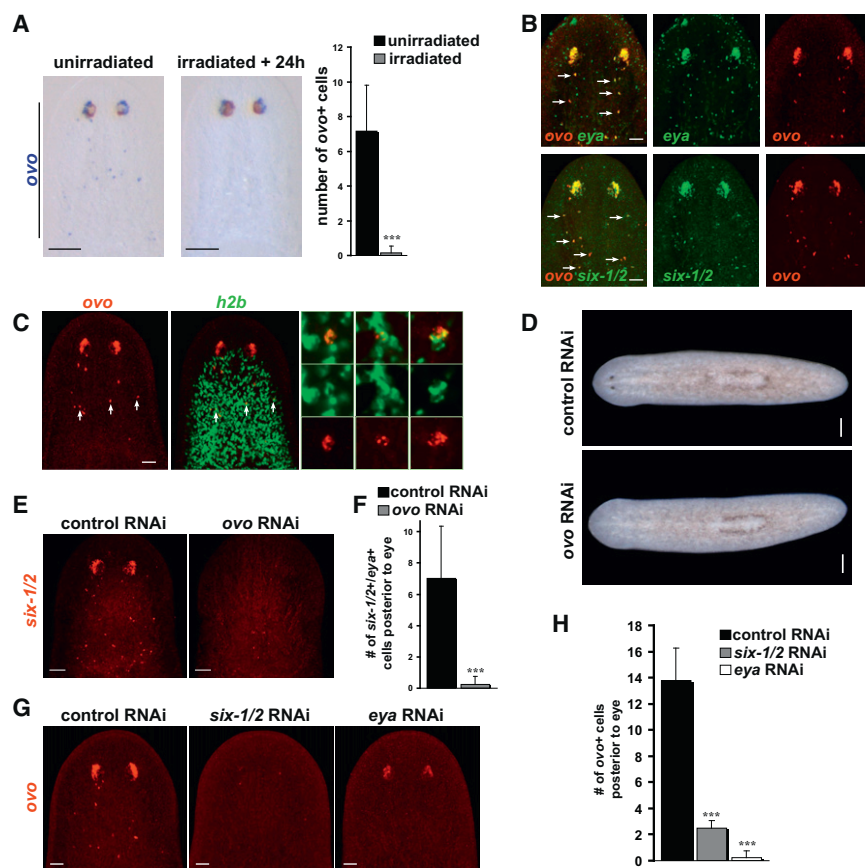


Figure 5. *ovo* Is Expressed in a Population of Homeostatic Eye Progenitors

(A) In starved intact animals, *ovo* is expressed in isolated dorsal cells between the eyes and pharynx. These cells are eliminated within 24 hr of irradiation.

(B) *ovo*⁺ cells posterior to the eyes in intact animals also express *six-1/2* and *eya*.

(C) *ovo*⁺ cells posterior to the eyes in intact animals also express the neoblast marker histone *h2b* (60% ± 15%, $\alpha = 0.05$).

(D) *ovo(RNAi)* animals that are not amputated fail to maintain eyes (n = 10/10).

(E and F) *ovo(RNAi)* animals that are not amputated fail to maintain *six-1/2*⁺/*eya*⁺ cells posterior to the eyes (n = 4 animals). For image clarity only *six-1/2* signal is shown.

(G and H) *six-1/2(RNAi)* and *eya(RNAi)* animals fail to maintain *ovo*⁺ cells posterior to the eyes (n = 4 animals).

Arrows indicated double-positive cells in (B) and (C). Error bars represent SD. ***p < .005. Scale bars, 100 μ m (A), 50 μ m (B, C, F, and H), and 200 μ m (D).

See Figure S5 as well.

(Figure 5D). These un-amputated *ovo(RNAi)* animals also lacked *six-1/2*⁺/*eya*⁺ cells in the region of putative homeostatic eye progenitors (Figures 5E and 5F). Reciprocally, homeostatic RNAi of *six-1/2* and *eya*, in addition to causing loss of eye tissue, caused a loss of *ovo*⁺ cells in the prepharyngeal region (Figures 5G and 5H). Overall, these data suggest that *ovo*, *six-1/2*, and *eya* are expressed in a population of eye progenitors during homeostasis as well as regeneration and are required for maintenance of this population.

***Smed-soxB*, *Smed-foxQ2*, *Smed-klf*, and *Smed-meis* Regulate Eye Differentiation and Morphogenesis**

RNAi of genes encoding four additional conserved transcription factors, *Smed-soxB*, *Smed-foxQ2*, *Smed-klf*, and *Smed-meis* (Figure S3) resulted in PRN aggregates that were smaller in cross-section than in the control (Figures 6A–6C). All of these genes were expressed in PRNs and eye progenitors during regeneration (Figure S6A). Photoreceptor aggregates in *klf(RNAi)* animals were also displaced posteriorly relative to the pigment cup and descended unusually far toward the interior of the animal along the D-V axis, embedding within the brain (Figures S7A and S7B).

Small eye phenotypes were not accompanied by an evident decrease in the number of *ovo*⁺ progenitors in these RNAi animals (Figures S6B, S6C, and 6F), suggesting that these genes function primarily following progenitor specification. Because

soxB expression was restricted to anterior PRNs, we assessed the presence of photoreceptor neuron subsets in *soxB(RNAi)* animals. Anterior PRNs (*eye53-1*⁺ [Collins et al., 2010] and *best-b*⁺) were strongly reduced or eliminated in *soxB(RNAi)* animals (Figure 6D). In contrast, posterior PRNs (*pctaire*⁺) were not affected (Figure 6D). *soxB* RNAi did not significantly impact numbers of PC (*sp6-9*⁺/*ovo*⁺) or PRN (*sp6-9*⁺/*ovo*⁺) progenitors (Figures 6E and 6F), and *soxB* is not abundantly expressed in the *ovo*⁺ progenitor population (Figure S6A). However, *soxB* eye signal is eliminated by *ovo* RNAi (Figure S6D). These data indicate that whereas *ovo* (along with *six1/2*, *eya*, and *otxA*) acts in general PRN progenitor specification (Figures 4A and 4E), *soxB* acts subsequently to promote differentiation of an anterior subset of PRNs.

Several RNAi phenotypes affected the pigment cup shape. Loss of photoreceptor cells by RNAi of PRN-specific genes, such as *otxA*, results in pigment cups with diminished apertures (Lapan and Reddien, 2011). Accordingly, in *klf* and *foxQ2* RNAi animals, optic cups were more closed than in the control, or even completely circularized (Figures 6A and 6B), possibly explained if rhabdomeres provide support for optic cup opening. In *meis(RNAi)* animals, however, optic cups were also elongated and often more than one cupped structure was visible in each eye - a defect not characteristic of photoreceptor cell loss (Figures 6B and S7C). Although *meis* expression was not detected in the pigment cup, *meis* transcripts are detectable in *tyrosinase*⁺ PC progenitors (Figure S7D). *Meis1* loss in mouse also disrupts optic cup morphogenesis, with optic cup duplication observed (Hisa et al., 2004).

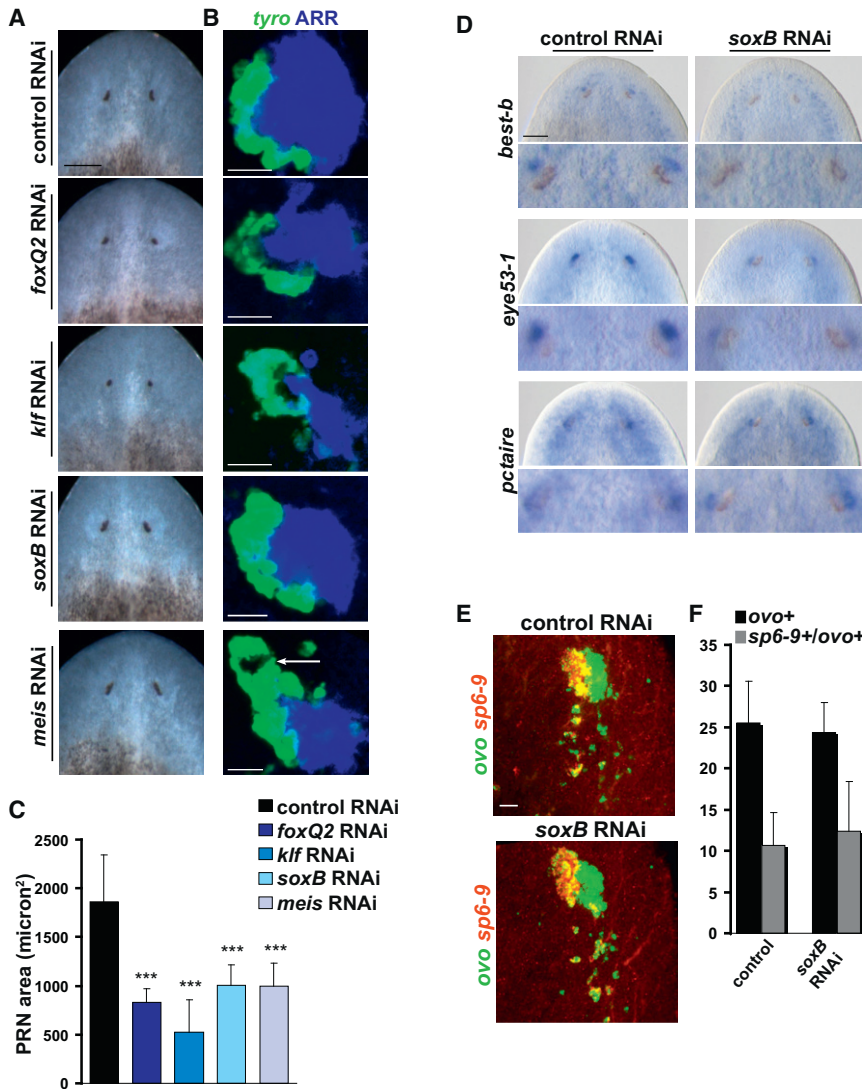


Figure 6. *Smed-soxB*, *Smed-meis*, *Smed-klf*, and *Smed-foxQ2* Are Required for Eye Regeneration

(A) Eye regeneration defects at 7 days of regeneration in live RNAi animals.

(B) Detection of *tyrosinase* transcript and Arrestin protein reveals defects in optic cup formation and photoreceptor neuron regeneration. Arrow indicates duplicated optic cup in *meis(RNAi)* animal.

(C) Quantification of cross-section area (μm^2) of photoreceptor neuron aggregate (mean + SD, $n = 8$ eyes).

(D) *soxB* RNAi results in loss of anterior subtypes of photoreceptor neurons, but not posterior subtypes ($n = 8$ eyes for each condition).

(E and F) *soxB* RNAi does not affect numbers of *ovo+* progenitors, nor the ratio of PC progenitors (*sp6-9⁺/ovo⁺*) to PRN progenitors (*sp6-9⁺/ovo⁺*). Error bars represent SD. *** $p < .005$. Scale bars, 200 μm (A), 100 μm (D), 20 μm (B and E). See Figures S6 and S7 as well.

Developing Eyes of Planarian Embryos Express Transcription Factors Involved in Eye Regeneration

Most eye formation studies in other organisms are performed in developing embryos. Therefore, we investigated whether the transcription factors that govern planarian eye regeneration might also be expressed during planarian embryonic development (Figure 7A). Recent work has shown that beginning at stage 6, *Schmidtea polychroa* embryos have expression of *eya*, *six-1/2*, *otxA* in the eyes, and lack expression of *pax6A*, *rax*, and *dachshund* in the eye domain (Martín-Durán et al., 2012), similar to the case in regeneration. We found that from early stages of *S. mediterranea* embryonic eye formation, *ovo* was expressed in the eyes and in mesenchymal cells posterior to the eye that were reminiscent of regenerative progenitor eye trails (Figure 7B). Double FISH (fluorescent in situ hybridization) with *ovo* enabled us to determine whether regeneration-related eye genes were expressed in the embryonic eye primordium and/or presumptive earlier progenitors. Embryonic

regulation of eye regeneration are relevant to planarian embryonic development.

DISCUSSION

Planarian Eye Formation in Regeneration, Homeostasis, and Embryogenesis

The cellular events bridging the pluripotent state and cellular differentiation during planarian regeneration and homeostasis are poorly understood, and the eye is emerging as a system to study adult lineage specification in this animal (Lapan and Reddien, 2011). *ovo* is the first identified gene that is expressed in all eye progenitors and is specifically expressed in eye cells, and therefore is a marker that facilitates study of progenitor dynamics during various modes of planarian eye formation. Our investigations of *ovo* expression lead to three conclusions concerning planarian eye formation. (1) Isolated eye progenitors are present early in regeneration (by 2 days after decapitation),

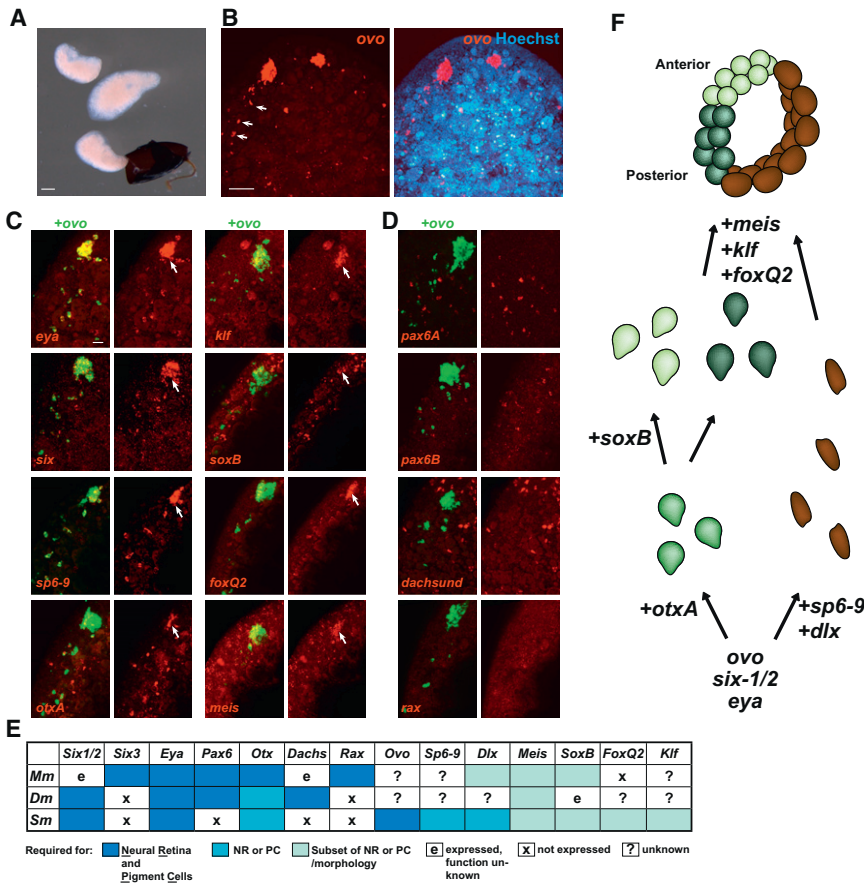


Figure 7. Expression of Transcription Factors in Planarian Embryos

(A) Freshly decapsulated planarian embryos of the stage used for expression analyses. The dark structure is the embryo capsule.

(B) *ovo* is expressed during embryonic development in eyes and trails posterior to eyes (arrows).

(C) Double-FISH with *ovo* is used to show presence of gene expression in eye primordium and eye progenitors during embryonic development. Arrows indicate the location of *ovo*⁺ eye primordia.

(D) Absence of gene expression for indicated genes in the eye primordium and eye progenitors during embryonic development.

(E) Table summarizing roles of transcription factors involved in eye formation in either mouse (*Mm*), *Drosophila* (*Dm*), or planarian (*Sm*). NR, neural retina; PC, optic pigment cells.

(F) Model of steps in planarian eye regeneration that are regulated by transcription factors.

Scale bars, 200 μm (A), 50 μm (B), 20 μm (C and D).

prior to the appearance of an aggregated eye primordium. Therefore, specification at a distance from the aggregated eye occurs from the onset of regeneration rather than only at later stages of eye growth (Lapan and Reddien, 2011). (2) Eye progenitors exist in uninjured intact animals in a dorsal prepharyngeal domain, similar to regenerative progenitors but at lower density. These data indicate that some neoblasts are specialized for specific lineages during normal tissue turnover conditions of adult life. (3) During embryogenesis, *ovo*⁺ cells are present in the eye and in a trail-like formation posterior to the eye.

Further experiments are required to formally determine whether, like regenerative progenitors (Lapan and Reddien, 2011), homeostatic and embryonic eye progenitors move toward the eye primordium. However, our *ovo* expression analyses indicate that migration of differentiating mesenchymal cells, rather than patterning of an epithelium, might be a feature of all modes of eye formation in planarians. Our findings advance the use of the planarian eye as a system for exploring the extent of similarity between embryonic, regenerative, and homeostatic modes of organ formation within a species and between species.

Eye Purification and Transcriptome Analysis

Canonical phototransduction cascades have been described for ciliary and rhabdomeric phototransduction based on functional investigations in vertebrates and *Drosophila*, respectively (Arendt, 2003). However, a diversity of phototransduction strat-

egies likely exists among animals. For instance, horseshoe crab (*Limulus*) and *Drosophila* light sensation both begin with an R-opsin/PLC cascade, but *Drosophila* phototransduction culminates in Trp channel opening (Katz and Minke, 2009). By contrast, most evidence from *Limulus* indicates that opening of cyclic nucleotide-gated (CNG) ion channels (preceded by IP3-dependent intracellular

calcium release and changes in cGMP levels) is responsible for photoreceptor depolarization (Garger et al., 2004). We observed robust expression for orthologs of nearly all central *Drosophila* phototransduction components in planarian photoreceptors, including two Trp channels. However, we also detected enrichment for intracellular calcium regulation and cGMP pathway-like components, including CNG channels. Based on the high levels of Trp channel gene expression, we hypothesize that these are the primary mediators of membrane depolarization following light detection. The roles of CNG channels in planarian eyes are unknown, but electrophysiological recordings combined with RNAi could be used to test possible functions, such as in the regulation of light response or light/dark adaptation.

The planarian pigmented optic cup has numerous similarities to the RPE of vertebrates. Melanin is not commonly found in protostome eyes; however, it is the primary pigment of both planarian PCs and the RPE. We expand upon this similarity by demonstrating that the enzymes used in melanin production and for protection from oxidative damage (a consequence of melanin production) are similar between these cell types. Because of the wide divergence in identity of ocular shading pigment among invertebrates (Vopalensky and Kozmik, 2009), the common synthesis of melanin in planarians and vertebrates need not indicate common ancestry of optic PCs. However, we identified additional similarities between planarian PCs and the vertebrate RPE with respect to expression of numerous

metabolite and solute transporters, consistent with a common function of these cell types as components of a transport epithelium. This similarity supports a model in which pigmented cells already existed as an accessory cell type separate from photoreceptor neurons in the ancestral bilaterian eye.

The RPE is commonly affected in eye diseases, including age-related macular degeneration (AMD), edema, and inherited retinal degeneration syndromes. A molecular explanation for pathogenesis is lacking for most of these diseases. Planarian PCs express orthologs of two RPE chloride channels underlying eye diseases. *Best-1* is mutated in Best vitelliform macular dystrophy, and *Cftr* mutations underlie vision defects in cystic fibrosis patients (Marmorstein et al., 2009; Xiao et al., 2010). Furthermore, RPE degeneration in AMD correlates with peroxide accumulation and declining Catalase function, which is also responsible for degeneration of other melanocytic cell types (Venza et al., 2012; Wood et al., 2009). *catalase-1* is very abundantly and specifically expressed in the planarian pigment cup. Molecular similarities between *Drosophila* ocular PCs and the vertebrate RPE have been difficult to identify (Charlton-Perkins and Cook, 2010). The planarian eye should therefore prove useful as an invertebrate model system for investigating orthologs of mammalian RPE genes involved in pathogenesis, as well as for inferring ancestral functions of ocular PCs.

Transcription Factor Control of Planarian Eye Formation

Comprehensive sequencing of RNA from intact eyes identified three genes that encode transcription factors and that are expressed in all cells of the eye and their progenitors—*six-1/2*, *eya*, and *ovo*. The requirement for *six-1/2* and *eya* in planarian eyes and eye progenitors is previously described (Lapan and Reddien, 2011; Mannini et al., 2004; Pineda et al., 2000) and both are homologs of well-known eye development genes (Figure 7E). We propose a model for eye regeneration in which *six-1/2*, *eya*, and *ovo* expression specifies all eye progenitor formation, with additional expression of *otxA* specifying PRNs and additional expression of *sp6-9* and *dlx* specifying PCs (Figure 7F).

How do available planarian eye data impact models about transcriptional regulators governing eye formation in the common ancestor of the Bilateria? Planarian data support the inclusion of *Otx* and *Eya* as ancestral bilaterian eye genes, and of *Six-1/2* as an ancestral eye gene for the protostomes (Figure 7E). Our data also support the possibility that *Meis* and *SoxB* family genes encode transcription factors with ancestral roles in eye biology. *Meis* genes regulate eye progenitor proliferation in *Drosophila* and vertebrates as well as optic cup morphology in mouse (Figure 7E) (Bessa et al., 2002; Heine et al., 2008; Hisa et al., 2004). The *SoxB* family *sox-neuro* and *fish-hook* genes are expressed in the *Drosophila* eye disc (Mukherjee et al., 2000), and *Sox2* is required for neural progenitor maintenance and differentiation of retinal ganglion cells in vertebrates (Matsushima et al., 2011).

In contrast to *Otx*, *Eya*, *Six-1/2*, *Meis*, and *SoxB*, several transcription factors that have prominent roles in *Drosophila* and/or vertebrate eye formation—*Pax6*, *Dachshund*, *Rax*, and *Six3*—have no apparent role in planarian eye formation (Figure 7E)

(Lapan and Reddien, 2011; Mannini et al., 2008; Martín-Durán et al., 2012; Pineda et al., 2002), a conclusion extended by our eye transcriptome and embryonic eye expression data. Therefore, planarian data does not support the argument that these genes are ancestral regulators of eyes. Loss of a role for any of these genes in eye biology during planarian evolution is clearly a reasonable possibility, especially in cases such as *Pax6* where data from multiple other phyla support an eye role for the gene. In addition to the case of the planarian eye, however, the development of *Drosophila* larval ocellus, branchiostome eye cups, *Limulus* eyes, and *Platynereis* adult eyes also does not depend on *Pax6* (Vopalensky and Kozmik, 2009). Furthermore, orthologs of the *Drosophila* eye regulator *Dachshund* do not have major functions in mouse eye development (Davis et al., 2008), and an ortholog of the vertebrate eye regulator *Rax* is not known to be involved in *Drosophila* eye development (Arendt et al., 2004). *Six3* family transcription factors predominate in vertebrate eye development, whereas most invertebrates depend on *Six-1/2* (Vopalensky and Kozmik, 2009). Analysis of additional animal eyes will therefore be an important direction for further testing evolutionary hypotheses related to these genes and eye evolution.

We describe a number of transcription factors conserved among the Bilateria that regulate planarian eye formation, but for which the functions in other animal eyes has not been extensively investigated (Figure 7E). These include *ovo*, *klf*, and *foxQ2*. Our prior work also indicated prominent roles for *dlx* and *sp6-9* in optic cup formation (Lapan and Reddien, 2011). One gene encoding an Ovo-like transcription factor, *shavenbaby*, is known to exist in *Drosophila*. This gene is well-studied in epidermal morphogenesis, but roles for this gene in the eye have not been reported. The chromosomal locus responsible for the blind-sterile phenotype in mouse contains the *ovo2* gene (Li et al., 2002). These mice exhibit small eye size as well as early onset cataracts; however, other genes are contained in this locus and no causal role for *ovo2* in this phenotype is known. Mouse *ovo2* is expressed in the surface ectoderm of the ventral forebrain at E8.75–9.5 (Mackay et al., 2006), the site of optical eminence formation. Interestingly, *ovo2* knockout mice die at E10.5 without displaying any sign of optic vesicle formation typical of this stage, despite the fact that D-V expression domains examined in the neural tube are maintained and overall embryonic development is not delayed (Mackay et al., 2006; Unezaki et al., 2007).

We demonstrate an unbiased technical approach to systematically survey the molecular basis of eye formation and function. This approach identified novel and conserved genetic features of eyes in planarians, further indicating the power of planarians as a system for study of animal eyes. Few transcription factors in animals display eye-specific expression and are required for formation of all eye cells, and *ovo* is a newly discovered one.

EXPERIMENTAL PROCEDURES

Tissue Isolation

Large asexual *Schmidtea mediterranea* were used for eye preparation. Animals were fed twice prior to eye harvest. Head tips were amputated

posterior to the eyes and collected into CMFB + 1 mg/mL collagenase (Sigma) + 0.05% NAC and agitated. Freed eyes were rinsed and transferred to Trizol for RNA collection. For control (ventral anterior) tissue, heads were amputated above the pharynx and then coronal amputation was made to remove dorsal tissue, including eyes.

cDNA Sequencing and Analysis

Approximately 1 μ g of RNA was used to construct Illumina libraries for 36bp single-end sequencing on the GAll platform. Libraries were constructed using Illumina reagents and according to commercial protocol. FPKM were determined using the Cufflinks program Cuffdiff. See [Extended Experimental Procedures](#) for further detail.

Gene Cloning

For cDNA library generation, RNA from heads was isolated using Trizol purification, and used as template for first-strand reverse transcription with Superscript III (Invitrogen). Cloning was otherwise performed as described ([Lapan and Reddien, 2011](#)).

RNA Synthesis and RNAi

For probes used in RNAi experiments, riboprobe template was produced with the same primers as were used for cloning, except that gateway adapters were not present on primers, and T7 promoter sequence was added to the 3' primer. Probe synthesis and RNAi were otherwise performed as described ([Lapan and Reddien, 2011](#)). In homeostasis RNAi experiments, animals were fed dsRNA food every 4 days for 6 weeks.

Histology and Imaging

Whole-mount FISH and antibody staining was performed as described ([Lapan and Reddien, 2011](#); [Pearson et al., 2009](#)). Embryos were killed in 2.7% HCl following manual decapsulation and were otherwise treated the same as adult worms for histologic analysis. Images were acquired with a Zeiss confocal microscope (LSM 700).

ACCESSION NUMBERS

The GenBank accession numbers for *Smed-ovo*, *Smed-klf*, *Smed-foxQ2*, *Smed-meis*, and *Smed-soxB* are JX091074, JX091075, JX091076, JX091077, and JX091078, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes an Extended Discussion, Extended Experimental Procedures, seven figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2012.06.018>.

LICENSING INFORMATION

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