Controlling gene loss of function in newts with emphasis on lens regeneration

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Here we describe a protocol for gene loss of function during regeneration in newts, specifically applied to lens regeneration. Knockdown with the use of morpholinos can be achieved both *in vitro* and *in vivo*, depending on the experimental design. These methods achieve desirable levels of gene knockdown, and thus can be compared with methods developed for use in other animals, such as zebrafish. The technology has been applied to study molecular mechanisms during the process of lens regeneration by knocking down genes at specific stages and examining their effects on other genes and lens differentiation. The protocol can take a few days or up to 20 d to complete, depending on the duration of the experiment.

INTRODUCTION

Among vertebrates, the newt (*Notophthalmus viridescens*) is indispensable to regeneration research. These animals are capable of regenerating body parts, organs and tissues such as limbs, tails and spinal cords, eye parts (such as the retina and the lens), brain, heart and jaw[1,2]. Most notably, it seems that regeneration is mediated by dedifferentiation of the somatic cells at the site of injury[3]. In the case of retina or lens regeneration, the process of transdifferentiation has also been documented[4,5]. This means that the regenerated tissues are produced by a different one—in the case of lens, from the iris pigmented epithelial cells (PECs) and in the case of retina, from the retinal PECs. Such events pose important biological questions that are closely relevant to reprogramming, stem cells, aging and of course gene regulation[6]. Despite the tremendous possibilities that the newt offers as a model organism, research has been considerably hindered until now by the difficulty of manipulating gene expression in adult animals.

In a companion protocol, we provide methods for expressing exogenous genes in newts by transgenesis[7]; here we describe methods to study gene loss of function during the process of lens regeneration that have been developed in our laboratory using the morpholinos, both *in vitro* and *in vivo*[8,9]. The choice to work with cells *in vitro* or *in vivo* depends on the experimental design (see below). Our protocols are presented with an emphasis on lens regeneration; however, although we have developed these techniques for lens regeneration, we believe that they can be applied to the regeneration of other tissues in the newt, such as limb tissues; morpholinos seem to work in a variety of animals and cells, so the only difference would be the experimental design. No other method has been established for efficient gene knockdown in the newt (such as small interfering RNA). Likewise, gene knockout technology is not available. However, the efficiency of our methods to delineate the function of genes is comparable to those used in other animals, such as zebrafish, frogs, chicks and sea urchins (see the Gene Tools Morpholino Publication Database, http://pubs.gene-tools.com/). One of the limitations of morpholino-mediated gene knockdown is the need for repeated administration of morpholinos, especially if their long-term effects are under investigation. Regenerative processes in newts take several weeks; thus, they can result in poor animal heath and deaths, and the process can be costly as well. However, certain precautions, especially in animal care (e.g., animals should be fed regularly, be separated from each other, have as clean an environment as possible and be observed every day), can help overcome these problems.

Experimental design

Regeneration of the lens occurs always from the PECs of the dorsal iris. The same cells of the ventral iris do not contribute to regeneration. Thus, one assay to study effects of genes and examine their function is to culture dorsal or ventral iris separately, transfect them and re-implant into a lentectomized eye as aggregates[10]. For this, one would elect to knockdown genes *in vitro*. It is however also important to examine the effects of genes *in vivo*. For this, it will be crucial to examine the role of genes in different stages of regeneration. Here again there are different approaches. If, for example, we wanted to study the effects of a regulator on lens differentiation, the morpholino directed against this particular regulator can be injected at a specific stage in the eye and the effects can be examined a few days later. However, if we wanted to study the effects of a gene over longer period of time, repeated injections would result in trauma that could possibly interfere with the regenerated lens. In this case, it is better to deliver the morpholino by intraperitoneal injections. Thus, if one wishes to study lens regeneration or any other regenerative process in the newt (see below), first a decision must be made on the particular gene, stage and duration. Then morpholinos can be chosen, administered and tissues examined for the outcome. Control morpholinos should be included in every study (see below).

Protocol overview. Here we describe three different options for efficient gene knockdown. The first describes knockdown *in vitro*, by using PECs that are responsible for lens transdifferentiation after lentectomy. The second describes a protocol for short-term knockdown by injection in the eye just before differentiation of the lens. For these two protocols, we have used morpholinos to inhibit the

expression of paired box 6 (*pax-6*), a major regulator of lens differentiation and of *crystallin* gene expression[8]. A third knockdown protocol is also presented for long-term regulation. For this, we have used morpholinos to inhibit the expression of histone B4, a reprogramming-related linker histone[9].

Morpholino design. The choice of morpholinos is important, depending on the experimental design. To study cells *in vitro*, special delivery morpholinos can be used. These antisense oligonucleotides contain morpholine rings replacing the ribose or deoxyribose sugar moieties and nonionic phosphorodiamidate linkages replacing the anionic phosphates of DNA or RNA. The same morpholinos can also be used for topical injection at the site of interest in live animals for short-term experiments. These oligonucleotides are injected in a solution containing carriers, reagents or peptides designed to deliver substances into the cytosol of cells by an endocytosis-mediated process without damaging the cell membrane. For long-term *in vivo* experiments, a different type of morpholino can be used (vivo-morpholinos). These morpholinos are linked to octaguanidinium groups that facilitate delivery and can also be used *in vitro* (http://www.gene-tools.com/). Depending on the experimental design and the nature of the target sequence (gene and tissue), administration of the morpholino can vary (as described above for lens regeneration or below for other applications). Usually, we select sequences at the beginning of the transcript. The length of the morpholino is ~25 nucleotides long.

Controls. To establish the effects of morpholinos, appropriate control morpholinos (usually scrambled/mismatched or standard controls provided by the manufacturer) should be used as well in an identical protocol. Controls should be included in every experiment to eliminate the possibility of artifact.

Other applications. Similarly, experiments can be designed to address problems in the study of limb regeneration. Limb regeneration is achieved through the production of the blastema by dedifferentiation of the somatic tissues at the stump. The formation of the blastema is mediated by certain steps. First is the closure of the wound by the wound epithelium, which takes ~1 d. Then, the process of dedifferentiation, which produces the blastema cells, ensues 1–2 weeks later[10]. To study the effects on wound epithelium, a single injection should suffice. However, to study the process of dedifferentiation, many injections would be necessary.

MATERIALS

REAGENTS

- *Notophthalmus viridescens* (Amphibia of North America, TN, USA)
- Pigmented dorsal iris epithelial cells prepared as described previously[11]
- L15 medium (Invitrogen, cat. no. 11415064)
- HEPES (pH 7.4; Invitrogen, cat. no. 15630-080)
- FBS (Invitrogen, cat. no. 12662011)
- Amphotericin B (Sigma, cat. no. A2942)
- Kanamycin sulfate (Sigma, cat. no. G0615)
- Ethanol (70% (vol/vol))
- PBS (10×; Ambion, cat. no. AM925)
- Sterile water
- Ethyl-3-aminobenzoate methanesulfonate (Sigma, cat. no. E10521)
  ! CAUTION It is harmful if inhaled or absorbed through skin. Dispose of waste according to institutional regulations.
- NaH_2PO_4 (Sigma, cat. no. S3139)
- Na_2HPO_4 (Sigma, cat. no. S3264)
- KH_2PO_4 (Sigma, cat. no. P9791)
- $NaHCO_3$ (Sigma, cat. no. S9888)
- KCl (Sigma, cat. no. P9541)
- $CaCl_2$ (Sigma, cat. no. C1016)
- $MgSO_4{\cdot}7H_2O$ (Sigma, cat. no. 10034-99-8)
- NaCl (Sigma, cat. no. S3014)
- Paraformaldehyde (Sigma, cat. no. 30525-89-4) **! CAUTION** It is harmful if inhaled or absorbed through skin. Dispose of waste according to institutional regulations.
- NaOH (Sigma, cat. no. 1310-73-2)
- OCT (optimal cutting temperature) compound (Electron Microscopy Sciences, cat. no. 62550-01)
- Sucrose (Sigma, cat. no. S0389)
- Special delivery morpholinos labeled with lissamine (Gene Tools; an experimental morpholino designed from the 5′ sequence at the start of the transcriptional unit plus a standard control morpholino or mismatch morpholino should be ordered.)
- Vivo-morpholino (Gene Tools; http://www.gene-tools.com/vivomorpholinos). A vivo-morpholino comprises a morpholino oligo with a unique covalently linked delivery moiety that consists of an octaguanidinium dendrimer. An experimental vivo-morpholino targeted at the gene of interest and a control vivo-morpholino should be ordered.
- Ethoxylated polyethylenimine (EPEI, 200 μM; Gene Tools)
- Endo-porter (Gene Tools)
- Primary antibody to detect targeted protein (pax-6, Developmental Studies Hybridoma Bank; histone B4, see ref. 9)
- H3 primary antibody (ab1791, Abcam)
- Secondary antibody corresponding to primary antibody (anti-mouse streptavidin-conjugated Alexa Fluor 350 from Molecular Probes (cat. no. S-11249) for pax-6; alkaline phosphatase-conjugated anti-rabbit IgG (Abcam, cat. no. ab6722-1) for B4 and H3)
- Fluorescent mounting medium (Dako, cat. no. S3023)
- 5-Bromo-2′-deoxyuridine (BrdU; Sigma, cat. no. B5002-1G)
- BrdU antibody (Millipore, cat. no. MAB3510)
- ApoTag Plus *In Situ* Apoptosis Fluorescein Detection kit (Millipore, cat. no. S7110)
- Nitrocellulose membranes (0.2-μm pore size, Invitrogen)
- NBT/BCIP stock solution (Roche, cat. no. 11681451001)
- iQ SYBR Green Supermix (Bio-Rad, cat. no. 170-8880)
- Milli-Q water (Millipore)
- Xylene (Fisher Scientific, cat. no. UN1307)

EQUIPMENT

- Tissue culture hood equipped with vertical laminar flow and UV light
- Vortex
- Incubator with temperature control
- Vacuum flask
- Water bath with temperature controls
- Micropipette
- Pulled glass pipette
- Mouth pipette
- Pasteur pipettes
- Tubes (5 ml)
- Tube rack (5 ml)
- Pipettes (2 ml)
- Scalpel with no. 11 blade
- Blade handle (Feather, no. 3)
- Surgical blade (Feather, no. 11)
- Forceps (Inox, no. 5)
- Insulin syringe (3/10 ml, 31 gauge; BD Biosciences, cat. no. 328438)

- Glass container with lid for storage of newts
- Temperature-controlled room set at 25 °C
- Plastic containers with lid for keeping newts (22 × 37 × 12 cm^3)
- Plastic containers with lid for anesthesia (11 × 11 × 3.5 cm^3)
- Dissecting microscope
- Stereoscopic microscope with dual arm illuminator (Nikon, SZ40)
- Fluorescence microscope
- Mini-PROTEAN 3 Electrophoresis Module (Bio-Rad)
- ImageJ software (version 1.40g)
- Parafilm (or other laboratory film), cut into 2 inch × 1 inch strips

REAGENT SETUP

Newt serum-free medium L-15 medium supplemented with 10 mM HEPES (pH 7.4), 1% (vol/vol) amphotericin B and 1% (vol/vol) kanamycin sulfate. Store at 4 °C for up to 1 month.

Newt complete medium L-15 medium supplemented with 10 mM HEPES (pH 7.4), 1% (vol/vol) amphotericin B, 1% (vol/vol) kanamycin sulfate and 10% (vol/vol) FBS. Store at 4 °C for up to 1 month.

PBS (10×) Combine 80 g NaCl, 3 g KCl, 0.7 g Na_2HPO_4, 0.2 g KH_2PO_4 dissolved in 1 liter of deionized water, 0.2 M phosphate buffer (pH 7.2), 16.6 g Na_2HPO_4 and 2.5 g NAH_2PO_4 in 400 ml of deionized water. Store at room temperature (25 °C) for several weeks.

Ethyl-3-aminobenzoate methanesulfonic acid (0.1% (wt/vol)) NaH_2PO_4 (5.1 g), 8.2 g Na_2HPO_4 and 0.01 g ethyl-3-aminobenzoate methanesulfonate in 1 liter of deionized water. Store at room temperature for several weeks.

Holtfreter's solution $NaHCO_3$ (0.2 g), 0.05 g KCl, 0.1 g $CaCl_2$, 0.2 g $MgSO_4$·$7H_2O$, 3.46 g NaCl in 2 liters of deionized water (pH 7.4). Store at room temperature for several weeks.

Morpholino mixture Special delivery morpholino (500 μM; 10 μl) plus 5.6 μl endo-porter. ▲ **CRITICAL** This solution should be prepared fresh.

Paraformaldehyde (4% (wt/vol)) Add 4 g of paraformaldehyde to 50 ml water and heat to 60–70 °C. Add 1 drop of 10 N NaOH and stir for 10 min to dissolve. Add 50 ml of 0.2 M PBS (10×). Filter-sterilize and store at 4 °C for no more than a month.

Sucrose (30% (wt/vol)) Sucrose (30 g), 10 ml PBS (10×) and make up to 100 ml using deionized water. ▲ **CRITICAL** This solution should be prepared fresh.

NBT/BCIP solution Add 200 μl of NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) stock solution to 10 ml of 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl and 0.05 M $MgCl_2$. ▲ **CRITICAL** This solution should be prepared fresh.

PROCEDURE

Knockdown of genes in cells involved in newt lens regeneration using morpholinos

1| Use option A if the goal is to knock down genes in cells *in vitro*, option B if the goal is to knock down genes *in vivo* for a short time or option C if a long-term *in vivo* experiment is required.

? TROUBLESHOOTING

(A) Knockdown of genes in cultured pigment epithelial cells ● TIMING 4 d

(i) Warm serum-free newt medium in 27 °C water bath.

(ii) Spray the micropipette, Pasteur pipettes, 2-ml pipettes, 5-ml tubes, rack for 5-ml tubes, vortex and strips of Parafilm with 70% (vol/vol) ethanol and place them in the tissue culture hood under the UV light for 15 min.

(iii) After 15 minutes, turn off the UV light.

(iv) In a 5-ml tube, add 188 μl of water to 5.6 μl of special delivery morpholino (500 μM stock). Mix and add 5.6 μl EPEI.

(v) Incubate for 20 min at room temperature.
▲ **CRITICAL STEP** This incubation time is critical and should not be increased or decreased. If several samples are being assayed, they should be staggered so that each is incubated for 20 min.

(vi) During the 20-min incubation, remove the medium from a culture of iris PECs (which have been cultured for 14 d) using a Pasteur pipette attached to a vacuum flask and discard. Rinse cells with 1 ml of serum-free medium right before adding the morpholino mixture in next step.
▲ **CRITICAL STEP** It is very easy to contaminate the primary cultures; hence, take extreme care when removing the medium. In addition, the timing must be right so that you are rinsing the cells right before you are ready to add the morpholino mixture. Do not leave the cells without any medium.

(vii) At the end of the 20-min incubation, vortex the morpholino mixture from Step 1A(v), add 1.8 ml of serum-free medium, vortex and add to the iris cells that were rinsed in Step 1A(vi).

(viii) Wrap each plate of cells with strips of Parafilm and incubate at 27 °C for 3 h. At the end of 3 h, remove the medium with a glass Pasteur pipette attached to a vacuum flask and replace with 2 ml of pre-warmed complete newt medium.

(ix) Incubate the transfected cells for 4 d at 27 °C.

(B) Short-term *in vivo* knockdown of gene expression during lens regeneration using morpholinos ● TIMING 4–5 d

(i) Anesthetize the newts by placing them in a glass container filled with 0.1% (wt/vol) ethyl-3-aminobenzoate methanesulfonic acid for 10–15 min.

(ii) Under a dissecting microscope, remove the lens from anesthetized newts by opening the cornea with a scalpel and applying light pressure to the anterior and posterior portion of the eye with forceps until the lens comes through the slit in the cornea.

(iii) At the desired stage after lentectomy, to inject the morpholino, make a small hole in the nasal side of the eye with a pulled glass pipette.

(iv) Add 1 µl of the morpholino mixture (as described in REAGENT SETUP) inserting a pipette into the temporal side until the mixture flows around the eye toward the nasal opening. As the morpholino is tagged with lissamine, it should be easy to see the morpholino mixture as it flows across the eye.
▲ CRITICAL STEP The morpholino mixture needs to be injected with the correct pressure; applying too much pressure leads to the damage of the structures of the interior eye, whereas too little pressure will not achieve efficient delivery of the morpholino. In addition, the morpholino is known to be effective for 3 d *in vivo*, but can be injected again for knockdown of gene expression over a longer period of time.
? TROUBLESHOOTING

(v) Place the newts in a glass dish lined with a paper towel soaked in Holtfreter's solution to recover. Maintain the newts in a temperature-controlled room at 25 °C.
? TROUBLESHOOTING

(vi) Euthanize the newts (by exposing them to the anesthesia solution for 30 min, followed by decapitation) 3 d after morpholino injection and fix the treated eyes in 4% (wt/vol) paraformaldehyde for ~10 h. If cryosections are preferred, incubate overnight in 30% (wt/vol) sucrose at 4 °C before embedding in OCT[12].

(vii) Process samples for paraffin embedding by incubating them in an ethanol dilution series (50, 60, 70, 80, 90 and 100% (vol/vol)), followed by xylene (100%), xylene/paraffin (50%/50%) and finally embedding them in paraffin. Each treatment should be carried out for at least 30 min.

(viii) Embed eyes in paraffin blocks[12].
■ PAUSE POINT Samples in paraffin blocks can be stored at 4 °C for days or even weeks before processing them for sectioning or for immunohistochemical analysis.

(ix) Section the eye into 10-µm-thick sections.
■ PAUSE POINT Sections can be stored at 4 °C for days or even weeks before processing them for immunohistochemical analysis.

(C) Long-term *in vivo* gene expression knockdown during lens regeneration using vivo morpholinos ● TIMING several days

(i) Anesthetize the newts by placing them in a glass container filled with 0.1% (wt/vol) ethyl-3-aminobenzoate methanesulfonic acid for 10–15 min, and measure body weight of newts.

(ii) Remove lenses under a stereoscopic microscope using scalpel and fine forceps.

(iii) Inject vivo-morpholino or control morpholino (0.5 mg ml^{-1} in 70% (vol/vol) PBS) into the abdominal cavity (12.5 µg g^{-1} newt weight) using insulin syringe. In the case of 1.3 g newts, 30 µl of morpholino solution is used.
? TROUBLESHOOTING

(iv) Keep newts in plastic containers lined with wet paper towels.
? TROUBLESHOOTING

(v) In the case of B4 knockdown, inject vivo-morpholino every day after lentectomy, up to the day of sample collection. The experiment was run for 20 d. Proceed to Step 2B if analyzing gene knockdown by western blotting; alternatively, if results are to be analyzed by immunohistochemistry (Step 2A), Euthanize the animals and proceed as in Step 1B(vi–ix) before proceeding with Step 2A.

Analysis of the efficiency of gene knockdown

2| At the end of each experiment, perform immunohistochemistry (option A) or western blotting (option B) using an antibody against the product of the targeted gene to determine the efficiency of gene knockdown by the morpholino. The choice of option A or B depends on the desired analysis and the antibody to be used. For example, if proliferation is also examined, immunohistochemistry is best. In addition, some antibodies work better on sections than on a western blot and vice versa. Either of these techniques could be used to analyze gene expression from all options in Step 1; however, we have used immunohistochemistry to analyze pax-6 expression following knockdown *in vitro* and short-term knockdown *in vivo* (options A and B of Step 1, respectively) and western blotting to analyze histone B4 expression following long-term knockdown *in vivo* (option C of Step 1), as outlined below.

(A) Immunohistochemistry ● TIMING 2 d

(i) Deparaffinize sections (from Steps 1B(ix) or 1C(v)) in 100% xylene for 10 min. For cells (from Step 1A(ix)), fix in 2% (wt/vol) PFA for 1 h; follow by washing in 1× PBS.

(ii) Apply primary antibody (in our case, we used pax-6) at 1:10 dilution to the deparaffinized sections and incubate overnight at 4 °C. Add 100 µl of the antibody, which is enough to cover the sections. Keep the samples in a moist environment.
? TROUBLESHOOTING

(iii) Wash with 1× PBS, three times (15 min each wash).

(iv) Apply secondary antibody at 1:100 dilution and incubate for 2 h at RT. Add 100 µl of the solution, which is enough to cover the sections. Keep the samples in a moist environment.
(v) Wash with 1× PBS, three times (15 min each wash).
(vi) Mount by applying 50 µl of mounting medium and cover with a cover slip. Observe under a fluorescence microscope. Cells are observed for reactivity to pax-6 antibody. Count the cells with reactivity in the control (control morpholino transfection) and in the experimental (pax-6 morpholino transfection) samples.

(B) Western blotting ● TIMING 2 d

(i) In the experiment with B4, the eyeballs were isolated 20 d after lentectomy (Step 1C(v)) and the dorsal and ventral irises were dissected out.
(ii) Homogenize tissues (isolated irises from the newt eyes) in 10 volumes of 70% PBS. Add two volumes of 0.6 N HCl (final 0.2 N) to the homogenate and keep it on ice for 30 min to extract histones.
(iii) Recover the supernatant after centrifugation at 10,000*g* for 10 min at 4 °C and dialyze it twice, for 30 min each, with 0.1 N acetic acid and twice with Milli Q water, for 30 min each, followed by once more overnight at 4 °C.
(iv) Separate the extracted proteins by SDS-PAGE and blot onto a nitrocellulose membrane (0.2-µm pore size, Invitrogen) by standard procedures suggested by the manufacturer (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/M1703930.pdf).
(v) Probe the B4 antigen with anti-B4 antibody at 1:25 dilution (overnight at 4 °C) followed by alkaline phosphatase-conjugated anti-rabbit IgG (1:250 dilution; 2 h). Incubations are performed in plastic bags in 2 ml of solution. Any other antibody suitable for detection on a western blot can be used here.
(vi) Probe the histone H3 antigen with anti-histone H3 antibody (1:250 dilution) followed by alkaline phosphatase-conjugated anti-rabbit IgG (1:250 dilution) using the same conditions as in Step 2B(iv).
(vii) Visualize the antigens by incubating the membrane in 10 ml NBT/BCIP solution for several minutes until the bands appear.
? TROUBLESHOOTING
(viii) Measure the intensities of the detected bands using ImageJ software (version 1.40g) and calibrate the amount of protein from its band intensity. Normalize the amount of B4 protein to that of histone H3.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1	Variable efficiency of morpholino	Targeted genes have variable expression rates	Even though morpholinos directed against any target should work, the timing, frequency and amount of injection of vivo-morpholino should be determined for different target genes, because their rates of expression can vary
1B(iv) and 1C(iii)	High newt death rate	Needle size too large	An ultrafine needle must be used to avoid newt tissue damage and death
1B(v) and 1C(iv)	Condition of newts is poor	Insufficient moisture in the container	Appropriate moisture in plastic container is important. In the case of a 22 × 37 × 12 cm^3 container, a 20 × 90 cm^2 paper towel is folded into two and put in the containers and ~10 ml of tap water is poured. The paper towel is changed every 3 d
1C(iii)	High newt death rate	Injection volume too high	Because a large injection volume can damage newts, do not inject more than 50 µl for 1.3-g newts
2A(ii) and 2B(vii)	Signal reactivity of antibody	Inappropriate antibody incubation conditions	For different antibodies, conditions can vary and should be established

● TIMING

Step 1A: 4 d—4.5–5 h for the transfection process (Step 1A(i–viii)) and 4 d for incubation of cells (Step 1A(ix))
Step 1B: 4–5 d—10–15 min for anesthetizing newts (Step 1B(i)); 1 h for morpholino injection (Step 1B(ii–iv)); 3 d for incubation (Step 1B(v)); and 1–2 d for tissue processing (Step 1B(vi–ix))
Step 1C: several days—10–15 min for anesthetizing newts (Step 1C(i)); 1 h for morpholino injection (Step 1C(ii,iii)); several days for incubation depending on the experimental design (Step 1C(iv–v)); and 1–2 d for tissue processing as in Step 1B(vi–ix)
Step 2A: 2 d
Step 2B: 2 d

ANTICIPATED RESULTS

We have studied the effects of *pax-6* knockdown *in vitro* and *in vivo*. *Pax-6* is an eye master gene and a regulator of lens differentiation. Almost complete gene knockdown was seen with morpholinos for *pax-6 in vitro* (**Fig. 1**). The specificity of the morpholinos was shown by the inability of a nonspecific standard control and a mismatch morpholino to knockdown targeted gene expression, as well as showing that the expression of two non-targeted genes was not affected by the specific morpholinos[8].

Lens regeneration takes 30 d to be complete in the newt. To inhibit the induction of lens regeneration in the short term, morpholino injections of *pax-6* morpholinos were given to the eye on days 4 and 10 of the regeneration process. In newts not injected with morpholinos, regeneration occurred in 90% of the animals. Newts injected with the standard control or mismatch morpholino showed regeneration in 78–88% of the animals, compared with the specific morpholinos that only

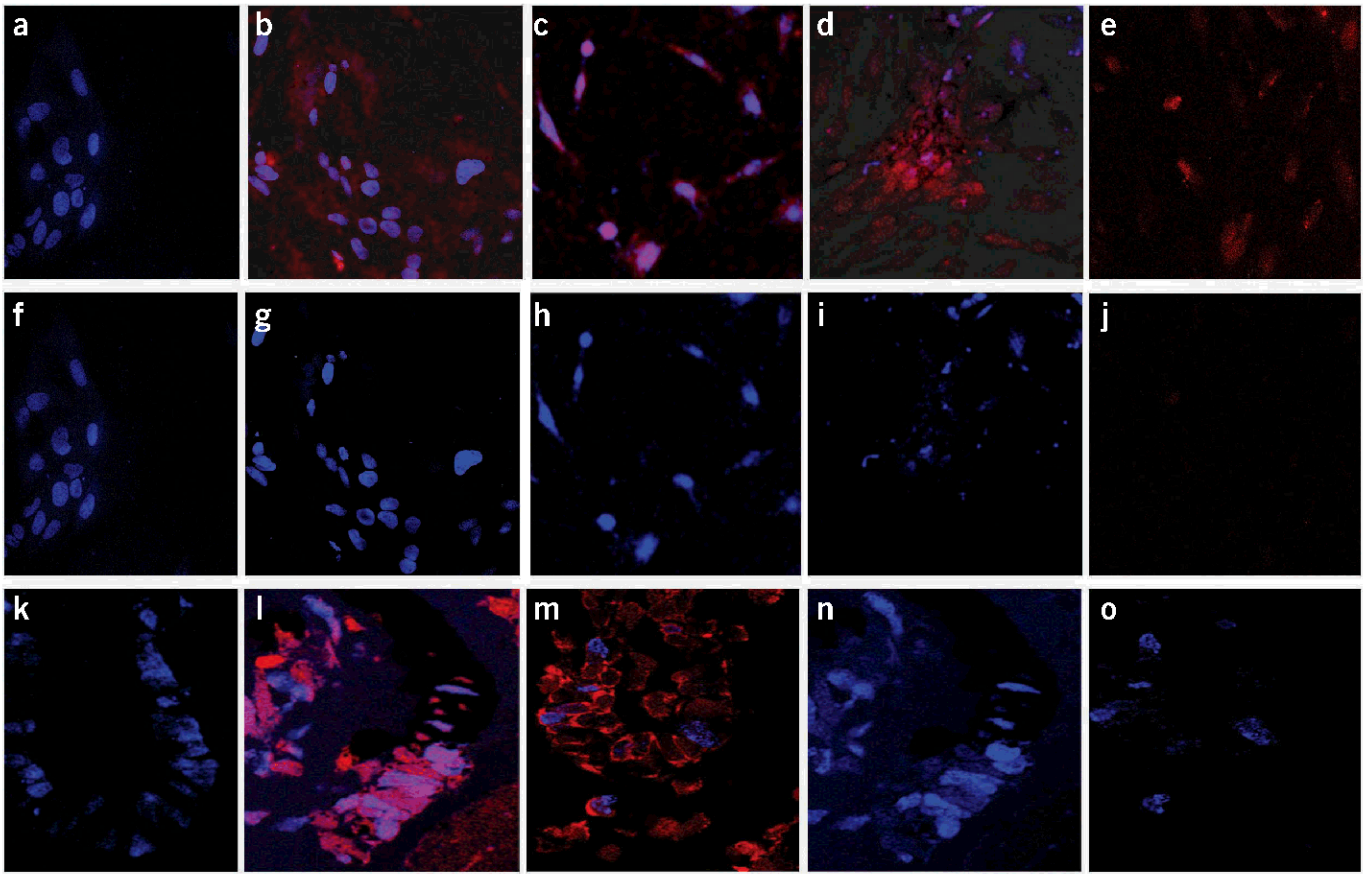

Figure 1 | *Pax-6* morpholinos decrease expression of pax-6 in cultured PECs and during *in vivo* lens regeneration in the newt *N. viridescens*. (**a**–**o**) Morpholino fluorescence is red, and Pax-6 immunofluorescence is blue. (**a**,**f**) Untreated cells; (**b**,**c**) cells treated with control or mismatched morpholino, respectively, show robust pax-6 immunofluorescence. (**d**,**e**) Cells treated with morpholinos against *pax-6* (*pax-6*-Mo1 and *pax-6*-Mo2) show reduced expression of pax-6. (**g**–**j**) The same images as shown in **b**–**e**, but showing only pax-6 immunofluorescence. (**k**–**m**) A regenerating lens collected from a newt 13 d after lentectomy shows high pax-6 expression in the absence of morpholino treatment (**k**) or in the presence of a standard control morpholino 3 d before collection (**l**). However, a 13-d-old newt regenerating lens treated with a morpholino that was designed to downregulate pax-6 for 3 d before collection, substantially downregulates pax-6 expression (**m**). (**n**,**o**) The same images as shown in **l**,**m**, but showing only pax-6 immunofluorescence. Appropriate IACUC permission was obtained for these experiments. Reprinted with permission from ref. 8.

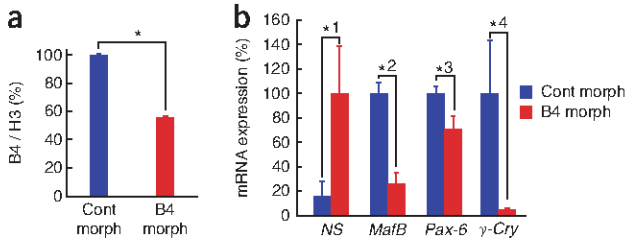

Figure 2 | Effects of B4 knockdown. (**a**) Decrease of B4 protein after B4 morpholino injection. After lentectomy, B4 vivo morpholino was intraperitoneally injected every day in *N. viridescens*. On day 10, dorsal irises were collected and amount of B4 protein in the irises was measured by western blotting. The amount of B4 was normalized with that of histone H3. *P = 0.000310 (n = 2 (cont morph, control morpholino), n = 2 (B4 morph, B4 morpholino)). (**b**) The effects of B4 knockdown on gene expression were analyzed by quantitative PCR. For this experiment, morpholino was injected every day for 20 d and the irises were collected at day 20. There is a clear indication that B4 knockdown significantly downregulates lens differentiation–specific markers, such as *MafB*, *pax-6* and *γ-crystallin* (*γ-Cry*). On the other hand, *nucleostemin* (*NS*), encoding a nucleolar protein related to a stem cell–like state, is upregulated. Error bars are means ± s.d. P values are from Student's t-test (two-tailed). *1, P = 0.0350 (n = 4); *2, P = 0.0000249 (n = 4); *3, P = 0.00342 (n = 4); *4, P = 0.0298 (n = 4). Appropriate IACUC permission was obtained for these experiments. Reprinted with permission from ref. 9.

showed regeneration in 25–42% of injected animals (**Fig. 1**). Apoptosis analysis showed that an increase in cell death was not a factor in the decrease in regeneration[8].

For a long-term experiments, we targeted histone B4 (ref. 9). B4 is known to be an oocyte-type linker histone, which is a counterpart of the somatic linker histone H1; it participates in nuclear reprogramming after somatic nuclear transplantation into oocyte. It has been elucidated that B4 is expressed in PECs in newt lens transdifferentiation. During dedifferentiation of PECs, the ratio of B4 to H1 increased by ~13 times. The effect of vivo-morpholino on B4 protein expression was analyzed by western blotting. The ratio of B4 to H3 was decreased by nearly 50% when compared with the levels in irises from newts injected with control morpholino 10 d after lentectomy (**Fig. 2a**). Knockdown of B4 decreased proliferation and increased apoptosis, which resulted in considerably smaller lenses. On day 20, the number of BrdU-positive cells (which are proliferating cells) in B4 morpholino-injected newts was substantially decreased to 63% of that in control morpholino-injected newts (control, n = 7; B4, n = 9). BrdU was administered 1 d before fixation of the eyes. We injected BrdU intraperitoneally (25 mg g^{-1} of body weight of a 10 mg ml^{-1} BrdU solution prepared in 70% (vol/vol) PBS). The number of apoptotic cells in B4 morpholino-injected newts was considerably increased by 43% in comparison to that of control morpholino-injected newts (control, n = 6; B4, n = 6). To examine apoptosis, we used the ApopTag Plus kit. Both the BrdU and apoptosis analysis have been described in detail[8,9]. Moreover, B4 knockdown altered gene expression of key genes of lens differentiation, such as *pax-6* and *mafB*, and nearly abolished expression of *γ-crystallin*, a key lens fiber marker. On the contrary, *nucleostemin* (an early dedifferentiation marker) was upregulated (**Fig. 2b**). Expression was determined by standard quantitative PCR technology outlined in detail in reference 9. Briefly, quantitative PCR was performed using a iQ SYBR Green Supermix and specific primers for the genes to be analyzed. To quantify the expression of each gene, threshold cycle (C_t) values were compared with standard curve generated using a series of dilutions of cloned cDNAs. The amount of RNA was normalized to that of the ribosomal protein L27.

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AUTHOR CONTRIBUTIONS P.A.T. and K.D.R.-T. designed, directed and analyzed data pertaining to loss of function experiments. T.H. and N.M. performed experiments and wrote part of the protocols. M.M.C.-R., S.Y., T.M. and K.N. wrote part of the protocols. P.A.T. and C.C. co-wrote the final version of the paper.

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