LETTERS

BMP inhibition-driven regulation of *six-3* underlies induction of newt lens regeneration

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Lens regeneration in adult newts is a classic example of how cells can faithfully regenerate a complete organ through the process of transdifferentiation[1–6]. After lens removal, the pigment epithelial cells of the dorsal, but not the ventral, iris dedifferentiate and then differentiate to form a new lens. Understanding how this process is regulated might provide clues about why lens regeneration does not occur in higher vertebrates. The genes *six-3* and *pax-6* are known to induce ectopic lenses during embryogenesis[7,8]. Here we tested these genes, as well as members of the bone morphogenetic protein (BMP) pathway that regulate establishment of the dorsal–ventral axis in embryos[9], for their ability to induce lens regeneration. We show that the lens can be regenerated from the ventral iris when the BMP pathway is inhibited and when the iris is transfected with *six-3* and treated with retinoic acid. In intact irises, *six-3* is expressed at higher levels in the ventral than in the dorsal iris. During regeneration, however, only expression in the dorsal iris is significantly increased. Such an increase is seen in ventral irises only when they are induced to transdifferentiate by *six-3* and retinoic acid or by BMP inhibitors. These data suggest that lens regeneration can be achieved in noncompetent adult tissues and that this regeneration occurs through a gene regulatory mechanism that is more complex than the dorsal expression of lens regeneration-specific genes.

To determine the role of *six-3* and *pax-6* in the induction of transdifferentiation of the ventral iris, ventral iris cells were transfected in the presence or absence of retinoic acid with the appropriate constructs and examined for induction by using an *in vitro* transfection and *in vivo* transplantation system that reproduces the conditions seen *in vivo*[10–12]. Retinoids have been shown to affect regeneration and to determine morphogenesis and differentiation of several tissues including the eye and limb[13–16]. In addition, dorsal or ventral iris explants were treated with soluble BMP-4, BMP-7, chordin and a soluble competitor for the receptor BMPR-IA.

After transfection and implantation of aggregated pigment epithelial cells (PECs), scores of eyes were examined (see Supplementary Information). As a rule, untransfected dorsal PEC aggregates transdifferentiate to lens, whereas the ventral ones do not. Under the conditions outlined in the Methods, short-term culturing of cells does not interfere with the potential for lens transdifferentiation. Dorsal aggregates produced a lens in over 83% of tests (10/12), whereas the ventral ones, as expected, did not (0/11; Fig. 1a–c). It has been shown, through β-galactosidase staining, that the lens is indeed derived from the aggregate[12]. Dorsal aggregates transfected with the constructs with retinoic acid treatment also transdifferentiated to lens (data not shown). With ventral PECs, however, only one protocol—namely, transfection of PECs with *six-3* in the presence

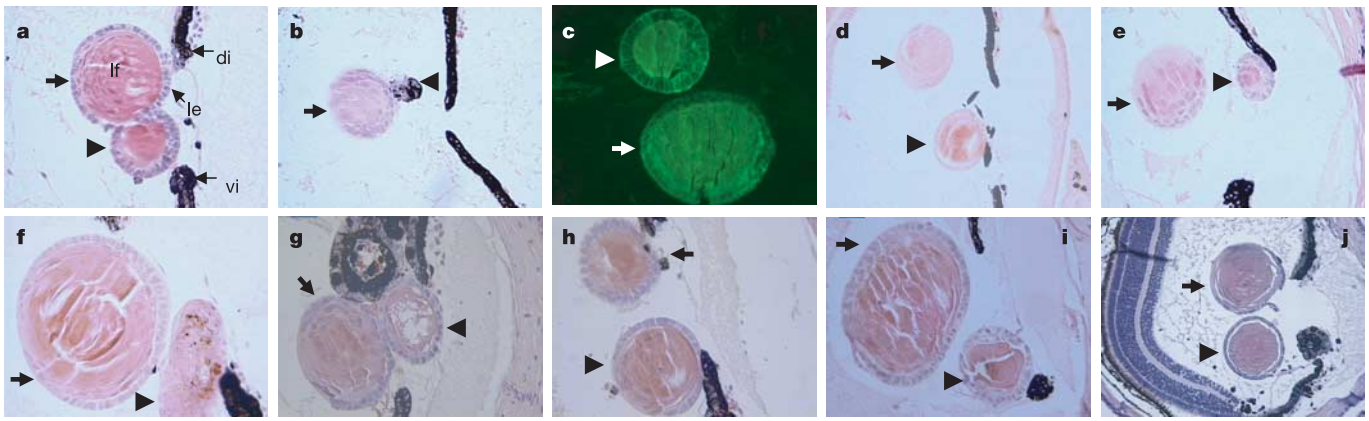

Figure 1 | Lens induction from ventral PECs. a–f, Lens induction by transplantation of PEC aggregates examined 30 d after transplantation. Thick arrows indicate the host regenerated lens; arrowheads indicate the PEC aggregate or the lens induced from the PEC aggregate. **a**, A control untransfected dorsal PEC aggregate that has transdifferentiated to lens. le, lens epithelium; lf, lens fibres; di, dorsal iris; vi, ventral iris. **b**, A control untransfected ventral PEC aggregate (arrowhead) that has remained pigmented and failed to transdifferentiate to lens. **c**, Detection of crystallin synthesis in both a host lens and an induced lens with a lens fibre-specific antibody to β-crystallin[23]. **d–f**, Induced lenses from ventral PEC aggregates transfected with *six-3* and treated with retinoic acid (RA). **g–j**, Induced lenses from ventral iris explants treated with BMPR-IA inhibitor (**g–i**) and with chordin (**j**).

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of retinoic acid—led to the induction of lens transdifferentiation (Fig. 1d–f). This induction occurred at a rate (3/4; 75%) comparable to that seen in the dorsal aggregates. Neither treatment with retinoic acid alone nor transfection of ventral PEC cultures with *six*-3 alone induced transdifferentiation.

In the BMP series we found that inhibition of the pathway by either the BMPR-IA competitor or chordin resulted in the induction of a lens from the ventral explants (3/15 and 1/8 respectively; Fig. 1g–j). The incidence of induction was low (17%); however, we regard this as highly significant because no untreated ventral explants differentiated to lens (0/27; 0% induction). This is in agreement with the established role of BMPs in maintaining ventral identity during embryogenesis and the fact that inhibiting the binding of BMPs to receptors results in dorsalization[9]. Notably, treatment of the dorsal iris explants with BMP-7, and to a lesser degree with BMP-4, considerably inhibited their ability to transdifferentiate to lens (1/12 (8.3%) and 5/12 (41.6%), respectively). Such results clearly indicate that BMPs maintain ventral identity and that inhibition of the pathway dorsalizes the ventral iris, allowing transdifferentiation.

To probe further the mechanism of induction, we undertook gene expression profiling of *six-3* and *BMPR-IA* during lens regeneration and during the experimental treatments that lead to the induction of lens regeneration from the ventral iris. Expression of *pax-6* was also assessed because of its known association with *six-3*. We examined samples of iris isolated 2, 4 and 8 d after lentectomy, as during this time dedifferentiation events that lead to regeneration from the dorsal iris have been initiated. Moreover, at later stages the vesicle starts expressing crystallins and differentiating to lens. Because these genes are also expressed in the differentiating lens, their induction-related expression might be 'contaminated'. Several unexpected points emerged from the expression patterns that call for a revision of our view of the mechanism of lens regeneration.

First, both dorsal and ventral iris showed expression of all three genes. Analysis of the data to compare expression between the dorsal and ventral iris showed that the three genes were expressed more highly in the intact ventral iris. This pattern was maintained at day 8 but with a lesser relative fold change (Fig. 2a). However, analysis of the data to compare expression in the 2-, 4- and 8-d dorsal iris relative to the intact dorsal iris, and the 2-, 4- and 8-d ventral iris relative to the intact ventral iris, to correlate expression with the process of regeneration, revealed another pattern. The expression of *six-3* was increased only in the dorsal iris and seemed comparable at day 8 to that in the ventral iris. *BMPR-IA* and *pax-6* were also slightly upregulated (Fig. 2b–d). Upregulation of *six-3* in the dorsal iris started at day 4 (Fig. 2c), whereas that of *pax-6* and *BMPR-IA* started at day 8 (Fig. 2d). Thus, an increase in *six-3* seems to be important during the dedifferentiation process in the dorsal iris. Because regeneration occurs only from the dorsal iris and because the ventral iris also expresses these genes, our data suggest that gene regulation associated with the competency for lens regeneration aims to increase expression over a particular threshold and not simply to render a regulatory gene as dorsal-specific. Such a pattern for *six-3* is clearly shown when its expression at the different time points is directly compared with that in the intact dorsal iris (Fig. 2e).

Treatment of ventral iris cells with *six-3* and retinoic acid, which resulted in induction of transdifferentiation, showed a similar pattern of upregulation of *six-3*, *pax-6* and *BMPR-IA* when compared with the untransfected ventral cells (Fig. 2f). Treatment of the cells with retinoic acid alone or transfection of *six-3* alone, which did not induce the irises to differentiate to lens, did not show such a pattern (data not shown). Similarly, treatment of ventral iris explants with chordin, which also resulted in induction, invoked a marked upregulation of *six-3*, *pax-6* and, to a lesser degree, *BMPR-IA* in the treated ventral irises, as compared with the increase in untreated irises (Fig. 2g). *BMPR-IA* transcriptional regulation might not be that important for the induction. Notably, the rate of increase (as a relative fold change) in the treated ventral irises was comparable to the increase in the regenerating 8-d dorsal iris. Thus, the treated ventral irises that transdifferentiated to lens adopted a gene expression profile (especially for *six-3*) that was seen only in the dorsal iris during dedifferentiation and regeneration. This observation indicates that when the ventral irises are coaxed to mimic patterns of regulatory events seen in the dorsal iris they become 'dorsalized' and thus can transdifferentiate into lens.

These expression patterns of *six-3* led us to examine whether there are subpopulations of cells in the dorsal or ventral iris that might account for these differences by using immunostaining to assess the distribution of Six-3-expressing cells. We stained serial sections along the nasal–temporal axis that spanned the whole iris (with distinct dorsal and ventral portions) and we counted the positive cells. Six-3-positive cells were found throughout the 8-d dorsal and ventral irises examined without apparent differences in their distribution (Fig. 3a), showing that the upregulation of *six-3* is not due to its expression in more cells. When the aggregates (or explants) transdifferentiated to lens, nearly all cells participated, also arguing against the existence of Six-3-expressing subpopulations (Fig. 1). We also examined the expression of Six-3 and BMPR-IA throughout the lens regeneration process. Figure 3b shows expression in dorsal and ventral iris at early stages (before vesicle formation) and Fig. 3c shows expression in

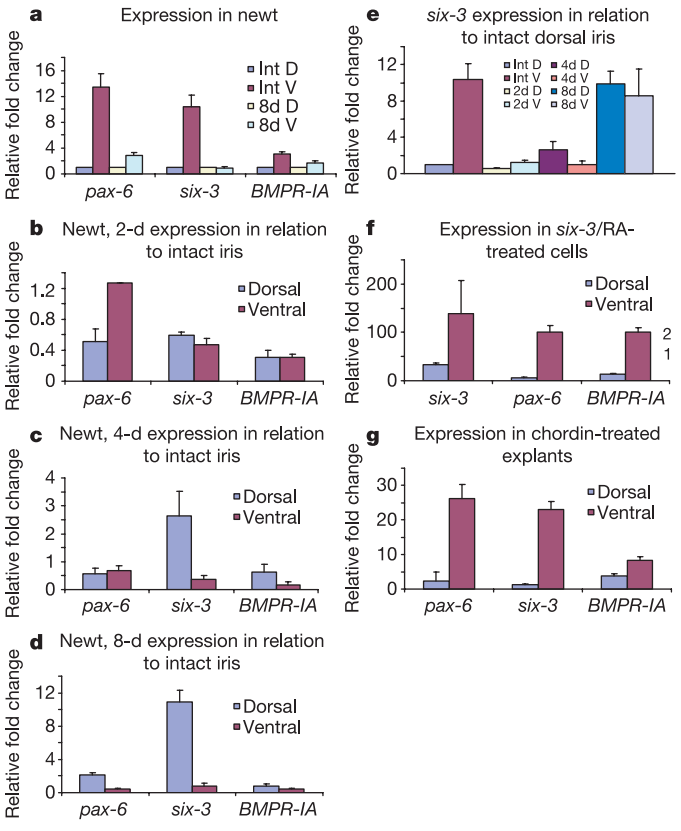

Figure 2 | Expression during lens regeneration and induction. **a**, Comparison of *pax-6*, *six-3* and *BMPR-IA* expression between the newt dorsal and ventral iris. Expression is shown in intact irises and 8 d after lentectomy. Values in the dorsal iris have been set to 1 and those in the ventral iris are shown as relative fold changes. **b**–**d**, Comparison of *pax-6*, *six-3* and *BMPR-IA* expression in 2-, 4- and 8-d irises with that in intact irises. **e**, Comparison of *six-3* expression at all time points with that in the intact dorsal iris. **f**, Expression in PECs treated with *six-3* plus retinoic acid (RA) relative to untransfected cells. Note the considerable increase in exogenous *six-3* levels in the ventral PECs. Note that the levels of *pax-6* and *BMPR-IA* are much lower, as indicated by the numbers on the right *y* axis (used to accommodate the very high fold increase in *six-3*). **g**, Expression in chordin-treated iris explants relative to untreated explants. Data show mean ± s.d.

dorsal iris at later stages (in vesicles or regenerating lens). Expression in ventral iris at later stages was also positive (data not shown). The presence of Six-3 in dorsal and ventral iris is consistent with the polymerase chain reaction (PCR) data; however, the immunostaining data are not quantitative.

We also considered whether the above 'treatments' are unique to the newt or whether they could induce lens transdifferentiation in irises from other vertebrates by testing another salamander, the axolotl, which possesses the ability to regenerate limbs and tail, but not the lens. None of the treatments induced transdifferentiation, indicating that the treatments are most probably newt-specific. However, it might be too premature to preclude the participation of *six-3* and BMP inhibition in lens regeneration in other species. The lens is regenerated from the dorsal iris in the newt but from the cornea in the pre-metamorphic frog—two strategies that differ from the embryonic induction of lens development and that could argue against absolute conservation of the inductive mechanisms.

To gain insight into the axolotl data, we examined gene expression in intact irises, in irises 8 d after lentectomy and in treated irises. In contrast to what was observed in the newt, the expression profiles in the axolotl differed considerably in both intact and 8-d irises (Fig. 4a). Expression in the intact and 8-d ventral irises was not higher than in the dorsal ones. Moreover, *six-3* was severely downregulated in the irises 8 d after lentectomy as compared with the intact irises. *pax-6* and *BMPR-IA* were slightly upregulated in both dorsal and ventral irises (Fig. 4b). This expression pattern is diametrically opposite to what we observed in the newt and indicates that negative regulation of *six-3*, as well as regulation in establishing thresholds, might be responsible for the lack of lens regeneration in the axolotl. So, why did the treatments fail to induce lens transdifferentiation? We think that it was because repression of *six-3* persisted even after the treatments. Indeed, treatment with chordin, which mediated upregulation of *six-3* and *pax-6* in the induced newt ventral irises, did not do so in the axolotl (compare Fig. 4c with Fig. 2g). Several explanations can account for the axolotl results. First, *six-3*

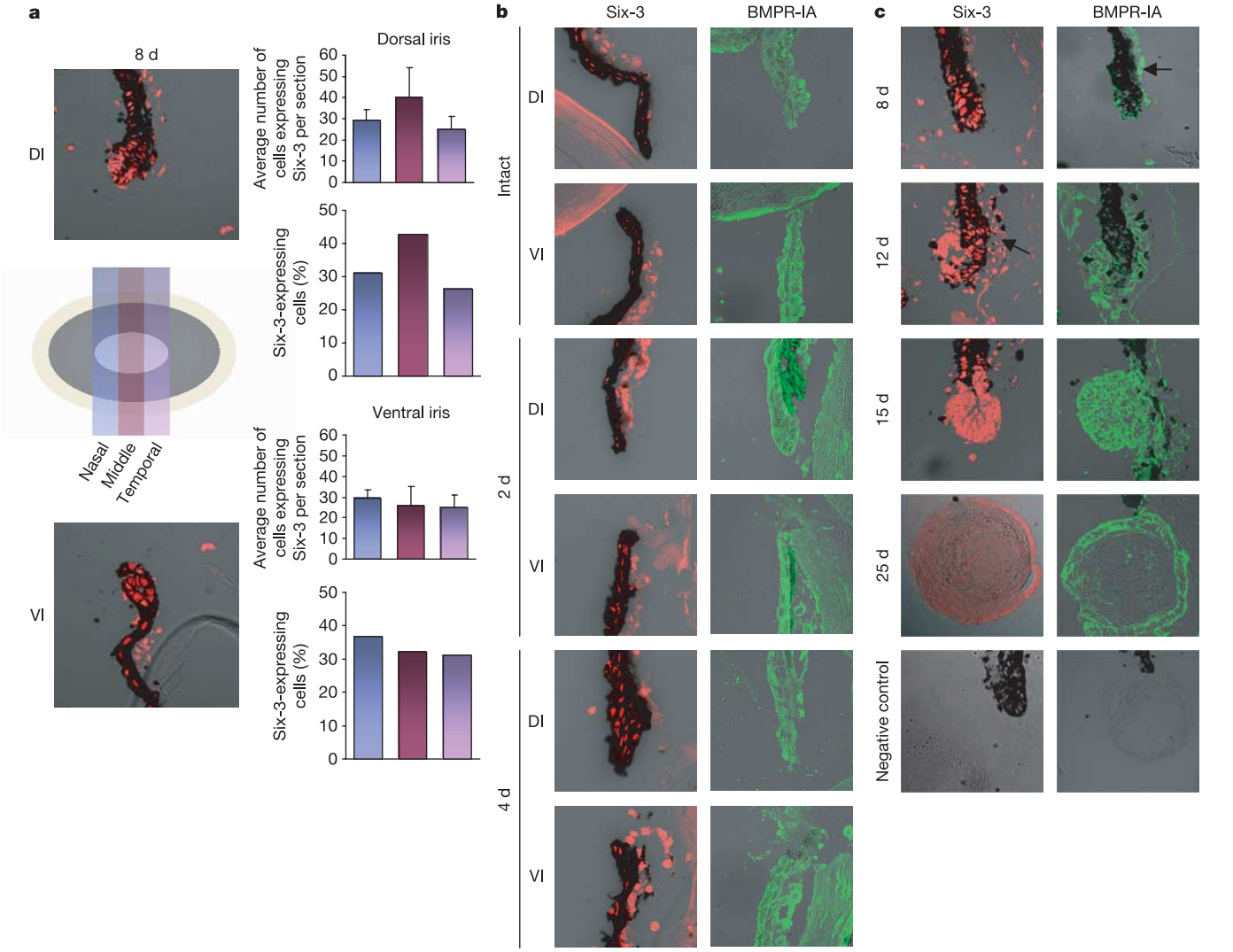

Figure 3 | Expression of Six-3 and BMPR-IA during regeneration. **a**, Identification of Six-3-positive cells in the dorsal (DI) and ventral (VI) iris 8 d after lentectomy, as assessed by immunostaining of serial sections along the nasal–temporal axis (see drawing). No apparent difference in cell numbers or distribution was seen (right). Graphical data are the mean ± s.d. **b**, Expression of Six-3 and BMPR-IA in dorsal and ventral iris at early stages (before vesicle formation). Owing to heavy pigmentation at these early stages for the BMPR-IA staining, sections were bleached. **c**, Expression of Six-3 and BMPR-IA at later stages. At 12 d after lentectomy, a definite lens vesicle has been formed. The lens vesicle and the differentiating lens fibres are positive at 15 d, and by 25 d only cells in the lens epithelium express Six-3. The same patterns can be seen for BMPR-IA. Negative controls are shown at the bottom. All images shown are mergers of the dark field and DIC images. The stroma (arrows) always shows unspecific fluorescence.

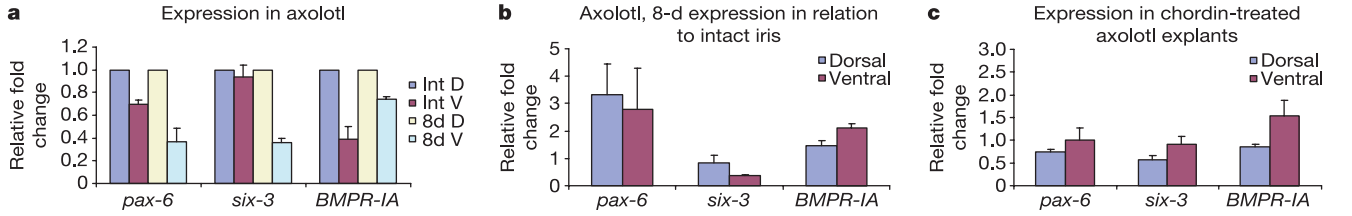

Figure 4 | Expression in axolotl. a, Comparison of *pax-6*, *six-3* and *BMPR-IA* expression between the axolotl dorsal and ventral iris. Expression is shown in intact irises and 8 d after lentectomy. Values in the dorsal iris have been set to 1 and those in the ventral iris are shown as relative fold changes. **b**, Comparison of expression in 8-d irises with intact irises. **c**, Expression in chordin-treated iris explants relative to untreated explants. Data show mean ± s.d.

repression could be regulated more tightly in the axolotl than in the newt, possibly by an inhibitor of its transcription and function. Second, the axolotl PECs do not respond to these treatments equally and they may require optimized conditions. Third, the mechanism of induction of lens regeneration in different vertebrates follows unique pathways.

Notably, *pax-6* did not induce transdifferentiation of the ventral iris, even though it was shown to be upregulated in the chordin-treated ventral irises. However, *six-3* was not upregulated in *pax-6*-transfected cells (data not shown), which might be the reason why *pax-6* could not induce transdifferentiation. On the basis of other results from our laboratories, we now consider that *pax-6* is involved in later events of lens regeneration, such as the proliferation of PECs in both the dorsal and ventral iris and the control of crystallin synthesis (unpublished data). The fact that retinoic acid was also necessary for the induction most probably indicates that other factors regulated by retinoic acid are involved, a synergism that has been shown in other studies[16–18]. Notably, both inhibitors of the BMP pathway and the *six-3*–*pax-6* loop are part of a network identified during induction of eye development[19]. The upregulation of *six-3* and *pax-6* in chordin-treated iris explants (Fig. 2g) suggests that the BMP signalling is upstream of the *six-3*–*pax-6* regulatory loop.

Previous work has shown that other important regulators of lens differentiation, such as FGFs, Sox2, MafB and members of the Hedgehog pathway, are expressed in both dorsal and ventral iris[20,21]. This expression goes against the commonly held belief that regulatory genes involved in lens regeneration should be dorsal-specific. However, our detailed quantitative studies suggest that previously unknown regulatory events are involved in the induction of lens regeneration. Collectively, our data show that induction of lens regeneration can be achieved in noncompetent adult tissues—an important finding because ectopic lens formation has not been previously shown in adults and it opens new avenues in the field of vertebrate lens regeneration.

METHODS

All methods not listed here can be found in Supplementary Information.

Cloning of newt *six-3* and *BMPR-IA* partial cDNAs. *BMPR-IA* was cloned with RNA isolated from newt forelimb blastema (~2 weeks after amputation) using Tri Reagent (Molecular Research Center) according to the manufacturer's instructions. Dorsal PECs were used to clone a partial cDNA for *six-3*. We used 1 μg of RNA to synthesize cDNA using an iScript cDNA synthesis kit (BioRad). For PCR, a portion of the DNA was used along with *Taq* polymerase, 200 μM dNTPs and 800 nM primers. We used the following primers: *BMPR-IA*, forward (5′-TGCTGYATTGCTGAYYTDGG-3′) and reverse (5′-GGRTCATTY GGCACCA-3′); *six-3*, forward (5′-CACTACCAGGAGGCCGAGAA-3′) and reverse (5′- TCCTTGAAGCAGTGCGTCTT-3′). DNA was purified with a MinElute gel extraction kit (Qiagen). The fragment was cloned by the pGEM-T Easy vector system (Promega) and sequenced.

Immunostaining. Affinity-purified polyclonal antibodies were made against Six-3 and BMPR-IA peptides. Antibody against Six-3 was made in rabbit (New England Peptide) and antibody against BMPR-IA in chick (Cocalico). Newts were anaesthetized and the lens was removed through a slit in the cornea. The newts were killed at 2, 4, 8, 12, 15 and 25 d after lentectomy. The eyeballs were enucleated and fixed in 4% formaldehyde for 4 h, washed in 1 × PBS buffer, cryoprotected in 30% sucrose, embedded in OCT (Andwin Scientific), frozen and sectioned at 10 μm. Slides with frozen serial sections were washed several times in PBS and 1% saponin (Sigma) and incubated in 10% goat serum in PBS. Occasionally, to reduce pigmentation, sections were bleached in 0.1% potassium permanganate for 10 min, immersed in 0.5% oxalic acid for 5 min and then rinsed in PBS.

The samples were incubated at 4 °C overnight with primary antibody against newt Six-3 diluted 1:10 in blocking solution or against newt BMPR-IA diluted 1:100 in blocking solution, washed in 0.3% PBST and PBS, and then incubated with the following secondary antibody for 2 h at 37 °C: Alexaflor 546-conjugated goat anti-rabbit (Molecular Probes) for Six-3, or FITC-conjugated rabbit antibody against chicken (Sigma) for BMPR-IA, diluted 1:200 in 10% goat serum in PBST. The sections were washed with PBST and PBS, and covered with coverslips using Vectashield (Vector Labs). Images were taken by confocal microscopy.

Real-time PCR. RNA was isolated from iris tissue and PECs by using Tri Reagent (Molecular Research Center) according to the manufacturer's instructions. We used the following tissues and cells: intact dorsal and ventral iris; 2-, 4-, 8-d dorsal and ventral iris; cells isolated from dorsal and ventral iris; dorsal and ventral iris cells transfected with *six-3* and treated with retinoic acid, transfected with *six-3* alone, or treated with retinoic acid alone; explants from dorsal and ventral iris; dorsal and ventral iris explants treated with chordin or BMPR-IA; axolotl intact and 8-d dorsal and ventral iris; and chordin-treated axolotl dorsal and ventral iris. The RNA isolated was used to evaluate expression of *six-3*, *BMPR-IA* and *pax-6* (along with a suitable reference gene) by real-time PCR, as well as by RT–PCR to verify that the correct fragment was amplified. Appropriate negative controls were included in all sets.

We used 0.75 μg of RNA to synthesize cDNA using an iScript cDNA synthesis kit (BioRad). All real-time PCRs were done with an iCycler (BioRad). For each real-time PCR reaction run in triplicate, 2 μl of cDNA, 800 nM primers and iQ SYBR Green Supermix (BioRad) were used. Primers were designed from the cloned cDNAs for *six-3* and *BMPR-IA* and from a published sequence of *pax-6*. The newt primers were *rpL27*, forward (5′-TACAACCACTTGATGCCA-3′) and reverse (5′-CAGTCTTGTATCGTTCCTCA-3′); *pax-6*, forward (5′-CTGGGC AGGTATTACGAG-3′) and reverse (5′-GTCTCTGATTTCCCAGGC-3′); *six-3*, forward (5′-CAAGAAGTTCCCGCTGC-3′) and reverse (5′-GGTAGGGG TCCTGTAGGTAC-3′); *BMPR-IA*, forward (5′-TGCTGTATTGCTGATTT AGG-3′) and reverse (5′-ATAGGTATCAAAGCAGTCCA-3′). The axolotl primers were *RP*, forward (5′-CATCAGATCAAGCAAGCAGTA-3′) and reverse (5′-CCAATGCAGCAGTTTAGATG-3′); *pax-6*, forward (5′-GAGTGCTCCGC AACCTG-3′) and reverse (5′-ATTCGTGTTCTCGCCTCC-3′); *six-3* (same as newt); *BMPR-IA*, forward (5′-CAGTGCTGCATTGCTGAT-3′) and reverse (5′-GGCTACTTCCCAAATAACC-3′).

For each real-time PCR the basic program was as follows: denaturation at 95 °C, annealing at 50.6 °C and extension at 72 °C (40 cycles). To minimize the background caused by primer–dimer formation, an extra step was added (78 °C for 6 s) at the end of each cycle. The readings were taken during this step. Data analysis was done with the Pfaffl method[22]. The reference genes were *rpL27* for newt and *RP* for axolotl, which both encode ribosomal proteins.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information Sequences for *six-3* and *BMPR-IA* have been deposited in GenBank under accession numbers AY799802 and AY795966, respectively. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to P.A.T. (panagiotis.tsonis@notes.udayton.edu).