Control of Maintenance and Regeneration of Planarian Eyes by *ovo*

Samuel D. Cross, ¹ Adiv A. Johnson, ¹ Benjamin J. Gilles, ¹ Lori A. Bachman, ¹ Takeshi Inoue, ² Kiyokazu Agata, ² Lihua Y. Marmorstein, ¹ and Alan D. Marmorstein

Correspondence: Alan D. Marmorstein, Department of Ophthalmology, Mayo Clinic, 200 1st Street SW, Rochester, MN 55905, USA; Marmorstein.Alan@mayo.edu.

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Purpose. Following decapitation, the planarian *Schmidtea mediterranea* regenerates its head and eyes. The gene *ovo* is required for eye maintenance and regeneration in response to wounding. In this study, we investigated whether eye regeneration in *S. mediterranea* could occur absent a wound healing response.

METHODS. One hundred twenty *S. mediterranea* were treated with *ovo* RNA interference (RNAi) or control (*unc-22*) RNAi by feeding double-stranded RNA (dsRNA). Following eye loss, *ovo* RNAi treatment was halted and replaced with control RNAi treatment. Quantitative real-time PCR (qPCR) was used to monitor *ovo* expression. Eye functionality was monitored via a phototaxis assay. Photoreceptor neurons were visualized via immunofluorescence staining of arrestin.

RESULTS. Treatment with *ovo* RNAi caused eyes to gradually shrink until they were completely absent. One hundred percent of *ovo* RNAi-treated planarians lost both eyes within 137 days of treatment onset. *ovo* RNAi-treated planarians were unable to regenerate eyes in response to decapitation. Upon removal of *ovo* RNAi, eyes became visible as small pigmented spots in the head within 28 days. The eyes slowly developed, appearing to gain pigmented cells first and then nonpigmented photoreceptors. Phototaxis assays demonstrated functional eye loss and eye restoration. *ovo* mRNA was significantly decreased following treatment with *ovo* RNAi and significantly increased following removal of *ovo* RNAi. Arrestin staining was present in the eyes, optic nerves, and optic chiasm of worms with regenerated eyes but not in eyeless worms

Conclusions. *S. mediterranea* have the ability to generate functional eyes in the absence of a wound healing response. This ability requires the expression of *ovo*.

Keywords: ovo, Schmidtea mediterranea, planarian, phototaxis, regeneration

Ocular trauma is the most common cause of monocular blindness and one of the most common causes of visual impairment in the United States, second only to cataracts. Over 2.4 million eye injuries occur in the United States each year, resulting in a rate of injury requiring medical attention of 3.15 per 1000 population. Although surgery can often restore functional sight, more than 500,000 people worldwide suffer from binocular blindness due to traumatic eye injury. For these patients, the best chance for recovering visual function may be through advancements in regenerative medicine and cell therapy.

The tissues of the human eye have very limited innate ability to regenerate, though there are several animal models of eye regeneration. Frogs and newts retain limited ocular regeneration through adulthood, and zebrafish have the ability to regenerate retina in response to chemical damage, mechanical damage, and phototoxicity.⁵⁻⁷ However, very few animals can regenerate whole eyes in response to injury, and none of these are vertebrates.⁸ One of these animals is the planarian flatworm, which has the ability to regenerate every tissue in its body, including a fully functioning set of eyes and a brain.⁹ The planarian is unique because it can regenerate a whole worm from a small fragment in just 9 days. This regenerative

ability is dependent on a population of adult pluripotent stem cells called neoblasts. ^{9,10} These qualities, in addition to the ease with which planarian genes can be knocked down with RNA interference (RNAi), ¹¹ make the planarian a very powerful model for understanding tissue regeneration.

The use of planarians as a model for eye regeneration in humans relies on similarity between tissues in planarians and humans. Although the planarian eye is far simpler than a human eye, there are significant similarities. The planarian eye is composed of a pigmented cell cup and photoreceptor cells, which extend rhabdomeres into the eye cup. 12 These photoreceptors have axons that project directly to the visual center of the planarian brain. 13 Planarian eyes express many genes homologous to those expressed in vertebrate photoreceptor cells and retinal pigment epithelium, such as opsin and bestrophin 1.¹¹ The transcription factor genes six-1/2, eya, ovo, and pbx have all been shown to be required for planarian eye regeneration. 11,14-16 If any of these genes are knocked down by RNAi, amputation of the head will result in the regeneration of a head with no eyes. Lapan and Reddien¹¹ demonstrated that homeostatically treating planarians with ovo RNAi causes the eyes to gradually shrink and eventually disappear completely. In addition to this, the gene ovo is the

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¹Department of Ophthalmology, Mayo Clinic, Rochester, Minnesota, United States

²Department of Biophysics, Graduate School of Science, Kyoto University, Kyoto, Japan

only known planarian transcription factor to be exclusively expressed in the eye and eye progenitor cells, 11 making this gene a good target for investigating eye regeneration.

The process of planarian regeneration as part of a wound healing in response to injury has been studied for over 100 years, and many of the mechanisms involved are being actively elucidated. However, it is crucial to also study regeneration in the absence of a wound healing response. This is critical for learning to regenerate lost tissue in patients who either are not healing from a wound or have wounds that have already healed. In this study we sought to determine whether planarians have the ability to regenerate eyes in the absence of the wound healing response typically associated with regeneration.

METHODS

Animals

In this study, an asexual, clonal strain of the planarian species *Schmidtea mediterranea* was obtained from Phillip Newmark (University of Illinois at Urbana-Champaign, Urbana, IL, USA) and cultured in Montjuic water¹⁷ at 20°C. We adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Phototaxis Assay

Planarians were transferred to a 10-cm petri dish in a dimly lit room. Half of the dish was covered with an opaque cover, and a light-emitting diode (LED) light source was placed 3 inches above the dish. The room lights were turned off and the LED was activated. After 6 minutes of light exposure, the planarians on the covered and uncovered sides of the dish were counted. ¹⁸ Counts were used to determine relative response values for each group of planarians. In this assay, a relative response value of 1 indicates that a planarian moved away from the light, and a value of –1 indicates that the planarian moved into the light. When averaged for all planarians in a given experiment, a value of 0 indicates that the planarians are equally distributed between the light and dark side of the dish.

Amputation

Planarians were fed 1 day before amputation. The planarians were cooled on ice and their heads were amputated using a No. 11 scalpel blade, immediately posterior to the eyes. The amputated planarians were then allowed to regenerate at 20°C.

RNA Interference

Double-stranded RNA (dsRNA) liver paste was prepared by expressing *ovo* dsRNA in *Escherichia coli* using the pPR244 vector. The bacterial culture was grown, induced via isopropyl β-D-1-thiogalactopyranoside, pelleted, and mixed with liver paste as described by others. ^{11,14} As a negative control, dsRNA targeting the *Caenorhabditis elegans* gene *unc-22* was used. For RNAi treatment, planarians were fed dsRNA liver paste every 3 to 4 days. Before each feeding the planarians were observed under a stereomicroscope and the numbers of eyes were counted.

Imaging

Worms were imaged live using a M165 FC Stereo Microscope, equipped with a DFC3000 G Camera and using the Leica Application Suite software (Leica Microsystems, Wetzlar, Germany). Immunofluorescent images were taken with a ×10

objective on a Nikon E600 research microscope (Nikon Instruments, Melville, NY, USA).

Quantitative PCR (qPCR)

Individual, whole planarians were homogenized using a Tissue Homogenizer TH01 (Omni International, Kennesaw, GA, USA). Total RNA was collected using the RNeasy Micro Kit (Qiagen, Venlo, The Netherlands). Reverse transcription was performed with the High Capacity cDNA RT Kit (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative PCR was carried out using the TaqMan Gene Expression Master Mix on a ViiA-7 qPCR machine (Thermo Fisher Scientific). For a reference gene we used glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), which was previously shown to be a stable reference gene to control for RNA sample variation in *S. mediterranea*. ¹⁹ Three independent qPCR measurements were performed for each probe on each planarian tested. Five nanograms of total planarian RNA was used for each sample. The primers used are as follows:

ovo.

Forward: 5'-CACTCTACTCGCAAACGATGA-3'
Reverse: 5'-ACAGGAAAGAAACTGACTGGAA-3'

GAPDH.

Forward: 5'-CTGCTGATGCACCAATGTATG-3' Reverse: 5'-CTTTCGCCAATGGAGCTAGA-3'

Whole-Mount Immunostaining

Planarians were immunostained according to previously published protocols.^{20,21} The rabbit anti-arrestin antibody was used at a dilution of 1:500 and has been previously characterized.^{20,21} An Alexa Fluor 488 chicken anti-rabbit IgG secondary antibody was used at a dilution of 1:500 (Life Technologies, Carlsbad, CA, USA). Worms were transferred to 35-mm coverslips in glass-bottom culture dishes (MatTek Corporation, Ashland, MA, USA) prior to imaging.

Statistics

Statistics were performed using t-tests. Significance was defined as P < 0.05.

RESULTS

ovo RNAi and Eye Loss

Homeostatic treatment of 60 planarians with ovo RNAi caused eyes to gradually disappear (Fig. 1). After 39 days, ovo RNAitreated planarians had a greatly reduced nonpigmented region and slightly reduced pigmented region of the eye (Fig. 1G). On day 48 we observed the first ovo RNAi-treated planarian to lose both eyes entirely (Fig. 2A). By day 64, no ovo RNAi-treated planarians had a discernible nonpigmented region, and the pigmented region of the eye was greatly reduced (Fig. 1H). A number of planarians treated with ovo RNAi had lost one eye entirely at this point, and some had lost both eyes (Fig. 2A). At day 80, most planarians treated with ovo RNAi had lost one or both eyes, and the eyes that were visible were notably diminished (Figs. 1I, 2A). At day 122, all but one ovo RNAitreated planarian had lost both eyes entirely (Fig. 1J), and by day 137 all ovo RNAi-treated planarians were eyeless (Fig. 2A). The rate at which the loss of eyes occurred was highly variable. Some planarians lost their eyes very quickly, while eyes in others very gradually shrank and didn't disappear completely until after 100 days of treatment (Fig. 2A). Most of the

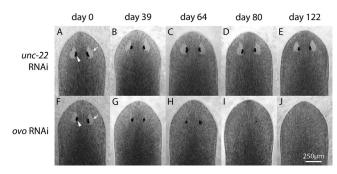


FIGURE 1. Knockdown of the gene *ovo* causes a gradual loss of eyes. Images of live worms following 0, 39, 64, 80, and 122 days of *ovo* (F-J) or control (unc-22) (A-E) RNAi treatment. After 122 days of treatment, the control planarians still had eyes (E) (n = 60), but almost all *ovo* RNAi planarians had no discernible eyes (J) (n = 60). Arrows are nonpigmented photoreceptors (A, F). Arrowheads are pigmented cells (A, F).

planarians had their eyes shrink asymmetrically, leaving them with one eye for a short period before losing the other (Figs. 1I, 2A). Despite the loss of eyes, the planarians were behaviorally normal and healthy. In addition to reacting to water currents, physical stimuli, and temperature changes, they continued to swim actively and eat normally. No changes were observed in the eyes of 60 control planarians treated with unc-22 RNAi in parallel (Figs. 1A–E). As determined by qPCR (Supplementary Fig. S1), treatment with ovo RNAi resulted in a significant, \sim 67% reduction in ovo expression compared to unc-22 RNAi-treated controls (P=0.035).

Functional Loss of Eyes

Normally, planarians avoid light during activities such as feeding and cleaning. After *ovo* RNAi treatment, eyeless *ovo* RNAi-treated planarians did not avoid light during these activities. In contrast, control planarians would all group in the darkest part of the dish or underneath the opaque label. To validate this quantitatively, the visual function of eyeless

planarians was assessed in a phototaxis assay. Control planarians very quickly moved away from the light source and into the shade with a normalized response of 0.64 ± 0.17 , n=20 (Fig. 2B). Instead of moving into the shade, the *ovo* RNAi-treated planarians exhibited no phototaxis response and had nearly equal numbers in the dark and light sides of the dish, with a normalized response of 0.06 ± 0.21 , n=20 (P < 0.002) (Fig. 2B). These data demonstrate that knocking down *ovo* expression causes a functional loss of both eyes.

Loss of Eye Regeneration in Response to Amputation

We examined the ability of planarians to regenerate eyes in response to wounding following knockdown of ovo. To accomplish this, we compared head regeneration in six eyeless, ovo RNAi-treated planarians to that of six control planarians. One day after head amputation, both groups of planarians developed a regeneration blastema, which continued to grow a new head (Figs. 3B, 3G). At day 4, very small eye spots were visible in the control group, but no eye spots were visible in the ovo RNAi group (Figs. 3C, 3H). At day 7, the eyes continued to grow to a normal size in the control group, while no eyes developed in the ovo RNAi group (Figs. 3D, 3I). By day 11, the regenerated heads began to form notable pigment, distinguishing the pigmented and nonpigmented regions of the regenerated eyes in the control group (Fig. 3E). In contrast, the ovo RNAi planarians remained eyeless (Fig. 3J). These data corroborate the findings of Lapan and Reddien¹¹ and demonstrate that, despite retaining the ability to regrow a head, knockdown of ovo renders planarians incapable of regenerating eyes in response to wounding.

Eye Regeneration in Intact Planarians

In order to determine whether or not planarians can regenerate eyes in the absence of a wound healing response, we removed the *ovo* RNAi treatment from a group of 20 *ovo* RNAi-treated planarians. Initially, there were no obvious effects. On day 28, after removal of treatment, very small eye

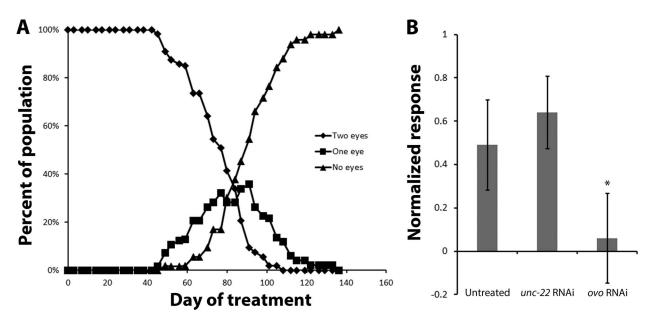


FIGURE 2. ovo RNAi causes a functional loss of eyes. (A) Population of ovo RNAi-treated planarians with two, one, or zero eyes versus days after beginning RNAi treatment. (B) Graphical representation of behavioral phototaxis assay of untreated, unc-22 RNAi-treated, and eyeless ovo RNAi-treated planarians (n=60). A normalized response of 1 indicates that all planarians travel away from light. A normalized response of 0 indicates that the planarians are randomly distributed in light and dark (n=20). *P<0.05.

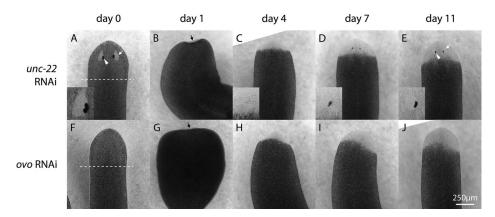


FIGURE 3. ovo RNAi causes loss of ability to regenerate eyes in response to injury. Heads of eyeless ovo RNAi-treated planarians were amputated as indicated by the dotted line in (A) and (F). Live worms were imaged 0, 1, 4, 7, and 11 days post amputation. After 1 day of regeneration, the regeneration blastema (black arrows in [B] and [G]) formed (control: n = 6/6, ovo RNAi: n = 6/6). For 11 days after amputation, regeneration of both the pigmented (white arrowbead in [A] and [E]) and nonpigmented (white arrows in [A] and [E]) cells of the eye were seen in the control planarians (A-E) (n = 6/6). No eye regeneration was seen in the ovo RNAi-treated planarians (F-J) (n = 0/6).

spots could be seen in the heads of some of the planarians (Fig. 4F). The eye spots initially consisted of only pigmented cells. By day 56, the eyes had gradually grown larger and began to form the nonpigmented photoreceptor cell region of the eye (Fig. 4G). Also by this time, all of the worms removed from *ovo* RNAi treatment had regenerated two eyes (Fig. 5A). By day 70 of regeneration, the eyes were restored to a normal size and shape (Fig. 4H). In contrast to the asymmetric loss of eyes induced by *ovo* RNAi treatment, the regenerating eyes grew symmetrically, with both eyes becoming visible at the same time (Figs. 4, 5A). As determined by qPCR (Supplementary Fig. S1), removal of *ovo* RNAi resulted in a significant, \sim 147% increase in *ovo* expression compared to *unc-22* RNAi controls (P = 0.0002).

Functionality of Regenerated Eyes

We wanted to confirm that eyes regenerated in an intact worm were functional. For this to be the case, the regenerated photoreceptors would have to connect to the existing brain rather than regenerating in concert with a regenerating brain.

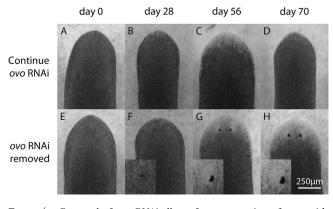


FIGURE 4. Removal of *ovo* RNAi allows for regeneration of eyes with no wound response. 137 days of feeding with *ovo* RNAi resulted in 100% of planarians becoming eyeless (A, E). We switched 20 eyeless planarians to the control *unc-22* RNAi (E-H) while maintaining 20 planarians on *ovo* RNAi (A-D). Planarians maintained on *ovo* RNAi remained eyeless for at least 70 days (D). 100% of planarians switched from *ovo* RNAi to *unc-22* RNAi regenerated eyes. Eyes became apparent as early as day 28 (F) with 100% of planarians regenerating complete eyes by day 56 (G). The eyes were of comparable size to those of control planarians by day 70 (H).

To determine the functionality of the regenerated eyes, the phototaxis assay was repeated for planarians that were removed from ovo RNAi treatment. The worms that were removed from ovo RNAi treatment demonstrated a significantly increased phototaxis response of 0.64 ± 0.17 , n = 9 (P < 0.009), indicating that the planarians moved away from the light. The worms that continued ovo RNAi treatment were found to have no change in light response, with a normalized response of 0.07 ± 0.12 , n = 11 (Fig. 5B). The phototaxis response of unc-22 RNAi-treated worms was 0.52 ± 0.10 , n = 20, and was not significantly different from that of the worms removed from ovo RNAi (P = 0.249) (Fig. 5B). These data demonstrate the functional restoration of eyes in response to removal of ovo RNAi treatment.

In planarians, the protein arrestin is found in photoreceptor neurons. Whole-mount immunostaining of planarian flatworms for arrestin reveals expression in eyes, optic nerves, and the optic chiasm.^{22,23} Although visual inspection revealed fully regrown eyes (Fig. 4) and our phototaxis assays demonstrated that the regrown eyes were functional (Fig. 5B), we wanted to further confirm that these regenerated eyes were rewired to the existing planarian nervous system. To do this, we stained control worms, eyeless worms, and worms with regenerated eyes for arrestin (Fig. 6). We found that, in control worms, arrestin staining was localized to the eyes (Fig. 6A) as well as the optic nerves and optic chiasm (Fig. 6B). In contrast, ovo RNAi-treated eyeless worms showed no discernible arrestin staining (Figs. 6C, 6D). Forty days after the switch from ovo RNAi to unc-22 RNAi, worms showed robust staining for arrestin in their regenerated eyes (Fig. 6E) as well as in their optic nerves and optic chiasm (Fig. 6F).

DISCUSSION

While previous studies have investigated planarian eye regeneration in response to head amputation, eye regeneration in the absence of wound healing has not previously been investigated. In this study, we sought to test the hypothesis that intact planarians could regenerate functional eyes in the absence of a wound healing response. We found that eyes eliminated by knockdown of the gene *ovo* were physically and functionally regenerated when *ovo* RNAi treatment was discontinued. These data demonstrate that the planarian has the ability to regenerate its eyes in the absence of cues induced by wounding.

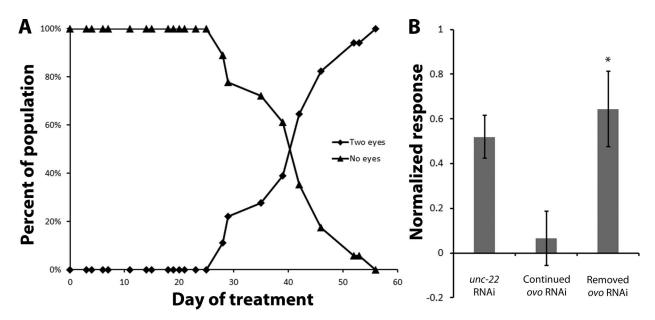


FIGURE 5. Restoration of *ovo* expression allows for recovery of visual function. (A) Population of *ovo* RNAi planarians with two or zero eyes versus days after cessation of RNAi treatment. Planarians removed from *ovo* RNAi treatment regrew eyes symmetrically, so the number of one-eyed planarians was zero throughout regeneration. (B) Phototaxis behavioral assay 63 days after cessation of treatment (n = 9) indicates restoration of light sensitivity and thus regeneration of functional eyes. *P < 0.05.

We reproduced the previous finding that long-term treatment of *ovo* RNAi results in the gradual loss of eyes in *S. mediterranea* in 100% of the population treated (Figs. 1, 2A). ¹¹ However, while total eye loss was previously reported after 2 months of treatment, we found that 86% of the population still had two eyes after 2 months (Fig. 2A). In our hands, total eye loss in the entire population occurred after 4.5 months of treatment (Fig. 2A). This is a large difference and may be due to our larger sample size or variation in the level of *ovo*

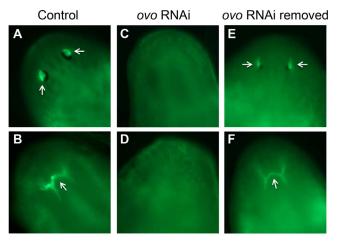


FIGURE 6. Regenerated eyes show robust arrestin staining specific to photoreceptor neurons. Whole-mount immunostaining using an antiarrestin antibody was used to stain for photoreceptor neurons in control worms, in eyeless *ovo* RNAi-treated worms, and in worms with regenerated eyes where *ovo* RNAi was relieved. In control worms, arrestin was localized to the eyes (A) as well as the optic nerves and the optic chiasm (B). Eyeless worms treated with *ovo* RNAi showed no discernible staining for arrestin (C, D). 40 days after switching eyeless worms from *ovo* RNAi to *unc-22* RNAi, robust arrestin staining was found in the regenerated eyes (E) as well as in the regenerated optic nerves and optic chiasm (F). *White arrows* in (A) and (E) highlight arrestin staining in eyes, while *white arrows* in (B) and (F) highlight arrestin staining in the optic nerves and optic chiasm.

knockdown between labs. ovo mRNA expression was found to be decreased by ~67% in the ovo RNAi-treated planarians using qPCR (Supplementary Fig. S1). Unlike creating a transgenic knockout, treatment with RNAi does not result in 100% knockdown. The data contained herein demonstrate that a two-thirds reduction in expression of the gene ovo is sufficient to cause eye loss and to inhibit eye regeneration. Interestingly, when ovo RNAi was ceased and planarians grew back their eyes, a \sim 147% increase in *ovo* expression over the value in controls was observed (Supplementary Fig. S1). The gene ovo is a master transcription factor that controls the development and regeneration of eyes in planarians.¹¹ Its expression is likely substantially increased over controls following relief of ovo RNAi due to a need for increased ovo to direct eye development. This stands in contrast to control worms, which already have eyes and require only enough ovo to sustain ocular maintenance.

Planarians have been shown to undergo whole body tissue turnover.²⁴ The gradual loss of eyes due to ovo RNAi can be explained by the loss of the ability of neoblasts to differentiate into eye cells, and the inability to replenish eye cells when they turn over. As the eyes diminished over time, the nonpigmented photoreceptors appeared to disappear before the pigmented cells (Fig. 1). This indicates that the turnover rate of planarian photoreceptors may be higher than that of pigmented cells. The rate at which the planarians lost their eyes was highly variable: The first planarian to lose both eyes did so after 49 days of treatment, and the last was after 136 days of treatment (Fig. 2A). Also, many of the planarians had one eye disappear before the other, leaving a population of temporarily one-eyed planarians during eye loss (Fig. 2A). The difference between eyes might be due to larger eyes taking longer to disappear. It is also possible that, given a dearth of eye progenitor cells following ovo knockdown, the remaining cells will preferentially migrate to the larger eye, allowing for monocular vision to be maintained. A significant reduction in the negative phototaxis response was observed in eyeless ovo RNAi-treated planarians (Fig. 2B), confirming the loss of functional vision in ovo RNAi-treated worms. These ovo RNAi-treated planarians were unable to regenerate eyes in response to wounding and formed a normal regeneration blastema and a regenerated head with no eyes (Fig. 3). This is consistent with a previous study, which demonstrated that short-term treatment of *ovo* RNAi followed by head amputation causes regeneration of a head with no eyes.¹¹

The restoration of ovo expression resulted in eye regeneration in the absence of wounding (Fig. 4). This is a novel finding and confirms that the regeneration of new eyes in an intact planarian is possible. The eyes first became visible after approximately 28 days as small pigmented spots (Figs. 4F, 5A). They gradually grew larger and soon began to form a nonpigmented region. After 70 days from the removal of ovo RNAi treatment, the eyes were of comparable size to those of control planarians (Fig. 4H). The regeneration of the pigment cells appeared to occur prior to the regeneration of photoreceptor cells, the same order as occurs when regenerating a whole head following amputation.²⁵ This indicates that the same process may be used to regenerate eyes in both cases, though it is interesting to note that eye regeneration seems to occur more slowly in an intact planarian. The observed difference in timing might be due to the absence of cues induced by a wound healing response. Amputation, leading to the formation of a blastema and regeneration, causes the proliferation of stem cells,26 which could allow for more rapid regeneration. It is also possible that signaling from the wound or regeneration blastema causes an increased rate of stem cell differentiation, leading to more rapid replenishment of eye

The phototaxis assay was repeated in order to determine whether the planarians removed from ovo RNAi treatment regained visual function (Fig. 5B). The worms removed from ovo RNAi were found to have a significantly increased light response compared to those continued on ovo RNAi treatment and were found to have a comparable light response to that of control planarians (Fig. 5B). For this to occur, the photoreceptor cells from the newly formed eyes would have to project axons through mature tissue, cross over properly at a newly formed optic chiasm,²⁷ and connect to an existing brain in the absence of developmental cues that would be present during head regeneration following amputation. Our whole-mount immunostaining of the photoreceptor-specific protein arrestin confirmed this, demonstrating the expression of arrestin in eyes, optic nerves, and the optic chiasm in worms with regenerated eyes where ovo RNAi treatment was terminated (Figs. 6E, 6F). Arrestin staining in control worms (Figs. 6A, 6B) was similar to the arrestin staining in worms that regenerated eyes independent of a wound healing response (Figs. 6E, 6F). In contrast, eyeless worms maintained on ovo RNAi showed no detectable arrestin staining (Figs. 6C, 6D).

Our data demonstrate that planarians have the ability to regenerate whole eyes independent of wounding and demonstrates for the first time, to our knowledge in any species, the ability to functionally connect regenerated eyes with an existing brain. This solidifies planarians as a powerful and unique model for studying eye regeneration and in particular how to reconnect and rewire the visual system in an intact organism. Understanding how to regenerate eyes would greatly benefit treatment of victims of ocular trauma and all diseases that involve the damage or loss of eye tissue. For this purpose, further study is required to investigate the interactions between the regenerating planarian photoreceptors and the visual center of the planarian brain.

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