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## The $\alpha 1$ isoform of the $\text{Na}^+/\text{K}^+$ ATPase is up-regulated in dedifferentiated progenitor cells that mediate lens and retina regeneration in adult newts\*

M. Natalia Vergara<sup>a</sup>, Laura K. Smiley<sup>a</sup>, Katia Del Rio-Tsonis<sup>a,+</sup>, and Panagiotis A. Tsonis<sup>b,+</sup>

<sup>a</sup>Department of Zoology, Miami University, Oxford, OH 45056, U.S.A.

<sup>b</sup>Center for Tissue Regeneration and Engineering at Dayton and Department of Biology, University of Dayton, Dayton, OH 45469-2320, U.S.A.

### Abstract

Adult newts are able to regenerate their retina and lens after injury or complete removal through transdifferentiation of the pigmented epithelial tissues of the eye. This process needs to be tightly controlled, and several different mechanisms are likely to be recruited for this function. The  $\text{Na}^+/\text{K}^+$  ATPase is a transmembrane protein that establishes electrochemical gradients through the transport of  $\text{Na}^+$  and  $\text{K}^+$  and has been implicated in the modulation of key cellular processes such as cell division, migration and adhesion. Even though it is expressed in all cells, its isoform composition varies with cell type and is tightly controlled during development and regeneration. In the present study we characterize the expression pattern of  $\text{Na}^+/\text{K}^+$  ATPase  $\alpha 1$  in the adult newt eye and during the process of lens and retina regeneration. We show that this isoform is up-regulated in undifferentiated cells during transdifferentiation. Such change in composition could be one of the mechanisms that newt cells utilize to modulate this process.

### Keywords

$\text{Na}^+/\text{K}^+$  ATPase  $\alpha 1$ ; newt; retina; lens; regeneration

## 1. Introduction

The phenomenon of tissue regeneration has been a topic of scientific research for centuries, and the reason is clear: knowledge of the mechanisms involved in this complex process provides the exciting possibility of manipulating it for the restoration of lost or damaged organs. However, despite the numerous advances in this area, the precise regulation of regeneration of different tissues remains largely unknown.

The capacity for restoration and functional recovery of injured tissues varies among animal species and, within them, among tissue type and developmental time (Sanchez Alvarado and

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<sup>+</sup>Equally contributing corresponding authors. E-mail address: delriok@muohio.edu Fax: (513)-529-6100, panagiotis.tsonis@notes.udayton.edu Fax: (937)-229-2021

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Tsonis, 2006). However, certain Urodele amphibians such as newts stand out for their exceptional regenerative abilities. These animals are able to restore almost any injured organ including limbs, jaw, brain, heart, and the retina and lens of the eye, at any point in their lifetime, constituting ideal model organisms for research in this area (Reviewed by Brockes and Kumar, 2002; Tsonis, 2000).

The ability of newts to regenerate their retina and lens was first documented more than 100 years ago, in studies by Philipeaux (1880), Griffini and Marcchio (1889), Colucci (1891), and Wolff (1895). The cellular source for regeneration of the retina is the retinal pigmented epithelium (RPE) in the posterior part of the eye, whereas the lens regenerates from the dorsal portion of the iris. Upon injury or complete removal of the retina or lens, these pigmented tissues are able to transdifferentiate to restore the missing structures. This process involves the dedifferentiation of mature cell types with loss of their characteristics including pigmentation, regression to an undifferentiated “stem cell-like” state, proliferation, and differentiation into all the different cell types that were lost, with the correct re-establishment of patterns and connections (Reviewed by Del Rio-Tsonis and Tsonis, 2003; Haynes and Del Rio-Tsonis, 2004; Mitashov, 1996, 1997; Reh and Pittack, 1995). Of course, such an intricate process has to be tightly controlled, and it is likely that several different mechanisms are recruited for this function.

In recent years, many of the key regulators of newt eye regeneration have been identified. Morphogens such as BMP, Hedgehog, Retinoic Acid and Fibroblast Growth Factors have been shown to be involved in different aspects of lens regeneration, including induction of dedifferentiation, control of cell proliferation and differentiation, through the activity of transcription factors such as *prox1*, *pax6* and *six3* (Grogg et al., 2005; Madhavan et al., 2006; Tsonis et al., 2004). Regarding retina regeneration, studies have pinpointed the role of Fibroblast Growth Factors and Notch signaling and the expression of the aforementioned transcription factors during different stages of the regeneration process. (Makar’ev et al., 2002; Markitantova et al., 2004; Nakamura and Chiba, 2007; reviewed by Vergara et al., 2005). Despite these important advances, the regulation of regeneration of eye tissues in the adult newt is far from being completely elucidated, and other mechanisms deserve consideration.

There is an increasing body of knowledge about the importance of biophysical signals as regulators of development, regeneration and neoplasia in different animals (Reviewed in Levin, 2007; Seegers et al., 2001; Song et al., 2004). Ion transporters are involved in the establishment of intracellular pH, ionic currents and voltage gradients across cells and epithelia, and have been implicated in the control of processes such as cell growth, proliferation, directional migration, wound healing and regeneration (Adams et al., 2007; Levin, 2007; Reid et al., 2005; Song et al., 2002). But the possibility of their involvement in eye tissue regeneration in the newt has not yet been analyzed.

One such transporter is the  $\text{Na}^+/\text{K}^+$  ATPase, a heterodimeric enzyme that establishes and maintains electrochemical gradients across the plasma membrane through the transport of  $\text{Na}^+$  and  $\text{K}^+$ , powered by the hydrolysis of ATP. It is composed of a catalytic  $\alpha$  subunit (of about 112 kDa) that contains the binding sites for ATP, ions, and serves as the receptor for cardiac glycosides such as ouabain, and a glycosylated  $\beta$  subunit (of MW 35-60 kDa), required for the normal activity of the enzyme. Four isoforms of the  $\alpha$  subunit and three isoforms of the  $\beta$  subunit have been identified in vertebrates, and their expression patterns differ across tissues and developmental stages (Cameron et al, 1994; Orłowski and Lingrel, 1988). Depending on its isoform composition, the enzyme will vary in its sensitivity to ions, ATP, and ligands, providing a mechanism for the cells to control their physiological properties through the regulation of isoform expression.

Partial inhibition of Na<sup>+</sup>/K<sup>+</sup> ATPase with ouabain has been shown to decrease neurite growth and alter its direction in response to wounding. Such treatment can also decrease wound healing rate and cell proliferation and affect orientation of cell division in response to injury in the rat cornea (Song et al., 2002;2004). In addition, Na<sup>+</sup>/K<sup>+</sup> ATPase  $\alpha$ 1 and  $\beta$ 1 subunits have been implicated in the regulation of cell motility by a mechanism that is independent of their role in ion transport and that involves the activation of the PI3 Kinase pathway (Barwe et al., 2005). Moreover, the  $\alpha$ 1 isoform of this enzyme has been implicated in intracellular signal transduction mechanisms (Aydemir-Koksoy et al., 2001;Chen et al., 2008; Haas et al., 2000, 2002;Kometiani et al., 1998;Liang et al., 2006;Liu et al., 2000;Tian et al., 2006; reviewed by Xie and Askari, 2002;Li and Xie, 2008).

Specifically in regeneration, changes in Na<sup>+</sup>/K<sup>+</sup> ATPase isoform expression have been reported during nerve regrowth in the rat peripheral nervous system. That is, after axotomy of the dorsal root ganglion and the sciatic nerve, there is a decrease in the expression levels of  $\alpha$ 1 and  $\alpha$ 2, whereas there is a marked, transient increase in  $\alpha$ 3 during the regeneration phase (Arteaga et al., 2004). This increase in  $\alpha$ 3 isoform expression is also observed in the ganglion cells of the retina during optic nerve regeneration in goldfish (Liu et al., 2002).

It is interesting to mention that despite the similar function of the different isoforms of this enzyme in the establishment of Na<sup>+</sup> and K<sup>+</sup> gradients, there is ample evidence pointing at specific, differential functions of each isoform in various cell types that lead to different physiological outcomes.

No studies have been performed to our knowledge on the expression of Na<sup>+</sup>/K<sup>+</sup> ATPase isoforms in newts, or their regulation during the process of transdifferentiation in lens or retina regeneration. In the present study we show for the first time the immunolocalization of the  $\alpha$ 1 isoform of Na<sup>+</sup>/K<sup>+</sup> ATPase in specific subsets of cells in the intact newt eye and its variation during lens and retina regeneration. We show that during dedifferentiation of the iris and retinal pigmented epithelium this isoform is transiently up-regulated in multipotent cells, and its expression decreases as differentiation progresses. We hypothesize that the regulation of  $\alpha$ 1 isoform expression is likely to be an important component in the modulation of transdifferentiation in adult newt tissues.

## 2. Materials and methods

### 2.1 Animals

Adult newts (*Notophthalmus viridescens*) were obtained from Mike Tolley Newt Farm (Nashville, TN). They were kept in half strength Holtfretter's solution at room temperature and handled according to the guidelines established by the Animal Care and Use Committee at Miami University. For surgical procedures and euthanasia the animals were anesthetized using a 0.1% 3-aminobenzoic ethyl ester solution.

### 2.2 Surgeries and tissue collection

For lentectomies, animals were anesthetized and a slit was made in the cornea using a scalpel. The lens was then removed through the pupil with the aid of forceps. For retinectomies, animals were lentectomized and a 50% Holtfretter's solution was gently blown inside the vitreous cavity using a pulled glass pipette to promote retina detachment. The retina was then removed through the pupil with forceps and the eye was rinsed by a stream of Holtfretter's solution. After surgery the animals were allowed to recover until the day of collection. Intact and operated eyes collected at different times after surgery (at least 6 eyes per time point for each type of surgery) were fixed in a 4% formaldehyde solution in PBS overnight, followed by

washes in PBS and immersion in 30% sucrose for cryoprotection. They were then embedded in OCT, frozen and cryosectioned at 10 $\mu$ m thickness for immunohistochemistry.

### 2.3 Culture of Na<sup>+</sup>/K<sup>+</sup> ATPase alpha1 ( $\alpha$ 6F) hybridoma cells and antibody purification

$\alpha$ 6F, an antibody that is specific for the  $\alpha$ 1 isoform of the Na<sup>+</sup>/K<sup>+</sup> ATPase (Arteaga et al., 2004; Drummond et al., 1998; Hlivko et al., 2006; Mobasheri et al., 2003; Wetzel et al., 1999; Zhang and Ng, 2007) was obtained from a hybridoma cell culture to be used for Western blot and immunohistochemical studies. Briefly,  $\alpha$ 6F hybridoma cells developed by Dr. D.M. Fambrough were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. The cells were cultured in high glucose DMEM supplemented with 20% FBS and 1% penicillin/streptomycin. The supernatant was collected by centrifugation at 157g. Then, 27.4g ammonium sulfate were added to 100ml of supernatant and incubated for 30 minutes at 4°C, followed by a 10 minute centrifugation at 10,000g. The supernatant was discarded and the pellet was resuspended in 10 ml of PBS. This solution was dialyzed for 3 days in cold PBS. The final concentration of this protein was 5.1  $\mu$ g/ $\mu$ l.

### 2.4 Protein extraction and Western blot

Newt kidneys, liver, brain, eyes and heart were isolated for protein extraction and ground in a tissue homogenizer, followed by the addition of RIPA buffer and protease inhibitors and sonication. The homogenates were centrifuged at 10,000g and the supernatants were collected. Protein concentrations were analyzed using the BCA method (Pierce). Fifty micrograms of protein from each tissue were mixed with 2X sample loading buffer (Pierce), incubated for 1 hour at 37°C and separated by SDS-PAGE on a 7.5% gel. The proteins were transferred to Immobilon-P membrane (Millipore), blocked with 5% non-fat dry milk in TBST for 2 hours, and incubated overnight at 4°C with a 1/1000 dilution of  $\alpha$ 6F antibody and a 1/5000 dilution of mouse anti- $\beta$ -actin antibody (Sigma). After several washes with TBST the membrane was incubated with HRP-conjugated anti-mouse secondary antibody for 1 hour at room temperature, followed by further TBST washes. The SuperSignal West Pico chemiluminescence system was used to visualize the protein bands, which were scanned in a FluorChem machine (Alpha Innotech). Quantification of  $\alpha$ 6F band intensities relative to  $\beta$ -actin was performed using an ImageQuant version 5.2 software (Molecular dynamics).

### 2.5. Protein sequencing

Newt kidney protein was used for sequence analysis. After proteolytic digestion the fragments were analyzed by microcapillary reverse-phase HPLC, directly coupled to the nano-electrospray ionization source of an ion trap and/or orbitrap spectrometer. These instruments are capable of acquiring individual sequences spectra on line at high sensitivity ( $\lll 1$  femtomole) for multiple peptides in the chromatographic run. These spectra are correlated with known sequences using the algorithm Sequest (Eng et al., 1994) and programs developed by Chittum et al., 1998. The sequence analysis was performed at the Harvard Microchemistry and Proteomics Analysis facility by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectroscopy (LC/MS/MS) on a Thermo LTQ-Orbitrap mass spectrometer. The obtained newt polypeptide sequences were compared to the ATPase, Na<sup>+</sup>/K<sup>+</sup> transporting, alpha 1 polypeptide sequence from *Xenopus tropicalis* obtained from NCBI (Accession AAH80463, version AAH80463.1 GI:51513488) using GeneRunner software (supplementary figure 1).

### 2.6 RPE explant cultures

Newt eyeballs were isolated and sterilized in 5% lugol followed by washes in Ca<sup>++</sup> Mg<sup>++</sup> free Hank's solution. The anterior portion of the eye was dissected out with microscissors and the

retina was removed from the posterior eye-cup using forceps. Then the RPE-Choroid layer was peeled off from the sclera using forceps and placed in 24-well plates containing L-15 medium supplemented with 10% FBS. The explants were maintained at 24°C with gentle rocking for 4 days. Finally, the transdifferentiating explants were collected, washed in PBS, cryoprotected in 30% sucrose and embedded in OCT for cryosectioning.

## 2.7 Immunohistochemistry

Four independent trials were performed to analyze the immunohistochemical expression pattern of  $\alpha 6F$  antibody in the intact eye and at different time points during both lens and retina regeneration, as well as in cultured RPE explants. Cryosections were rinsed in PBS and incubated with the  $\alpha 6F$  antibody solution overnight at 4°C. They were then washed 3 times for 10 minutes in PBS and incubated in secondary antibody anti-mouse IgG conjugated to Alexafluor 488 (Molecular Probes) diluted 1/100 in 0.3% Triton X-100, 0.5% NGS in PBS-solution for 2 hours at room temperature. They were washed in 0.3% PBS-Triton X-100 followed by PBS washes, mounted using Vectashield (Fisher) and analyzed using a confocal microscope. Corresponding negative controls were performed by incubating the sections in PBS instead of the primary antibody.

## 3. Results

### 3.1 Western blot analysis of the alpha1 isoform of $\text{Na}^+/\text{K}^+$ ATPase in newt tissues

In a previous study in our laboratory, while investigating a molecule that we suspected was involved in lens regeneration in the newt (Eguchi, 1988; Imokawa et al., 1992), we co-purified a protein whose mass spectrometry analysis showed high homology (10 polypeptides) to the *Xenopus tropicalis*  $\text{Na}^+/\text{K}^+$  ATPase isoform  $\alpha 1$ . These sequences, shown in figure 1A, correspond to actual newt polypeptides, and their localization within the *Xenopus tropicalis*  $\text{Na}^+/\text{K}^+$  ATPase  $\alpha 1$  sequence is presented in supplementary figure 1. Aware of the recent discoveries on the regulation of  $\text{Na}^+/\text{K}^+$  ATPase isoform expression during development and nerve regeneration, their involvement in intracellular signaling mechanisms, and the literature describing the importance of endogenous electrochemical gradients in the regenerative process, we set out to investigate if the expression of this protein was regulated during newt lens and retina regeneration as well.

No studies had been reported to our knowledge on either protein or mRNA expression of any  $\text{Na}^+/\text{K}^+$  ATPase isoform in newts. Therefore we started by analyzing the expression profile of the  $\alpha 1$  isoform of this protein in various adult newt (*Notophthalmus viridescens*) tissues by Western blot. We used a monoclonal antibody ( $\alpha 6F$ ) that is specific for this isoform. This antibody has been widely employed to distinguish  $\text{Na}^+/\text{K}^+$  ATPase  $\alpha 1$  from the other isoforms by immunohistochemistry and Western blot in different tissues, including the retina, of various animal models (Arteaga et al., 2004; Drummond et al., 1998; Hlivko et al., 2006; Mobasher et al., 2003; Wetzell et al., 1999; Zhang and Ng, 2007). Moreover, the epitope that is recognized by this antibody includes one of the polypeptide sequences that we had identified in our mass spectrometry analysis (highlighted in figure 1A and red in supplementary figure 1). Using this technique we were able to detect a protein band of about 100 kDa, similar to the size of  $\text{Na}^+/\text{K}^+$  ATPase  $\alpha 1$  in other vertebrates, in all the different tissues analyzed, including the eye (Figure 1B). This protein seemed to be most abundant in the kidney, coinciding with reports on the expression levels of this isoform in other vertebrates (Farman et al., 1991; Orłowski and Lingrel, 1988; Tumlin et al., 1994). In order to further validate the identity of this protein, we performed immunohistochemistry on newt kidney cryosections, confirming its strong staining pattern on the plasma membrane of the kidney tubules (Figure 1C). This is consistent with reports of  $\alpha 1$  being the only isoform that could be detected in the kidney tubules in other animal species (Orłowski and Lingrel, 1988; Tumlin et al., 1994).

### 3.2 Expression pattern of Na<sup>+</sup>/K<sup>+</sup> ATPase alpha1 in the intact newt eye

We then investigated the expression pattern of Na<sup>+</sup>/K<sup>+</sup> ATPase  $\alpha$ 1 in the intact newt eye by immunohistochemistry, using the same  $\alpha$ 6F antibody (Figure 2). We found a strong immunostaining for this protein in the photoreceptors (Figures 2A and 2C), lens epithelium (Figures 2A and 2D) and non-pigmented ciliary body (Figures 2A and 2B). We could not detect the expression of this protein in any of the other neural or glial cell types of the retina, the lens fiber cells, pigmented ciliary body or RPE using this method. Such expression pattern was consistent throughout four different trials performed for this experiment, and is different from that reported for this isoform in other animal species (see discussion).

### 3.3 Expression of Na<sup>+</sup>/K<sup>+</sup> ATPase alpha1 during lens regeneration

In order to assess the expression of this protein during lens regeneration, we performed lentectomies on adult newts and collected the eyes at different days post-operation, processing them for immunohistochemistry. In these animals, upon lens removal, the pigmented epithelial cells of the dorsal iris (and never the ventral) start to dedifferentiate, and eventually form a vesicle of clear, multipotent cells. These cells then proliferate and ultimately re-differentiate to form a lens epithelium and elongated lens fiber cells that lose their nuclei and organelles and express high levels of crystallin proteins (Eguchi 1963,1964, reviewed by Del Rio-Tsonis and Tsonis, 2003). By the end of the process, the regenerated lens is histologically and functionally normal and does not develop cataracts. We found that Na<sup>+</sup>/K<sup>+</sup> ATPase  $\alpha$ 1 was not detected in the heavily pigmented iris cells, dorsal or ventral (Figure 3). However by 9 days post-operation, when depigmented cells start to be observed at the tip of the dorsal iris, this protein was immunolocalized to the plasma membrane of those undifferentiated cells (Figure 3C). In the subsequent proliferating lens vesicle stages, staining was observed in all of the undifferentiated vesicle cells (Figure 3D-G). Later, as the lens vesicle started to differentiate, the staining for this protein became progressively restricted to the lens epithelial cells and disappeared from the differentiating lens fibers (Figure 3H-K). At the end of the regeneration process, the staining pattern resembled that of the intact lens. The results described here for each time point were consistent throughout four independent trials.

### 3.4 Expression of Na<sup>+</sup>/K<sup>+</sup> ATPase alpha1 during retina regeneration

In order to investigate if the expression of  $\alpha$ 1 in undifferentiated cells is a common feature in the process of regeneration by transdifferentiation, we analyzed the immunolocalization of this protein at different time points after retinectomy.

Newts are able to regenerate a complete, functional retina that reconnects to the correct places in the brain and restores vision, even after complete removal of the neural retina. The cellular source of regeneration is the retinal pigmented epithelium (RPE), whose cells dedifferentiate and proliferate, forming a neuroepithelium that grows in size and finally splits into the three nuclear layers and two plexiform layers that compose the normal eye, restoring photoreceptors, Müller glia, and all the different neuronal cell types (reviewed by Fischer and Reh, 2001; Mitashov, 1996, 1997; Raymond and Hitchcock, 2000; Del Rio-Tsonis and Tsonis, 2003). Moreover, the RPE layer is restored in this process, as opposed to what is the case for other animal models such as the embryonic chick. Cheon and Saito (1999) classified this process into early, intermediate and late stages, to facilitate its analysis.

Despite the fact that we had not detected Na<sup>+</sup>/K<sup>+</sup> ATPase  $\alpha$ 1 in the intact RPE, we found expression of this protein in all of the depigmented cells since the early stages of retina regeneration (when the retina is 1-2 cells thick, figure 4A-C). At intermediate stages (when the neuroepithelium is 3-7 cells thick and before the formation of synaptic layers), staining for this protein was observed in the plasma membrane of all the progenitor cells (Figure 4D-E). Finally, at late regeneration stages (after laminarization), the expression of this molecule became

progressively restricted to photoreceptor cells, with some staining persisting in the plexiform layers (Figure 4F-H). At the end of the process the staining pattern was similar to that of the intact retina. No variations from the described patterns were observed through the four trials performed.

Even though transdifferentiation is the main mechanism that contributes to retina regeneration in the newt, there is still some contribution at the periphery of the eye from the stem/progenitor cells located at the ciliary body/ciliary marginal zone. As described above, the non-pigmented ciliary body stains strongly for this protein in the intact eye. During regeneration, these cells thicken and proliferate, to add to the neuroepithelium that is being formed. We found that the undifferentiated cells at the ciliary margin expressed  $\alpha 1$  during the whole process of regeneration (Figure 5A-E).

Finally, to further support our results we decided to investigate if the expression of  $\alpha 1$  in undifferentiated cells would also occur in an in vitro model of RPE transdifferentiation. For this purpose we devised an assay in which a sheet of RPE-choroid was cultured in suspension through gentle agitation, without attaching to the culture dish, in a medium that contains FBS but no other added growth factors. Under these conditions, the pigmented cells were able to transdifferentiate as early as 4 days in vitro. At this time,  $\alpha 1$  started to be detected in the depigmented cells (Figure 5F). It is likely that such cells arise from transdifferentiation of the RPE, since the choroid has never been shown to transdifferentiate in any in vitro or in vivo conditions.

Our results taken together suggest that  $\text{Na}^+/\text{K}^+$  ATPase  $\alpha 1$  might play a role in the early stages of transdifferentiation during both lens and retina regeneration.

#### 4. Discussion

The  $\text{Na}^+/\text{K}^+$  ATPase has a vital function in all cells, establishing the gradient of  $\text{Na}^+$  and  $\text{K}^+$  across cells membranes. However the distribution of the different isoforms of this protein varies across tissue types and is tightly regulated during development. In the mouse retina, the  $\alpha 1$  isoform has been immunohistochemically detected in the retinal pigmented epithelium, Müller glia, and ganglion and horizontal cells, whereas  $\alpha 2$  has been localized mainly in Müller glia cells and non-pigmented ciliary body (Wetzel et al., 1999). The  $\alpha 3$  isoform, on the other hand, has been found in all retinal neurons, and seems to be crucial for the proper organization and synaptic structure of photoreceptors and bipolar cells in the retina (Molday et al., 2007). In addition, these three  $\alpha$  isoforms are expressed in the ciliary processes of the human, bovine, rabbit and rat eyes, where they are proposed to be involved in the production of aqueous humor (Flugel and Lutjen-Drecoll, 1988; Ghosh et al., 1990; Martin-Vasallo et al., 1989; Mori et al., 1991; Okami et al., 1989). In the lens, expression of the  $\alpha$  isoform seems to be species-dependent,  $\alpha 1$  being the common (but not the only) isoform expressed in the lens epithelial cells of rats, rabbits, pigs, cows, frogs and humans, with even more variability in isoform expression reported for lens fibers (For a detailed review see Delamere and Tamiya, 2004).

In the mature newt eye, we have immunohistochemically detected the  $\alpha 1$  isoform only in photoreceptors, and not in the other neural cell types, glia or RPE. In addition, we have found a strong expression of this protein in the non-pigmented ciliary body but not in the pigmented layer of this structure (as is the case in other animals). Regarding the lens, we have observed expression of  $\alpha 1$  only in the lens epithelium and not in the fiber cells. The discrepancies observed between the newt and other vertebrates seem to point to differences in the physiological regulation of eye structures across species. The expression of this isoform in the ciliary body could point to its involvement in vitreous humor production, although it could also be related to the presence of stem/progenitor cells in this area of the eye. From its retinal

expression it could be speculated that it may play a role in photoreceptor stabilization similar to that of  $\alpha 3$  in rodents. On the other hand, the difference in distribution could mean that this protein is performing novel functions in newt tissues that are distinct from those reported for other species. In any case, the precise function of this isoform in the newt eye remains to be investigated.

In regards to the regeneration of eye structures, it has been well established that the newt regenerates its retina and lens through transdifferentiation of the pigmented epithelia of the eye. In the present study we have reported high expression levels of  $\alpha 1$  protein in all the undifferentiated, multipotent cells that are generated from the pigmented epithelia during regeneration of both, the lens and retina structures. However, the intact RPE and iris do not express detectable levels of  $\text{Na}^+/\text{K}^+$  ATPase  $\alpha 1$  protein. Such tight regulation suggests the possibility of a functional role for this isoform in the physiology of undifferentiated cells or the regulation of transdifferentiation, since otherwise it would be more energetically sensible for the regenerating tissues to start expressing high levels of this protein only at the end of the transdifferentiation process, as the cell types that normally express it in the mature eye are formed.

This view is supported by the several reports of  $\text{Na}^+/\text{K}^+$  ATPase  $\alpha 1$  expression in undifferentiated, multipotent cells in other systems. For example,  $\alpha 1$  has been shown to be the main isoform detected in embryonic stem cells, which, upon induction of differentiation into neurons, start expressing the  $\alpha 3$  isoform (Reviewed by Habiba and Mercer, 2000). It has also been reported that  $\alpha 1$  is abundant in leukemia cell lines, and that induction of maturation of these cells decreases the levels of mRNA for this molecule (Gilmore-Hebert et al., 1989). Interestingly, novel functions beyond the establishment of ion gradients have been recently discovered for this protein. In a study by Moseley et al. (2005), microarray analysis of hearts of mice that lack one copy of the  $\alpha 1$  isoform of  $\text{Na}^+/\text{K}^+$  ATPase has shown that this isoform is linked to multiple biological pathways. Moreover, multiple reports have demonstrated a role for this subunit in intracellular signal transduction mechanisms through the regulation of Src and the transactivation of EGF receptors (Aydemir-Koksoy et al., 2001; Chen et al., 2008; Haas et al., 2000, 2002; Kometiani et al., 1998; Liang et al., 2006; Liu et al., 2000; Tian et al., 2006; reviewed by Xie and Askari, 2002; Li and Xie, 2008).

Alteration in the expression levels of  $\text{Na}^+/\text{K}^+$  ATPase isoforms during regeneration has been previously reported. During nerve regeneration in the rat peripheral nervous system there is a decrease in the expression of  $\alpha 1$  and  $\alpha 2$  isoforms, whereas there is a marked, transient increase in  $\alpha 3$  during axonal regrowth (Arteaga et al., 2004). This increase in  $\alpha 3$  isoform expression has also been reported in a study by Liu et al. (2002) on optic nerve regeneration in goldfish. The upregulation of the  $\alpha 3$  isoform as opposed to  $\alpha 1$  in these cases could be due to species differences, but more likely, it could be attributed to the fact that axonal re-growth is the main mechanism behind nerve regeneration, rather than transdifferentiation, and therefore in such cases there is no need for a regression to an undifferentiated state of the cells.

We believe that the variation in  $\text{Na}^+/\text{K}^+$  ATPase isoform expression in all the aforementioned physiological contexts is far from trivial. There are many reasons to believe that the different isoforms of this protein are linked to specific cellular functions, and that the idea of a simple, redundant role for the various isoforms in  $\text{Na}^+$  and  $\text{K}^+$  exchange seems naïve in the light of new discoveries. For example, both  $\alpha 1$  and  $\alpha 2$  isoforms are expressed in skeletal muscle, but analysis of contractile parameters in the muscles of mice that lack one copy of either gene has demonstrated opposite roles in muscle contractility for these isoforms (He et al., 2001). In addition, even though the  $\alpha 1$  and  $\alpha 4$  isoforms of  $\text{Na}^+/\text{K}^+$  ATPase co-localize to the mid-piece of the flagellum in rat sperm cells, only  $\alpha 4$  seems to play a role in sperm motility through the interaction with a  $\text{Na}^+/\text{H}^+$  exchanger (Woo et al., 2000, 2002). It is likely that further research

will demonstrate other mechanisms for functional specificity of the various isoforms of this enzyme in different contexts.

Finally, it is interesting to point out that Rx, a gene that is crucial for retinal development, has an expression pattern in many vertebrates that is very similar to that observed for Na<sup>+</sup>/K<sup>+</sup> ATPase  $\alpha$ 1 in this study, being expressed in the undifferentiated neuroepithelium as the retina develops, and being restricted to the ciliary body and photoreceptors as differentiation of the eye progresses (reviewed by Bailey et al., 2004).

In conclusion, our findings suggest that the  $\alpha$ 1 isoform of Na<sup>+</sup>/K<sup>+</sup> ATPase is a good candidate molecule to play a role in the modulation of transdifferentiation and regeneration of adult newt tissues. The possibility  $\alpha$ 1 regulating cell multipotentiality opens new avenues for research that certainly deserve to be explored.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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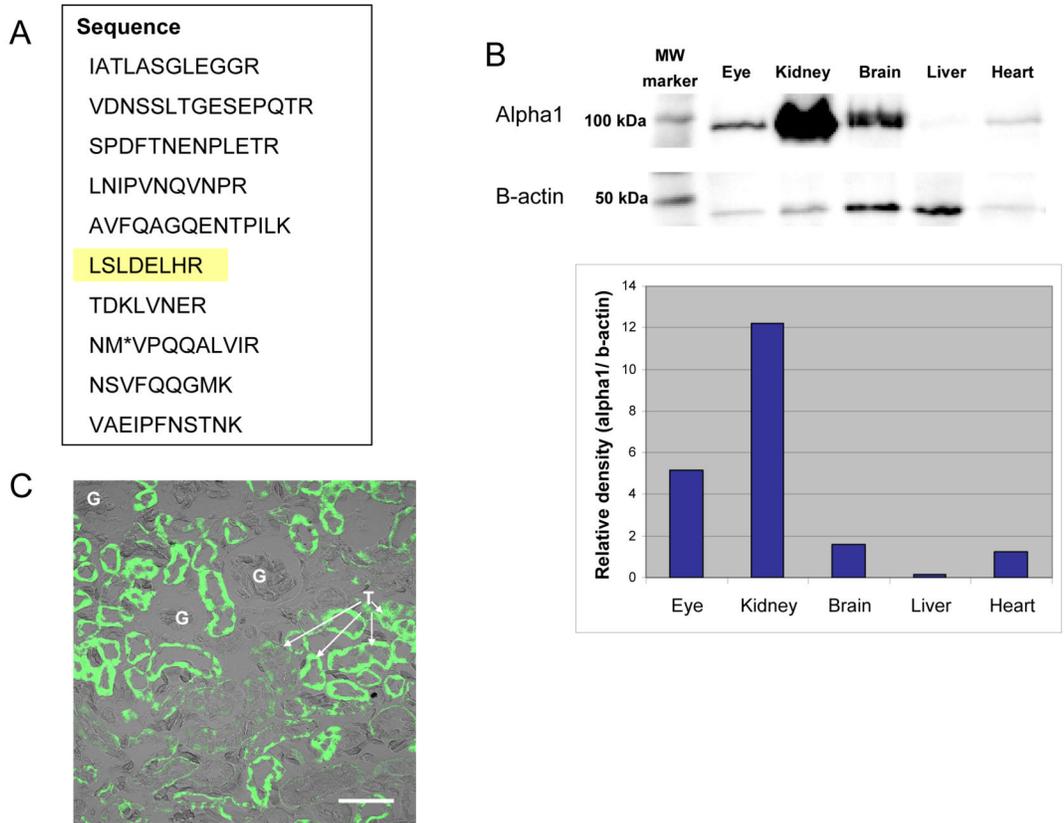
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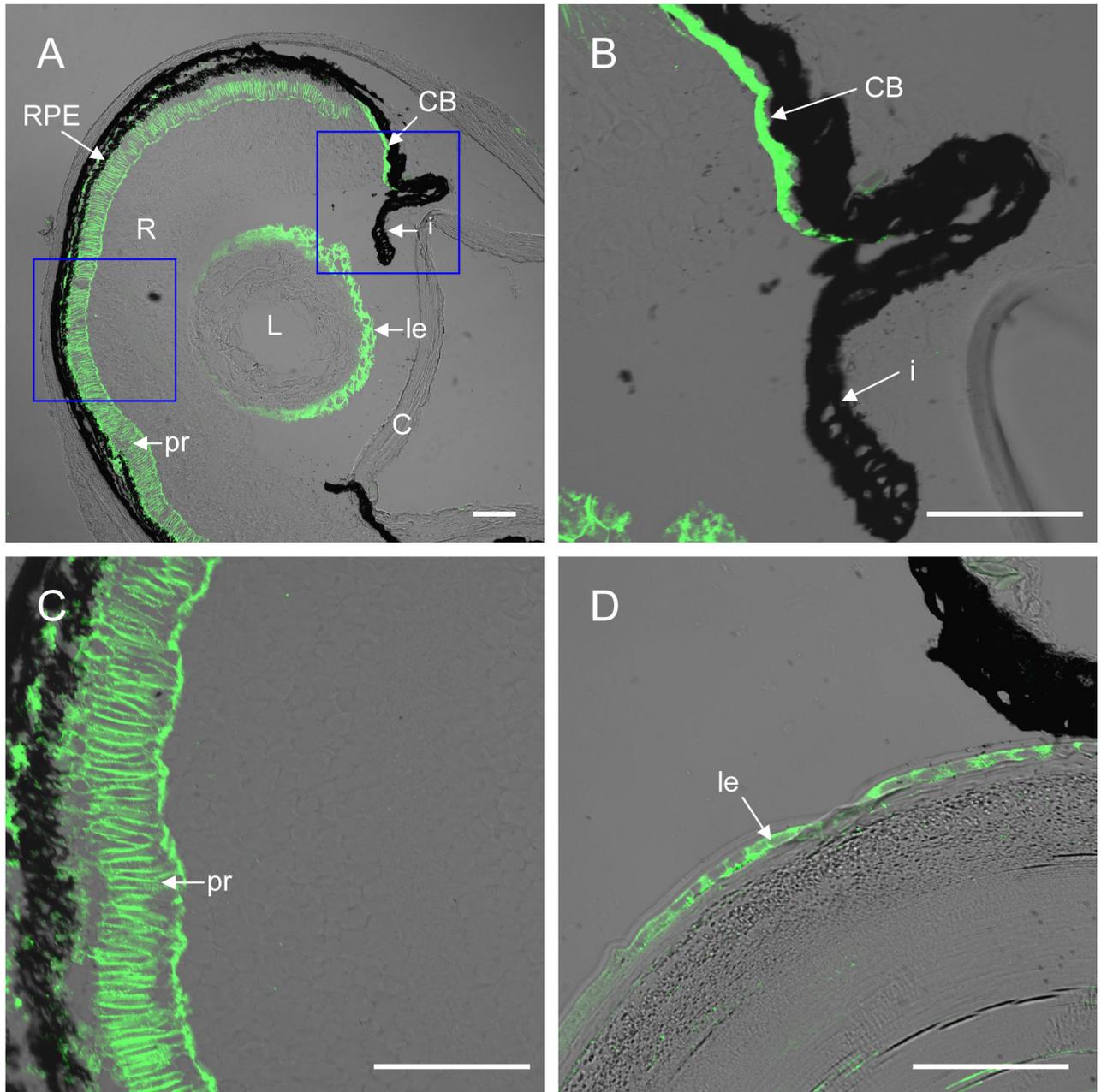
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**Figure 1.**

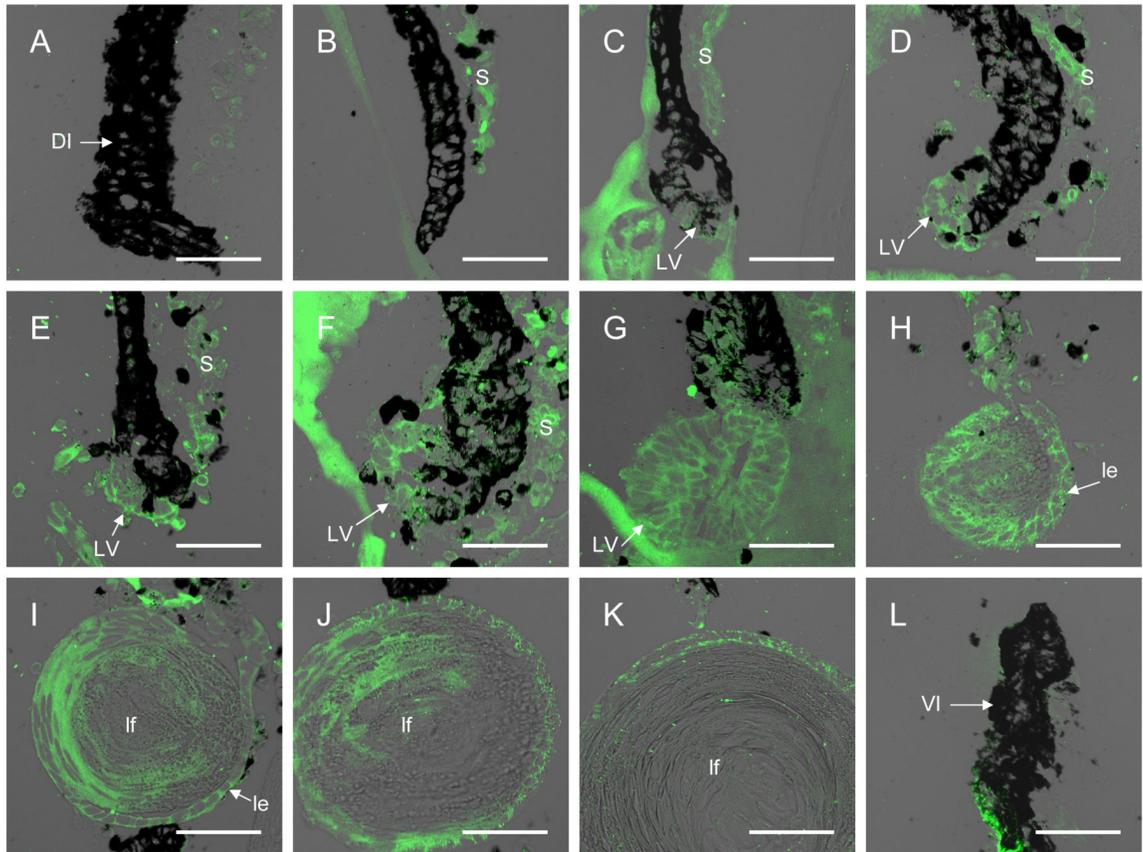
Expression of  $\text{Na}^+/\text{K}^+$  ATPase alpha1 protein in adult newt tissues. (A) Polypeptide sequences corresponding to the newt  $\text{Na}^+/\text{K}^+$  ATPase alpha1 protein, obtained by mass spectrometry. The highlighted sequence is part of the epitope recognized by the  $\alpha 6\text{F}$  monoclonal antibody used in this study. (B) Top: Western blot showing the distribution of  $\text{Na}^+/\text{K}^+$  ATPase alpha1 protein in different adult newt tissues. A protein band of  $\sim 100$  kDa was detected in all tissues. Bottom: quantification of the band intensity for  $\text{Na}^+/\text{K}^+$  ATPase alpha1 relative to that of  $\beta$ -actin for the different tissues tested. (C) Immunohistochemical localization of  $\text{Na}^+/\text{K}^+$  ATPase alpha1 in newt kidney sections using the  $\alpha 6\text{F}$  antibody. Notice that the different sections of the kidney tubules are labeled by this antibody in different levels of intensity, whereas the glomeruli are not labeled. Abbreviations: G: glomerulus; T: kidney tubules. Scale bar represents  $100 \mu\text{m}$ .



**Figure 2.**

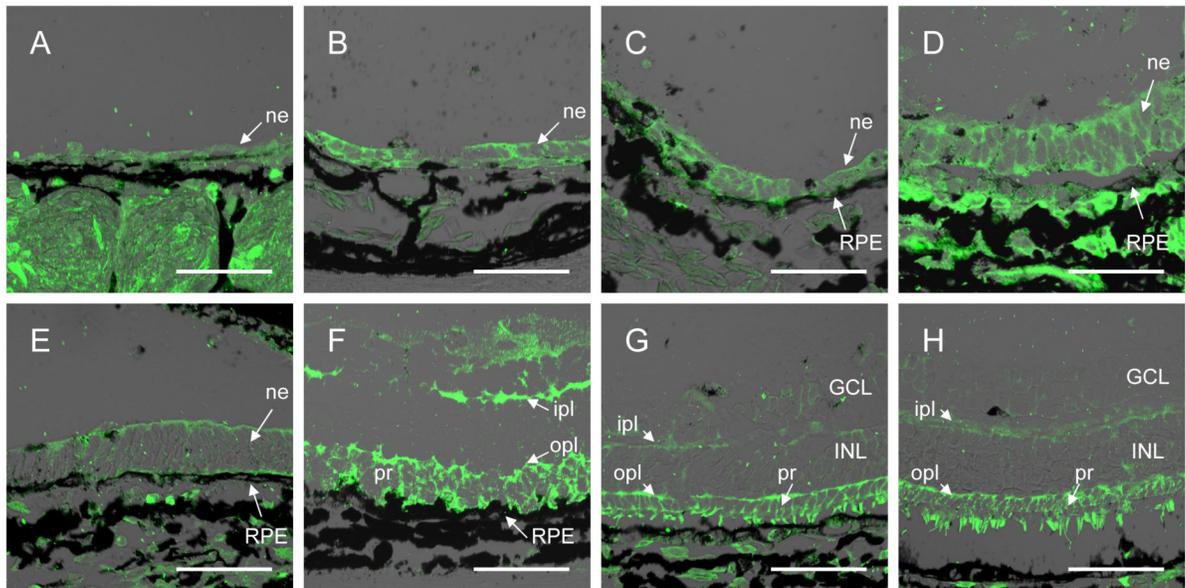
Expression of Na<sup>+</sup>/K<sup>+</sup> ATPase alpha1 in the mature newt eye. (A) Immunohistochemistry was performed on adult newt eye sections using the  $\alpha 6F$  antibody (shown in green). Notice the staining in the ciliary body, lens epithelium and photoreceptor layer of the retina (fluorescence between the RPE and choroid layers is nonspecific and is also present in the negative controls, whereas the RPE cells are not stained by this antibody). (B) Higher magnification of the boxed area in the anterior region of the eye in (A) showing the immunostaining of the ciliary body and the lack of staining in the iris. (C) Higher magnification of the boxed area in the posterior region of the eye in (A) showing that only the photoreceptor cell membranes (inner and outer segments) are stained by this antibody. (D) High magnification image of a section of the lens through the middle of the eye. Notice that only the lens epithelial cells are labeled by this

antibody whereas there is no staining of the lens fibers (fluorescence towards the middle of the lens is due to trapping of the antibody that occurs also in the negative controls). The anterior side of the eye is to the right and the dorsal side at the top of every picture. Abbreviations: RPE: retinal pigmented epithelium; CB: ciliary body; i: iris; R: retina; L: lens; le: lens epithelium; C: cornea; pr: photoreceptors. Scale bars represent 100  $\mu\text{m}$ .



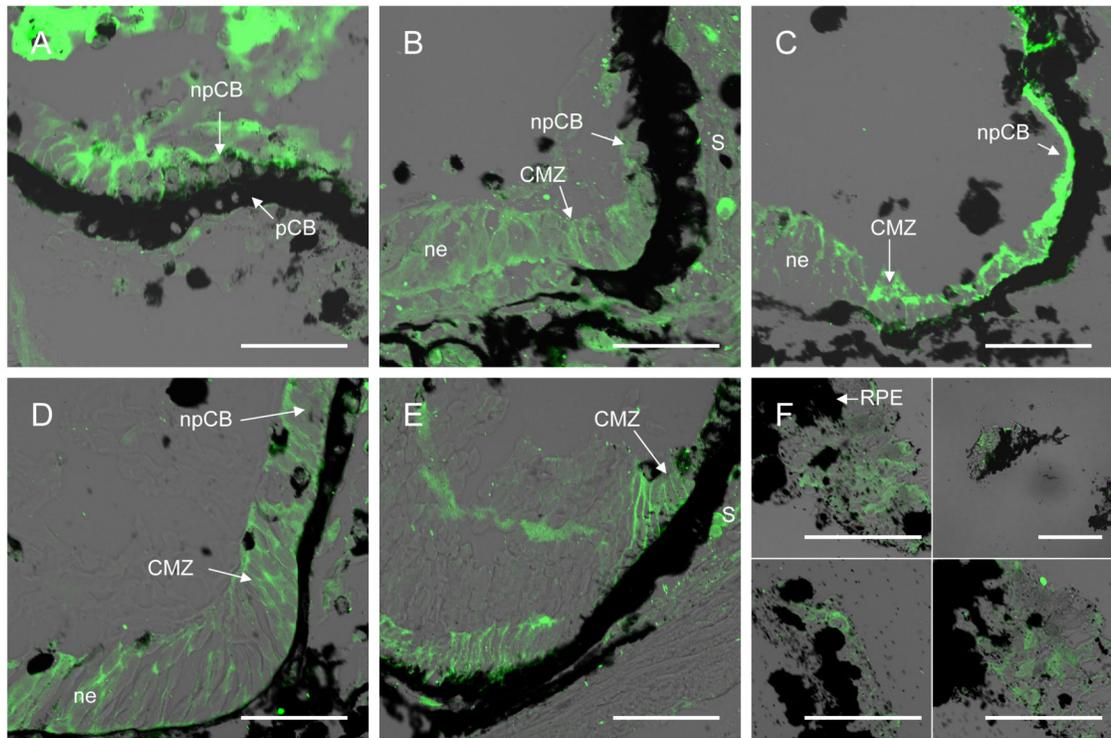
**Figure 3.**

Expression of  $\text{Na}^+/\text{K}^+$  ATPase alpha1 during lens regeneration. Immunohistochemistry was performed on newt eye sections obtained at different time points post-lentectomy. (A-K) Dorsal iris-lens vesicle view at 5, 7, 9, 10, 12, 15, 20, 25, 30, 35 and 45 days post-lentectomy (A, B, C, D, E, F, G, H, I, J and K respectively). Notice the immunolabeling with  $\alpha 6\text{F}$  antibody (green) only in depigmented cells during lens vesicle stages, and becoming progressively more restricted to the lens epithelium as differentiation continues (fluorescence seen in the stroma of the iris is nonspecific and is also observed in the negative controls). (L) Ventral iris view of a newt eye 12 days post-lentectomy. Antibody staining was not observed in the ventral iris at any of the assayed time points. The anterior side of the eye is to the right and the dorsal side at the top of every picture. Abbreviations: DI: dorsal iris; S: stroma; LV: lens vesicle; le: lens epithelium; lf: lens fibers; VI: ventral iris. Scale bars represent 100  $\mu\text{m}$ .



**Figure 4.**

Expression of  $\text{Na}^+/\text{K}^+$  ATPase alpha1 during retina regeneration. Immunohistochemical analysis was performed on newt eye sections obtained at different time points post-retinectomy. (A-C) During early stages of regeneration (regenerate is 1-2 cells thick), at 15, 17 and 20 days post-operation (A, B and C respectively), this protein is localized in the dedifferentiated cells derived from the RPE. Notice that only a monolayer of dedifferentiating cells is observed in (A), which later becomes a bilayer (B) with the inner cells giving rise to retinal progenitors and the outer cells to progenitors that will replenish the RPE, and that all of these progenitors are immunolabeled by the  $\alpha 6\text{F}$  antibody. (D-E) During intermediate stages of regeneration, as the cell number increases and the neuroepithelium becomes more compact (23 and 25 days post-operation, D and E respectively), immunostaining is seen in the plasma membrane of all the cells that form this still immature neuroepithelium. (F-H) At late regeneration stages, 30, 35 and 40 days post-operation (F, G and H respectively), the expression of this protein becomes restricted to the plexiform layers and photoreceptors. The fluorescence observed between the RPE and choroid layers at the different stages is nonspecific and corresponds to red blood cells that are autofluorescent also in the negative controls. Abbreviations: RPE: retinal pigmented epithelium; ne: regenerating neuroepithelium; pr: photoreceptors; ipl: inner plexiform layer; opl: outer plexiform layer; GCL: ganglion cell layer; INL: inner nuclear layer. Scale bars represent  $100\ \mu\text{m}$ .



**Figure 5.**

Expression of  $\text{Na}^+/\text{K}^+$  ATPase alpha1 in the ciliary region during retina regeneration, and in vitro localization. (A-E) Ciliary margin view of the regenerating newt retinas at 15, 20, 23, 25 and 30 days post-retinectomy immunostained with the  $\alpha 6\text{F}$  antibody (A, B, C, D and E respectively). The ciliary body (one cell thick, closest to the iris) and the CMZ (multiple cells thick, adjacent to the retina) are always positive for this staining. The fluorescence of the stroma and red blood cells is nonspecific and is present in the negative controls as well. (F) Immunohistochemistry for  $\alpha 6\text{F}$  on RPE explants transdifferentiating in culture. Four different cases of explants are shown. Notice the plasma membrane staining pattern in the depigmented tissue. Abbreviations: npCB: non-pigmented ciliary body; pCB: pigmented ciliary body; CMZ: ciliary marginal zone; ne: neuroepithelium; RPE: retinal pigmented epithelium; S: stroma. Scale bars represent 100  $\mu\text{m}$ .