REGULATION OF EMBRYONIC EYE REGROWTH IN *XENOPUS LAEVIS*

By

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Bachelor of Science-Biochemistry
University of New England
2013

A dissertation submitted in partial fulfillment
of the requirements for the

Doctor of Philosophy – Biological Sciences

School of Life Sciences
College of Sciences
The Graduate College

University of Nevada, Las Vegas
May 2024

UNLV | GRADUATE COLLEGE

Dissertation Approval

The Graduate College
The University of Nevada, Las Vegas

November 29, 2023

This dissertation prepared by

Dylan Guerin

entitled

Regulation of Embryonic Eye Regrowth in *Xenopus Laevis*

is approved in partial fulfillment of the requirements for the degree of

Doctor of Philosophy – Biological Sciences
School of Life Sciences

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ABSTRACT

MECHANICS AND SIGNALING IN XENOPUS LAEVIS EMBRYONIC EYE REGROWTH

By

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Regrowth of lost organs and tissues is an amazing ability that some animals possess. For regrowth to be considered full, the organism must regrow the organ or tissue to a state that is structurally and functionally similar to that which was lost. The ability of an organism to regrow lost or damaged tissue varies among animals, both in the mechanisms utilized to achieve regrowth, and in what body parts can be regrown. Potentially even closely related species can vary wildly in regenerative ability. Therefore, regenerative research can look to other tissues and organisms for direction in elucidating the mechanisms through which regrowth is achieved, we must individually determine these mechanisms for each unique organism and tissue.

The Tseng laboratory has determined that the African clawed frog, *Xenopus laevis* can regrow its embryonic eye. This regrowth is achieved in a period of five days following ablation of the developing optic cup and lens placode at developmental stage 27, and the regrown eye is physiologically indistinguishable from the normally developed eye and is functional. This ability is lost as the animal ages, with mature eyes being incapable of full regrowth. We are interested in determining how the embryonic eye achieves regrowth. What are the signals regulating regrowth

and does regrowth recapitulate development of the eye? This dissertation helps to elucidate some of the answers to these larger questions.

Within our model of regrowth, eye development and regrowth are taking place concurrently, creating interesting questions about timings and order of development/regrowth of the ablated eye. *Xenopus* eye development is well studied, which allows us to compare our regenerative mechanisms to well-known developmental mechanisms and timings. During *Xenopus* eye development, the retinal cells differentiate in a particular, overlapping order: ganglion cells, horizontal cells, cones, rods, amacrine, bipolar, and finally Müller glial cells. We determined that during eye regrowth, the retinal cells differentiate in this order as well. Additionally, the regrowing eye has a delay in eye formation compared to the normal developing eye (as determined by morphology and molecular markers) for two days, but by the third day post-surgery, it has caught up to the contralateral eye.

We have determined the overall morphology of the eye during regrowth. However we do not know many of the mechanisms regulating regrowth. Previous work in our laboratory determined that inhibition of apoptosis and Pax6 both independently inhibit eye regrowth, but there are likely other regulators at work. My work determined that the Notch signaling pathway is required for regrowth of the *Xenopus* embryonic eye.

The Notch signaling pathway is a conserved developmental pathway that regulates proliferation and differentiation. The pathway is activated through cell-to-cell signaling, with ligand binding triggering a cleavage of the receptor then acts as a transcription factor in the nucleus. I determined that Notch signaling during eye regrowth is required during the first day of the five-day period. Previous work demonstrated an increased amount of proliferation during that period, and by inhibiting Notch1 signaling during the regrowth period, a significant reduction in

mitotic cells observed. It does not seem however that inhibition affects differentiation in the regrowing eye, with Notch1 inhibited eyes showing the general retinal morphology of uninhibited eyes, as well as mature rod and ganglion cells in their appropriate places.

To determine the interaction of Notch with other known regenerative mechanisms, I overexpressed Notch1 during eye regrowth, while blocking V-ATPase activity. The vacuolar V-ATPase is a transmembrane enzyme that is integral to maintaining cell voltage. Membrane voltage is another regulator of regeneration, and V-ATPase is a regulator of Notch signaling in other systems. I demonstrated that overexpression of Notch1 signaling during regrowth is sufficient to rescue V-ATPase inhibition of regrowth, indicating a link between the two pathways in *Xenopus laevis* embryonic eye regrowth.

I have helped determine the general physiology of the eye during regrowth, as well as determining the necessity and function of Notch signaling. However, there are many questions still remaining regarding embryonic eye regrowth in *Xenopus laevis*. It is my hope that future generations of scientists utilize this research as a foundation to discover novel mechanisms governing eye regrowth, as well as regrowth of other organs in other systems.

ACKNOWLEDGEMENTS

Graduate school was difficult for me, and I would not have been able to complete it without the amazing people in my life. I would like to thank everyone in the Tseng lab, but first and foremost Cindy Kha. You're a great friend and an amazing teacher and I don't think I can convey how much your help was integral to my success. Also, for showing me all the great food in Las Vegas.

In a similar vein I would like to thank my partner, Cameron Exner. Your support and expertise mean the world to me. Thank you for all your editing as well as the emotional support. I love you.

I would also like to thank Keala Kiko for answering every question I had during my time here, and there were many. I'm not sure I've seen someone so knowledgeable about all the bureaucratic things that go into getting a degree. Thank you, Ala, from the bottom of my heart.

Thank you to all the amazing friends I made here. You really helped me grow and enjoy my time in Las Vegas. Also thank you to the lab for your support and help.

DEDICATION

To the frogs. Thank you for letting me care for you and for your sacrifice.

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LIST OF ACRONYMS

BMP……Bone Morphogenic Protein

CMZ……Ciliary Margin Zone

DMSO……..Dimethyl Sulfoxide

DNA……Deoxyribonucleic Acid

DPS……....Days Post-Surgery

EFTF……..Eye Field Transcription Factor

FGF……..Fibroblast Growth Factor

GCL……....Ganglion Cell Layer

GFP……..Green Fluorescent Protein

H3P……....Phosphorylated Histone 3

HIF……Hypoxia Inducible Factor

HPS……Hours Post-Surgery

IAP……..Inhibitors of Apoptosis Proteins

INL…….....Inner Nuclear Layer

JNK……...Jun-N Terminal Kinase

MAPK……Mitogen Activated Protein Kinase

MMR…….....Marc's Modified Ringer

MO……Morpholino

NICD…….Notch Intracellular Domain

ONL……..Outer Nuclear Layer

RGC……...Retinal Ganglion Cells

RI……..….Regrowth Index

RNA……..…….....Ribonucleic Acid

ROS……Reactive Oxygen Species

RPC……..Retinal Progenitor Cells

RPE…….....Retinal Pigmented Epithelium

RVM……..Retinal Vascular Membrane

V-ATPase……Vacuolar-type Adenosine Triphosphatase

CHAPTER 1

REGENERATION: A HISTORY, AN OVERVIEW, AND MECHANICS OF VERTEBRATE EYE REGROWTH

1.1 Definition of Regeneration

Regeneration is the process of regrowing lost or damaged tissue. Regenerative ability has obvious benefits, however, this ability is not present in many animals, including humans. However much we would like to be able to regrow lost body parts, we are limited to digit tips as infants, and regrowing portions of our liver (Illingworth, 1974; Fausto, Campbell, and Riehle, 2006), which pales in comparison to other organisms such as the *Hydra* or some species of planarian flatworm, which can grow entire bodies from dissociated cells, or even other vertebrate species such as zebrafish, which can regrow their fins, epithelial tissue, and retinas or axolotls which can regenerate its limbs, spinal cord, or even parts of its brain (Sarras et al., 1993; Wagner et al., 2011; Joven et al., 2019; Marques et al., 2019). Helpfully, for those working to implement regenerative therapies in humans, many of the drivers of regeneration in these other organisms are present in humans as well. It is through studying these organisms and learning how they can perform these regenerative feats that we can learn how to potentially implement regenerative therapies in humans.

1.2 History

Published research into regeneration dates back to the 1700s. René Réaumur in France, Lazzaro Spallanzani in Italy and Abraham Trembley in Switzerland determined that crayfish, salamanders, and *Hydra* could regenerate (de Réaumur, 1712; Trembley, 1744; Spallanzani, 1768. The experimental methodology they utilized to test the limits and abilities of regeneration in their chosen models was different from the more descriptive form biology took previously,

leading some to credit Trembley as the father of biology. In fact, Trembley was the first to describe stem cells and Spallanzani disproved spontaneous generation through his work with frog embryos. Regeneration research was at a high at this time, even prompting a dogmatic dispute in the Catholic church over the location of the soul in the body (if a bisected *Hydra* regrows as two, which one has the soul?) (Stott, R. 2012) over time interest in regeneration waned as new interest in embryonic development gained prominence. It was not until 1949 that Elmer Grinshaw Butler discovered that the mesoderm of the newt differentiates to contribute to the blastema, the mass of cells facilitating limb regeneration, that major interest in regeneration research took hold (Butler and Schotte, 1949). About thirty years later, Eguchi and Okada in Japan demonstrated transdifferentiation of newt iris to lens following lensectomy, and Niazi and Saxena demonstrated that tadpole limb regeneration could be disrupted through vitamin A (Okada, Eguchi, and Takeichi, 1973; Niazi and Saxena, 1979). New techniques of the modern age allowed for a more in-depth view of the mechanisms of regeneration, allowing for our modern focus not just on which animals can regenerate what body part, but what allows this regeneration to occur and how might we manipulate these processes.

1.3 *Xenopus,* Eye Development, Morphology, and Regeneration

Regeneration itself requires multiple interacting processes to succeed. Following damage to the tissue, first that damage must be healed if any regeneration is to occur. Following this, the lost tissue must be replaced through cell proliferation. These cells must then differentiate into the adult cell types required for that tissue. It is not enough for the tissue to have attained the correct number and identity of cells, but also that the tissue regains the shape of that which was lost. If a regrown eye has differentiated ganglion, rod, and cone cells in a large number, but those cells are not organized, the eye will not function. Therefore, it is necessary for the mechanisms regulating

eye regrowth to both properly recapitulate adult cell numbers and physiology (Tseng, 2017; Kakebeen and Wills, 2019; Guerin et al., 2021).

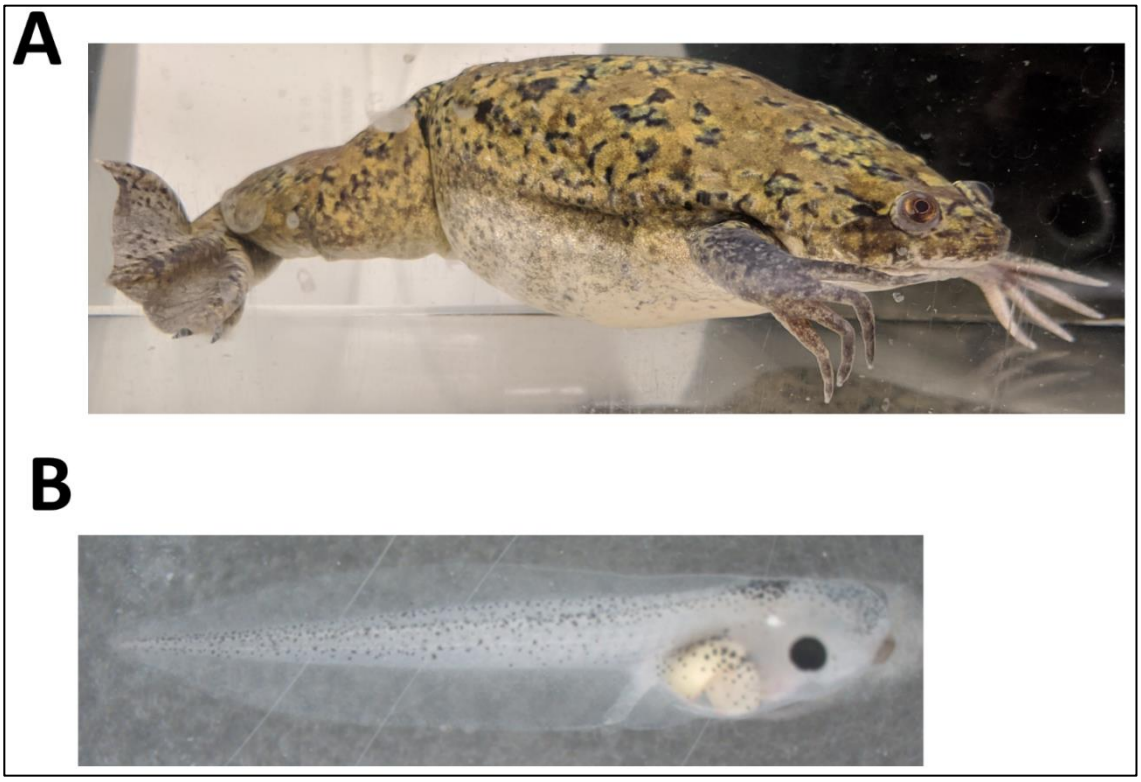


Figure 1.1 *Xenopus laevis*. (A) Adult frog. (B) Tadpole

The model organism that we utilize to study regrowth is the African clawed frog, *Xenopus laevis* (Fig 1.1). *Xenopus*, originally from South Africa were brought to Europe by Lancelot Hogben in the 1930s to act as living pregnancy tests (Hogben and Hogben, 1998). Injection of female *Xenopus* with a pregnant woman's urine would stimulate the frog to produce embryos while a non-pregnant woman's urine would not. It was not until after World War II that

Peter Nieuwkoop utilized *Xenopus* for developmental research. It is through his work that we have the developmental stages utilized in *Xenopus* research to this day (Nieuwkoop and Farber, 1994). *Xenopus* research took off due to the ease of rearing multiple animals, the external laying and relatively rapid development of their large embryos, as well as the large number of offspring obtained from a single mating. These large embryos make excellent protein factories through injection of RNAs into the embryo and harvesting the extract. Additionally, their embryos are remarkably hearty, making them easy to care for as well as excellent candidates for explant experiments (Exner and Willsey, 2021). *Xenopus* research picked up steam in 1971 when John Gurdon showed that ribosomal RNA was not synthesized by anucleolate embryos (Gurdon et al., 1971). Additionally, John Gurdon was the first to clone an animal, *Xenopus laevis*, earning him the Nobel Prize (Gurdon et al., 1958). *Xenopus laevis* are an excellent model for regenerative research and critically important to the field of development.

Xenopus are remarkable among regenerative models in that they display age-dependent regeneration. The larval stages of *Xenopus* are capable of regenerating their tails, limb buds, and eyes (Dent, 1962; Morgan and Davis, 1902; Beck et al., 2003; Kha and Tseng, 2018; Kha et al., 2018), but lose much of that ability following metamorphosis. The Tseng laboratory has determined that removal of the developing eye results in a regrown eye, complete by five-days post-surgery (Kha et al., 2018). The goal of my project is to determine through what mechanisms that eye will regrow.

The *Xenopus* eye (Fig 1.2) is a great model of the vertebrate eye and follows the same general developmental pattern as all vertebrate eyes. Eye development begins following gastrulation by the overlapping expression of various eye-field transcription factors in the forebrain. The transcription factors specify the presumptive optic vesicle and disruption of these

transcription factors can lead to eye malformations or even lack of eyes. Ectopic expression of these factors can induce eye development in other regions of the embryo (Zuber et al., 2003). The eye field begins as a single region that must be separated into two. This is achieved through Sonic Hedgehog and Six3 signaling, and failure to separate the eye field results in cyclopia (Ohtsuka et al., 2022). Following separation, invagination of the eye field occurs to form the optic cup, which allows for the further specification of the lens and retina.

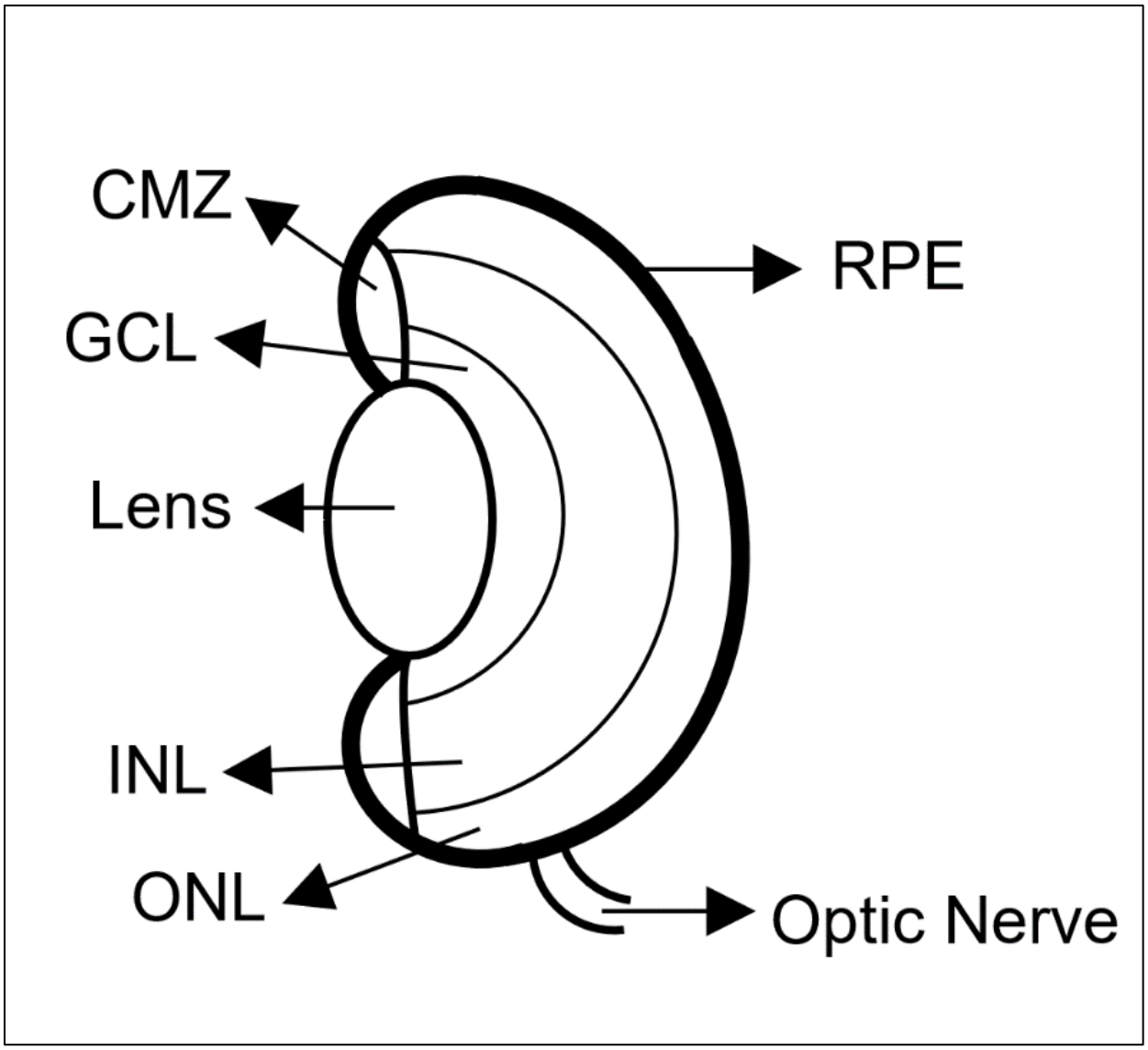


Figure 1.2 Representation of the vertebrate eye

The lens develops from the overlying ectoderm known as the lens placode, which thickens and then invaginates to form the lens vesicle. The retina forms from the posterior layer of the optic cup in two separate layers. The posterior layer is the retinal pigmented epithelium (RPE), while the anterior becomes the neural retina. This decision is mediated by FGF9, with ectopic expression of FGF9 in the RPE causing development of the retina instead (Marines-Morales et al., 2004). The neural retina is composed of three layers; the ganglion cell layer (GCL), composed of ganglion cells, the inner nuclear layer (INL), composed of bipolar cells, amacrine cells, and Müller glia, and the outer nuclear layer (ONL) containing the photoreceptors. Light enters through the lens and hits the photoreceptors at the posterior of the eye. The photoreceptors activate in response to light and relay that signal through the bipolar cells to the ganglion cells, then onwards to the brain (Graw, 2010; Heavner and Pevny, 2012).

The ability to regenerate such an important organ as the eye is an astounding feat. Animals such as newts, zebrafish, chick, rabbit, mouse, cat, and of course our frog *Xenopus laevis* are capable of regeneration of at least part of the eye (Gwon et al., 1989, Gwon et al., 1990; Kodama and Eguchi, 1995; Call et al., 2004). Eye regeneration is documented in invertebrates as well, though I am keeping the focus on vertebrate eye regeneration, as the invertebrate eye has a different structure from vertebrates, as well as variation in structure even among other invertebrates.

Lens regeneration has been documented in multiple animals but there is extensive study on lens regeneration in newts (Tsonis et al., 2004). The lens is the structure at the anterior of the eye composed of crystallin responsible for focusing light onto the retina. Following lensectomy in newts, the pigmented epithelial cells within the dorsal iris dedifferentiate in order to begin to form a new lens vesicle. These cells lose their iris identity, losing their pigmentation, and take on

the new identity of a lens progenitor. These cells then proliferate and differentiate, recapitulating lens development, completed at around nineteen days following lensectomy (Kodama and Eguchi, 1995). Amazingly, this ability is present throughout the life of the newt, up to eighteen lensectomies over sixteen years having no negative effect on regenerative ability (Eguchi et al., 2011).

A similar method of transdifferentiation is observed in *Xenopus laevis* where following lensectomy the new lens is derived from the outer cornea (Freeman 1963). In contrast to the newt however, this ability is lost completely following metamorphosis (Filoni et al., 1997). Interestingly, this seems to be enabled by the removal of the lens, as transdifferentiation of the cornea happens in response to exposure to the vitreous humor, creating a case where cornea cultured in the vitreous humor will transdifferentiate better than in vivo (Henry and Tsonis, 2010). The exposure of cornea to vitreous humor itself is the catalyst, as cornea implanted into the vitreous humor without removal of the lens, or the removal of barriers between the two without full removal of the lens or cornea epithelium still results in transdifferentiation (Reeve and Wild, 1978; Bosco et al., 1978, Bosco et al., 1980).

Newts and *Xenopus* are the most widely studied models of lens regeneration, however, there are other vertebrates capable of regenerating their lens. Chick have demonstrated some regenerative ability, but there is some debate as to if the regeneration is a result of transdifferentiation or of proliferation of residual competent ectoderm (McKeehan, 1961). Mammals such as mouse, rabbit, and cat are also capable of lens regeneration, albeit through a different mechanism. In these cases, regeneration of the lens is achieved through remnant lens epithelial cells in the capsular bag, a sack that supports the lens. Full removal of the capsular bag

results in the inability of these organisms to regenerate their lens (Gwon et al., 1989; Gwon et al., 1990; Call et al., 2004).

In addition to lens regeneration, some organisms can also regenerate their neural retina, the part of the eye responsible for responding to light. One of the most widely utilized models for retinal regeneration is the zebrafish *Danio rerio*. In zebrafish, damage to the retina releases cytokines from destroyed cells. These cytokines are engulfed by the Müller glia to initiate the regenerative response (Bailey et al., 2010). The Müller glia proceed to asymmetrically divide, dedifferentiating and reprograming to produce a retinal stem cell that is utilized to repair the damaged tissue (Ramachandran et al., 2010; Powell et al., 2013).

As with most instances of regeneration, canonical developmental pathways are utilized to regulate repair. For zebrafish retinal regeneration to proceed, the eye-field transcription factor Pax6 is required. Though dedifferentiation of the Müller glia still takes place in the absence of Pax6, the progenitors are unable to fully differentiate into mature retinal cells (Thummel et al., 2010). Additionally, these progenitors migrate to the ONL to divide, migrating back into the INL to form the requisite cells (Nagashima et al., 2013). While the reasons for this migration are unknown, it is hypothesized that a transcription factor gradient is required to establish proper cell identity (Lahne and Hyde, 2015).

Another well-studied signaling pathway playing an important role in zebrafish retinal regeneration is the Notch signaling pathway. In zebrafish retinal regeneration, Notch signaling is downregulated in a MAPK-dependent manner, and activation of Notch during this period results in regenerative failure (Wan et al., 2012; Conner et al., 2014).

In contrast to zebrafish, chicks achieve retinal regeneration through transdifferentiation of retinal pigmented epithelium (RPE) cells (Orts-Llorca and Genis-Galvez, 1960; Coulombre and Coulombre, 1965), the pigmented layer at the posterior of the eye that absorbs scattered light and provides maintenance for the neural retina. Following retina damage, the RPE cells dedifferentiate, lose pigment, and undergo proliferation to replenish the lost retina. Separation of the RPE from the neural retina results in the transdifferentiation of RPE cells to retinal cells (Coulombre and Coulobre 1965). This process is driven by FGF 1 and 2, and exogenous FGF is sufficient to transdifferentiate RPE into neural retina in vivo (Pittack, Jones, and Reh, 1991). This ability, however, is lost after embryonic stage 4.5 (Park and Hollenberg, 1993).

In post-metamorphosis *Xenopus laevis*, retinal regeneration transpires in a similar manner to the chick. Upon removal of the retina, cells in the RPE migrate to the retinal vascular membrane (RVM) and transdifferentiate to take on a stem-like role in order to regenerate the retina. For proper regeneration to occur, the RVM must remain at least partially attached, and within the RVM FGF-2 induces transdifferentiation, with the new cells using the RVM as a scaffold. Additionally, cells from the ciliary marginal zone (CMZ), a highly proliferative region of the eye, contribute to the regenerate retina (Yoshii et al., 2007).

In pre-metamorphic *Xenopus*, partial resection of the retina is regenerated by 30-days-post injury through repopulation of retinal progenitor cells (RPC's), which in this case are organized similarly to the CMZ (Martinez-De Luna et al., 2011). If the CMZ is removed, regeneration cannot naturally occur, instead only succeeding upon exogenous addition of FGF (Vergara and Del Rio-Tsonis, 2009). This reiterates the important role FGF plays in transdifferentiation of existing eye cells.

1.4 Model of *Xenopus laevis* Embryonic Eye Regrowth

In contrast to the previous cases of retinal regeneration taking place within the differentiated retina, the Tseng laboratory utilizes a model of eye regeneration occurring early in the process of eye development, allowing us to observe and test eye regeneration in the context of development. The Tseng laboratory has determined that *Xenopus laevis* embryos can regrow their eyes following removal of approximately 85% of the optic cup, the tissue that will develop into the eye, at developmental stage (st.) 27, by five days post-surgery (DPS) (Fig. 2.1) (Kha et al., 2018). Quantitation of removed optic cup tissue was performed through fixation immediately after surgery, sectioning and staining with anti-neural antibody (Xen1), to mark remaining eye cells.

At st. 27 the optic cup is a monolayer of cells, that though specified to an eye fate and beginning differentiation, is still relatively early in eye development, allowing us to view regeneration concurrent with development. Damage to the optic cup is followed by an increase in proliferation beginning at 6 hps and maintaining through 24 hps, peaking at 12 hps (Kha et al., 2018). By 3 dps the regrown eye is morphologically similar the contralateral, unoperated eye, containing the required cell types (rods, cones, ganglion cells, Müller glia) in the proper layers (Fig 1.2, 2.1), with an optic nerve extending to the brain. Importantly, the regrown eye is functional. To determine functionality 3 dps tadpoles were placed in a container where one side is black and the other is white. Tadpoles with one or two functional eyes spend the vast majority of their time (>95%) swimming in the white half of the container, turning around when they hit the black side, while blind tadpoles spend similar time in both colors (Kha et al., 2018). Tadpoles that had regrown an eye, then had the contralateral eye removed, making them dependent on the regrown eye for vision, showed similar behavior to sighted tadpoles, spending 88.2% of their time in the white background, which is not significantly different than controls that had one eye

removed (88.4% of time in white),which demonstrates that the regrown eye is functional (Kha et al., 2018). Regrowth quality declines if the surgery is performed after st. 27, with steep declines occurring as early as st. 29, as measured by the Regrowth Index (RI) (Kha and Tseng, 2018).

The Tseng laboratory has begun to determine the molecular mechanisms that regulate this process. We have determined that apoptosis is required for regeneration of the eye. Treatment of the embryos with either the apoptosis inhibitors M50054 or NS3694 post-surgery significantly reduce regrowth quality as compared to controls (Kha et al., 2018). The Tseng laboratory also see that apoptosis-inhibited tadpoles that have their contralateral eye removed, making them dependent on the regrowth-inhibited eye, behave more similarly to non-sighted tadpoles than those that successfully regrew their eye (Kha and Tseng, 2018). This finding is interesting because apoptosis is not required for development of the eye (Johnston et al., 2005). This means that there are potentially other mechanisms specific to regrowth that remain to be elucidated.

Additionally, the Tseng laboratory has determined that the proton pump V-ATPase is required for eye regrowth (Kha et al., 2023). V-ATPase activity is required for maintaining neural stem cells in mouse development (Lange et al., 2011), as well as required for zebrafish and *Xenopus* tail regeneration (Adams et al., 2007; Monteiro et al., 2014). *Xenopus* embryos treated with the V-ATPase inhibitor concanamycin A following surgery, or previously injected with RNA coding for a nonfunctional subunit within the V-ATPase enzyme, and then had their eye ablated at st. 27 both failed to regrow their eyes. The V-ATPase-inhibited eyes contained ganglion cells, rods, and the retinal pigmented epithelium (RPE), as well as the correct retinal patterning, they were significantly smaller than control regrown eyes. V-ATPase-inhibited eyes had a significant reduction in proliferating cells, as labeled by phosphorylated histone 3 (H3P) during the first day of regrowth, as compared to control regrowing eyes, likely contributing to

the reduced eyes size. It is the proton pump function of V-ATPase that is required for regrowth as overexpression of a yeast proton pump (PMA1.2) in V-ATPase-inhibited embryos was sufficient to rescue regrowth. However, overexpression of PMA1.2 was insufficient to rescue inhibition of regrowth caused by apoptotic inhibition, indicating a lack of a link between the two (Kha et al., 2023).

There are still many questions that remain unanswered within this model. We do not know to what degree this model of regeneration is recapitulating eye development. Development and regeneration are occurring concurrently, and we do not know to what degree the eye needs to restart the developmental process to achieve regeneration? Also, there could potentially be mechanisms unique to embryonic eye regrowth, distinct both from later eye regeneration, and from eye development. In this dissertation I will address some of these questions.

CHAPTER 2

STUDYING IN VIVO RETINAL PROGENITOR CELL PROLIFERATION IN *XENOPUS LAEVIS*

2.1 Preface

This chapter was published in Methods in Molecular Biology as:

Kha, C. X.*, Guerin, D. J.*, & Tseng, K. A. (2020). Studying In Vivo Retinal Progenitor Cell Proliferation in Xenopus laevis. Methods in molecular biology (Clifton, N.J.), 2092, 19–33.

*Authors contributed equally to this work.

Author Contributions: Cindy Kha and I both wrote an equal amount for this paper, with Cindy contributing the images, with the exception of the Notch images, and I created the table. Kelly Tseng contributed the conception and design of the study, as well as writing and editing.

Editorial note: The manuscript is reproduced here with permission from Springer. Changes were made to the figure numbers to fit with the overall formatting of the dissertation.

2.2 Abstract

The efficient generation and maintenance of retinal progenitor cells (RPCs) are key goals needed for developing strategies for productive eye repair. Although vertebrate eye development and retinogenesis are well characterized, the mechanisms that can initiate RPC proliferation following injury-induced regrowth and repair remain unknown. This is partly because endogenous RPC proliferation typically occurs during embryogenesis while studies of retinal regeneration have largely utilized adult (or mature) models. We found that embryos of the African clawed frog, Xenopus laevis, successfully regrew functional eyes after ablation. The

initiation of regrowth induced a robust RPC proliferative response with a concomitant delay of the endogenous RPC differentiation program. During eye regrowth, overall embryonic development proceeded normally. Here, we provide a protocol to study regrowth-dependent RPC proliferation in vivo. This system represents a robust and low-cost strategy to rapidly define fundamental mechanisms that regulate regrowth-initiated RPC proliferation, which will facilitate progress in identifying promising strategies for productive eye repair.

2.3 Introduction

Studies of neural development in the African clawed frog, *Xenopus laevis*, have contributed significantly to the existing knowledge on vertebrate eye formation , including the identification of the eye-field transcription factors (EFTFs) and retinogenesis (Chang and Harris, 1998; Holt et al., 1998; Peron et al., 1998; Viczian et al., 2003; Zuber et al., 2003; Zaghloul et al., 2005; Andreazzoli, 2009; Wong and Rapaport., 2009). There are several features that make *Xenopus* a versatile system to study the eye (Henry et al., 2008; Tseng, 2017). First, it is well suited for in vivo studies as *Xenopus* embryos develop rapidly and externally and can be generated in large numbers. Second, many molecular and cellular tools are available for investigating gene function (Amin et al., 2014; Tandon et al., 2017; Tseng, 2017). Third, *Xenopus* embryos have relatively low culture costs as compared to mammalian models. Lastly, the mature *Xenopus* eye and the human eye have comparable structures due to the close evolutionary relationship between Xenopus and humans (Wheeler and Brändli, 2009). Notably, *Xenopus laevis* is also an established model for retinal and lens regeneration (Freeman, 1963; Underwood et al., 1993; Yoshii et al., 2007; Vergara and Del Rio-Tsonis, 2009; Henry and Tsonis, 2010; Day and Beck, 2011; Martinez-De Luna et al., 2011; Araki, 2014; Martinez-De Luna and Zuber, 2018). The high regenerative ability of *Xenopus laevis*, coupled with its well-

understood eye developmental processes, makes it an ideal and unique platform for devising and testing strategies to promote productive eye repair.

Retinal progenitor cells (RPCs) are of strong interest because of their potential as treatment strategies for restoring visual function in the context of injury and/or disease (Selier et al., 2008). During eye development, the multipotent RPCs derive from cells of the optic cup and generate all retinal neuron cell types and the Müller glia (Wetts and Fraser, 1988). It is known that a number of developmental mechanisms are used during retinal regeneration (Shaefer et al., 1999; Haynes et al., 2007; Malloch et al., 2009; Martinez-De Luna et al., 2011). Thus, a key objective in building strategies for productive eye repair is to identify the differences and similarities between developmental and regenerative retinal progenitor cell (RPC) proliferation. However, current retinal regeneration studies are largely focused on mature eye models, making it challenging to undertake effective comparisons with developmental events, which occur in a very different context. To facilitate such studies, a developmental model of eye repair is needed. We found that *Xenopus* tailbud embryos at developmental stage (st.) 27 successfully regrew eyes after surgical ablation (Kha et al., 2018; Fig. 2.1). The regrowth process was rapid, completing within 5 days after ablation (Fig. 2.1a–h). The regrown eye was age-appropriate; contained the expected structures including the retina, lens, and pigmented epithelium; connected to the optic nerve; and showed visual function (Fig. 2.1i). Our studies also showed that eye regrowth is age-dependent, with st. 32 embryos losing this ability (Kha and Tseng, 2018).

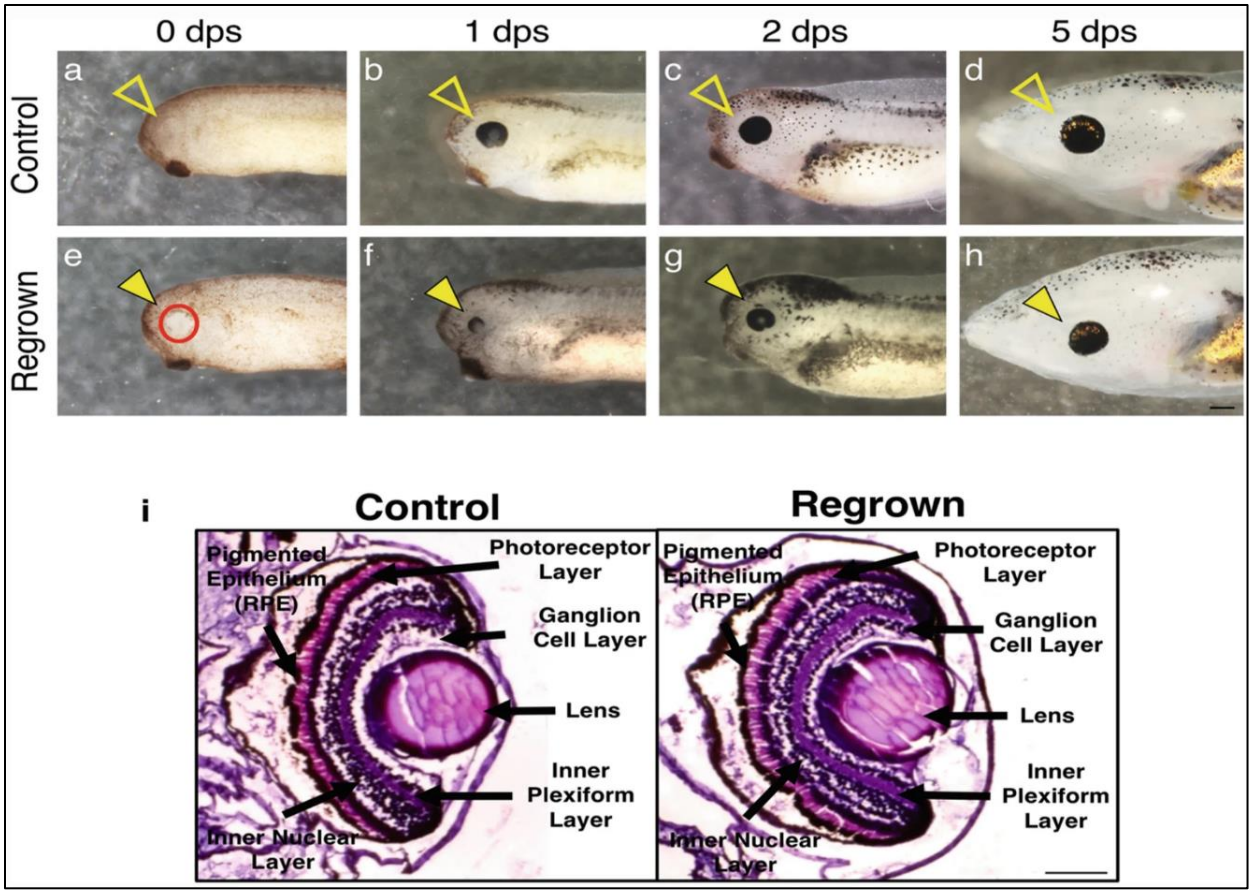


Figure 2.1 Eye regrowth following surgical ablation. Images show normal eye development (a–d) and eye regrowth progression (e–h) at 0, 1, 2, and 5 days post surgery. Closed arrowheads indicate surgery site; open arrowheads indicate age-matched unoperated eye. Regrown eyes have the same eye structures compared to an unoperated sibling control. (i) Hematoxylin- and eosin-stained section of an unoperated sibling control (left panel) and a regrown eye at 5 days post surgery (right panel). (a–h) Up = dorsal, down = ventral, left = anterior, right = posterior. (i) Up = dorsal, down = ventral. Scale bars: (a–h) = 200 µm, (i) = 50 µm (reprinted from Experimental Eye Research, 169/April, 2018, Kha, C.X., Son, P.H., Lauper, J., and Tseng K.A.-S., A model for investigating developmental eye repair in Xenopus laevis , 38–47

This new developmental model for eye repair now enables a detailed examination of how regenerative RPC proliferation can drive multi-tissue eye regrowth. Within the first 24 h after surgical ablation, there was a significant increase in proliferation in the regrowing eye but not in sham-operated eyes (Kha et al., 2018). This result showed that productive regrowth requires multipotent RPC proliferation after injury. Moreover, regenerative RPC proliferation was specific for regrowth and not due solely to injury. Another interesting finding is that the eye

regrowth observed after st. 27 ablation is not due to the retinal stem cells in the ciliary margin zone (CMZ) as these cells are only present much later at st. 35 (Straznicky and Gaze, 1971). Together, the results indicated it is most likely the embryonic RPCs that regulate eye regrowth.

Here, we provided detailed methods to study in vivo RPC proliferation in the context of *Xenopus* embryonic eye regrowth, including embryo culture, ablation surgery, and functional approaches to define cellular and molecular mechanisms that regulate this process. We have successfully used this model to identify apoptosis (programmed cell death) as a regeneration-specific mechanism that is required for eye regrowth (Kha et al., 2018).

In summary, the *Xenopus* developmental eye repair model described here represents a new and robust platform to interrogate in vivo retinal progenitor cell proliferation in a model vertebrate. It will enable rapid progress in distinguishing between developmental and regenerative eye mechanisms, facilitate new approaches toward stimulating RPC proliferation in vivo, and provide opportunities for translating these findings toward identifying suitable populations of stem cells for eye repair and promoting mammalian RPC proliferation in vitro and in vivo.

2.4 Materials

Instruments and Dissecting Tools

1. A dissecting stereo microscope

2. Two pairs of surgical forceps, No. 5 (Dumont)

3. Two pairs of AA-style forceps

4. Transfer pipets, disposable, 7.5 mL

5. Plastic Petri dishes, 60 mm × 15 mm

6. Plastic Petri dishes, 100 mm × 15 mm

7. Delicate task wipers (Kimwipes)

8. Vibratome, Leica VT1000 S or similar

General Solutions

1. 70% Ethanol in deionized water.

2. 0.1× Marc's Modified Ringer (MMR) medium: 0.1 M NaCl, 2.0 mM KCl, 1 mM MgSO4, 2 mM CaCl2, 5 mM HEPES, and pH 7.8.

3. 1% Agarose (Sigma-Aldrich) solution dissolved in 0.1× MMR. Heat to dissolve.

4. MEMFA fixative medium: 100 mM MOPS (pH 7.4), 2 mM EGTA, 1 mM MgSO4, and 3.7% (v/v) formaldehyde (Sive et al., 2000).

5. Dejellying solution: 3% cysteine solution in deionized water and pH 7.8.

6. 4% Low-melt agarose (VWR) solution dissolved in 0.1× MMR. Heat to dissolve.

Solutions for Eye Tissue Removal Surgery

1. 5% Tricaine methanesulfonate (MS222, Sigma-Aldrich): dissolved in deionized water and stored at 4 °C.

2.5 Methods

Embryo Culture

1. For general *Xenopus laevis* care, induction, and fertilization of embryos, follow published protocols.

2. A protective layer of jelly surrounds the eggs. The jelly is removed from the embryos after fertilization using a 3% cysteine dejellying solution (Sive et al., 2007). After dejellying is completed, wash the embryos several times with 0.1× MMR to completely remove the cysteine solution. Fill a 100 mm × 15 mm Petri dish with 0.1× MMR (30–35 mL). Use a transfer pipet to transfer 60–80 embryos into the dish (see Note 1).

3. Culture embryos to the desired stage. Embryos are staged using the Nieuwkoop and Faber developmental staging series (Nieuwkoop and Faber, 1994) and can be grown in temperatures ranging from 14 to 25 ℃. The rate of development is dependent on the culture temperature and embryo density. Here are general guidelines for developmental timeframe: one-cell embryos develop into st. 27 embryos in ~1.5 days at 22–25 ℃, ~2 days at 18 ℃, and ~3 days at 14 ℃ (see Note 2).

4. Monitor the growth of embryos daily. Use AA-style forceps to move and examine embryos under a stereo microscope. It is critical to maintain clean and healthy cultures. Use a transfer pipette to remove any dead embryos. Medium should remain clear. Replace cloudy medium with fresh 0.1× MMR as needed.

Preparations for Surgery

1. Set up a clean work area for surgery by wiping all surfaces with 70% ethanol, including the dissection microscope stage and surgical forceps. Spray 70% ethanol onto Kimwipes, and use the wipes to gently clean forceps tips.

2. An agarose-lined dish can be used as an aid to hold embryos in place for surgery (see Note 3). First, dissolve 1% agarose in 0.1× MMR using a microwave. Allow the solution to slightly cool before pouring the solution into a 60 mm × 15 mm Petri dish until the bottom is fully covered (~10 mL). After the agarose has solidified and cooled to room temperature, create an indentation using the tip of a 200 μL pipette tip to create a well in the agarose wide enough to hold the embryos in place. Next, fill the dish with 10–15 mL of 0.1× MMR. Add in one to two drops of 5% MS222 with a disposable transfer pipet, and then gently swirl the plate to mix to reach a final concentration of 0.02% (see Note 4).

3. Set aside two additional 60 mm × 15 mm Petri dishes for washing the animals out of the anesthetics used. Fill each dish with 10–15 mL of 0.1× MMR.

4. Fill one 100 mm × 15 mm Petri dish with 30–35 mL of 0.1× MMR to use as a culture plate for animals after surgery.

Eye Ablation Surgery

1. We have carefully studied the eye regrowth process at st. 27 tailbud embryo and observed robust retinal progenitor proliferation (Kha et al., 2018; see Note 5). Tailbud embryos at st. 27 can be identified by examining their external morphology (as described by Nieuwkoop and Faber, 1994). Our studies showed that eye regrowth ability is lost after st. 32 (Kha and Tseng, 2018).

2. To anesthetize the embryos, use a transfer pipette to gently transfer five to ten tailbud embryos to the 1% agarose dish containing 0.02% tricaine in 0.1× MMR. The embryos should become unresponsive within a few minutes.

3. Place the dish containing embryos underneath a stereo microscope to visualize the embryos and to perform surgical procedures.

4. A transparent vitelline membrane surrounds the tailbud embryo. This membrane needs to be removed prior to surgery to allow direct access to the eyes (Sive et al., 2007). To remove the vitelline membrane, first use a pair of No. 5 forceps to pinch the membrane in the middle posterior region of the embryo while holding the embryo in place. With a second pair of No. 5 surgical forceps, pinch the membrane at a location adjacent to the first pair. While holding the membrane with both pairs of forceps, pull the forceps in opposite directions to gently break apart the membrane and release the tailbud embryo (Fig. 2.2a). Allow the embryos 10–15 min to gradually straighten (Fig. 2.2b) out prior to beginning the next steps.

5. Place the embryos into the indentation(s) made in the agarose plate. Using a pair of AA-style forceps, orient the embryos laterally with the same side (either right or left) facing upward (see Note 6).

6. In st. 27 tailbud embryos, the eye is easily identified at the head region as it protrudes out. At this stage, the embryonic eye contains the differentiating lens placode and an eye cup, with retinogenesis having started at st. 24. Use a pair of sharp No. 5 surgical forceps to make an initial surgical incision into the eye. This can be done by angling the forceps tips to make a small incision at the protruding edge of the eye (Fig. 2.2c). At the same time, a second pair of forceps can be used to brace the body of the animal during surgery (see Note 7).

7. After the initial cut, some eye tissues will bulge out slightly (Fig. 2.2c, d). Using the sharp tips of the forceps, continue cutting around the outline of the eye until the protruding tissues are completely excised and removed from the embryo (Fig. 2.2e–g; see Note 8).

8. After surgery, allow the embryo to recover in 0.1× MMR for 3–5 min. Remove the embryo from the tricaine solution by gently transferring the operated embryo to a Petri dish containing 0.1× MMR using a transfer pipet (see Note 9).

9. Perform a second wash by transferring the operated tailbud embryos to a second Petri dish containing 0.1× MMR. Maintain animals in 0.1× MMR at all times. It is important to minimize the amount of solution transferred between dishes to avoid transferring residual tricaine during the wash steps.

10. After the second wash, transfer the operated tailbud embryo to the culture plate. Observe embryos for normal wound closing at the surgery site (2–3 h). Culture the embryos in a 22 ℃ incubator for 1–5 days as needed.

11. For individual experiments, generally, 20–30 embryos are needed. Set aside a similar number of age-matched unoperated embryos to serve as controls.

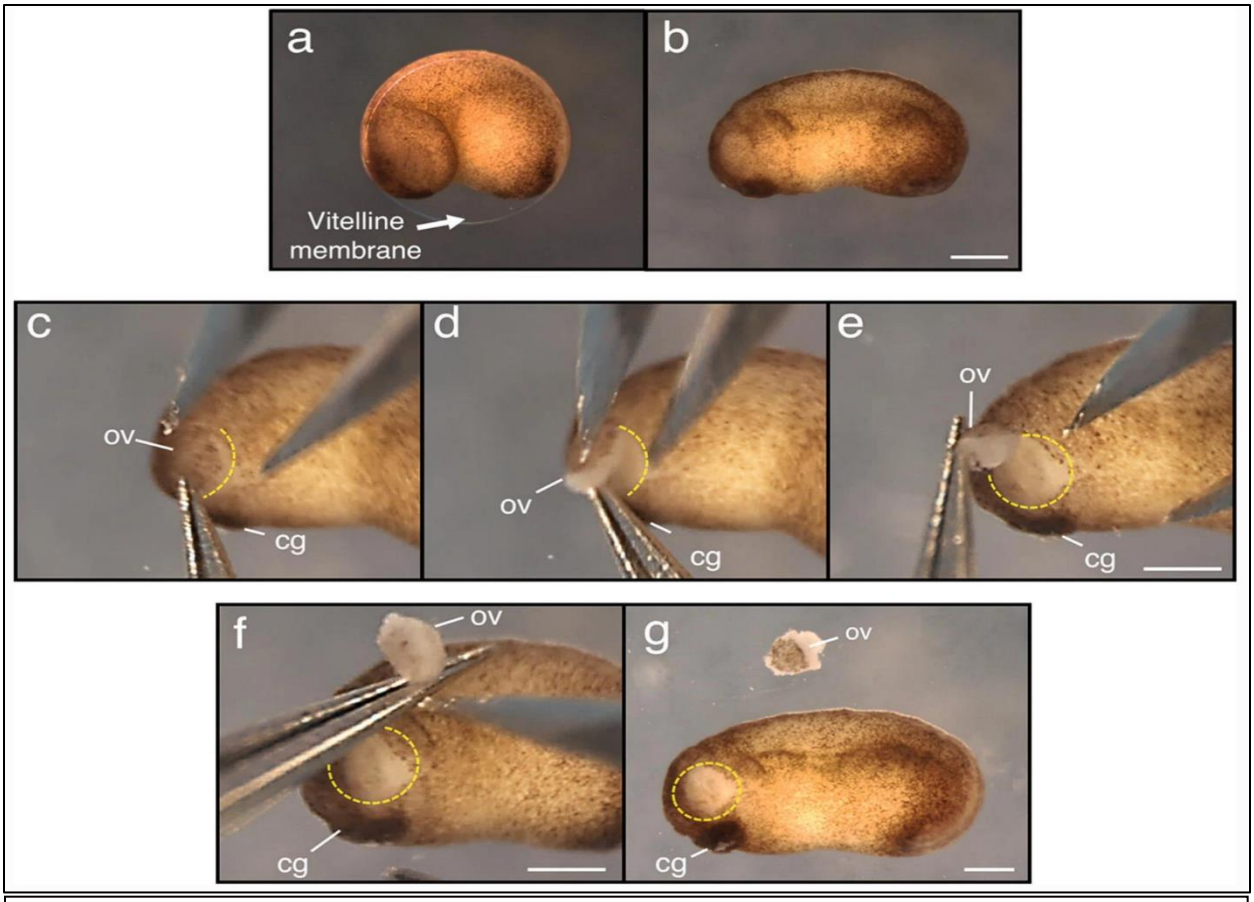


Figure 2.2 Key steps in the eye ablation protocol. (a) The vitelline membrane (indicated by an arrow) is translucent and encases the tailbud embryo. (b) Removal of the vitelline membrane enables the embryo to straighten and allows access to the eye. (c–g) Images showing eye ablation surgery. (c) An initial cut is made using sharp forceps. (d) The cut is continued around the outline of the eye. (e–g) The eye tissues protrude during the surgery, and the tissues can be removed as one intact embryonic eye. ov optic vesicle, cg cement gland. Up = dorsal, down = ventral, left = anterior, right = posterior. Scale bars: (a–g) = 500 μm

Assessment of Eye Tissue Removal

1. Assessment of eye surgery can be performed using a combination of tissue sectioning and immunofluorescence microscopy. To quantify the amount of tissue removed by surgery, first fix operated embryos after surgery in MEMFA for 1–3 h at room temperature or overnight at 4 °C. Embed fixed embryos in 4% low-melt agarose, and generate sections through the eye region using a vibratome as described in (Blackiston et al., 2010).

2. For each tailbud embryo, generate three to four transverse sections of 50 μm thickness through the surgery site. Immunostain sections with primary antibodies to identify eye tissues (Figs. 2.3a and 2.4a, b; Table 2.1). The pan-neural marker, Xen1, identifies neural tissues, including the eye cup (Kha et al., 2018). The basement membrane surrounding the eye can be visualized using an anti-laminin antibody (Kha et al., 2018). To assess the extent of the surgical ablation, obtain digital images of eye sections (Kha et al., 2018; see Note 10). Select the section containing the largest amount of remnant eye tissue (as labeled by the Xen1 antibody). Measure the area of the remnant eye tissue and the contralateral unoperated control individually to calculate the percentage of eye tissue ablated (Fig. 2.3a).

3. If the eye surgery is performed correctly, the embryonic eye tissues are removed without damage to the surrounding neural and mesodermal tissues (Fig. 2.3a). We consistently remove ~83% of the embryonic eye tissue and observe full eye regrowth by 5 days post surgery (Kha et al., 2018; see Note 11).

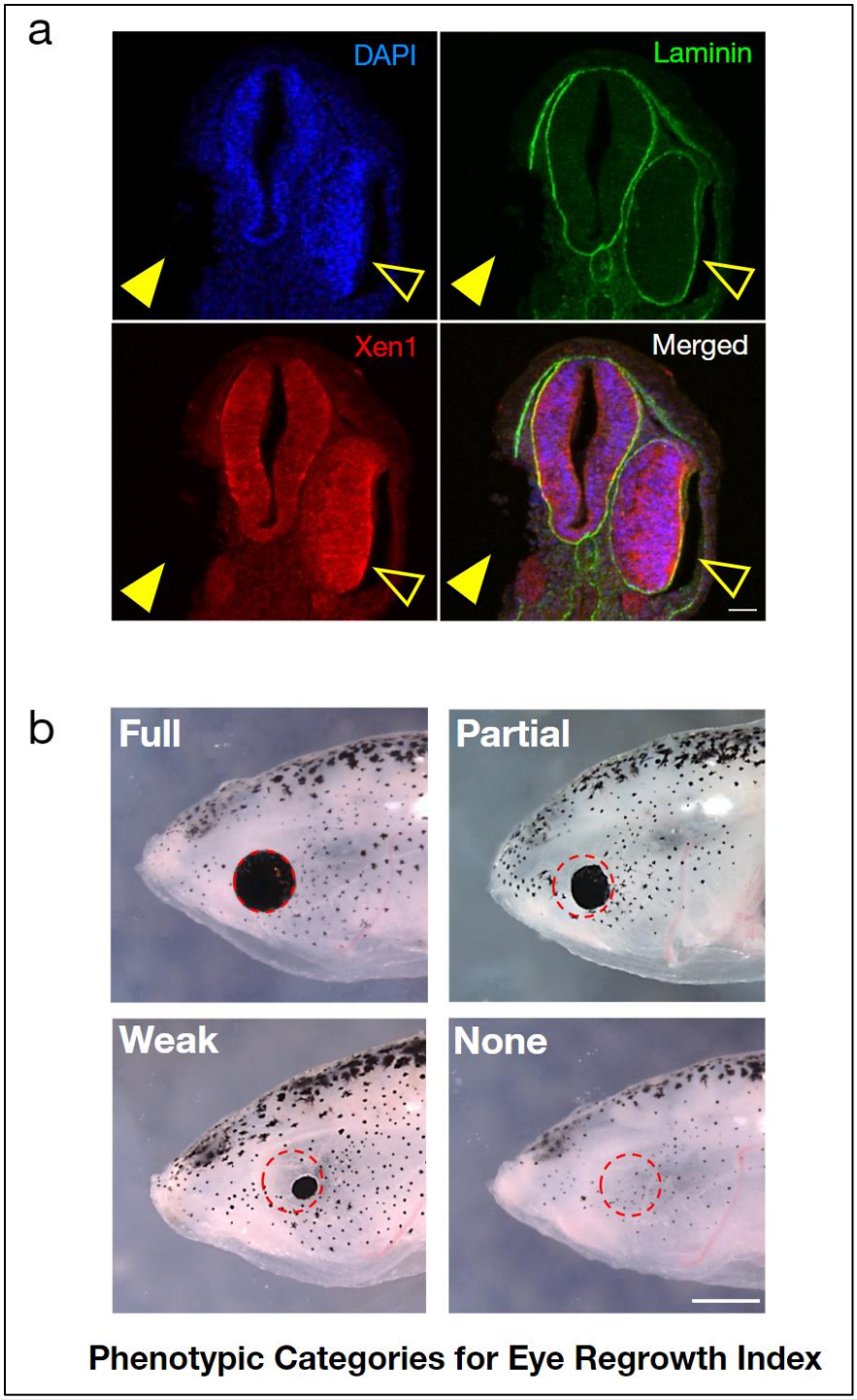


Figure 2.3. Assessment of eye ablation and eye regrowth. (a) Shown are representative images after surgery to quantify the extent of tissue removal. Images are immunostained, transverse sections through the eye of a st. 27 tailbud embryo after surgery. Closed arrowheads indicate surgery site; open arrowheads indicate unoperated eye. Blue color indicates nuclear staining (DAPI). Green color indicates the basal lamina (anti-lamina), and outlines the optic vesicle. Red color indicates neural tissues (Xen1). (b) Representative images of a regrown eye following 5 days post surgery. Each regrown eye was scored based on four phenotype categories. Full = eye of appropriate size with lens. Partial = eye with minor abnormalities and comparably smaller. Weak = eye tissues with abnormal and/or absence of most eye structures. None = no visible eye tissues. (a) Up = dorsal, down = ventral. (b) Up = dorsal, down = ventral, left = anterior, right = posterior. Scale bar: (a) = 25 µm, (b) = 300 µm (reprinted from Experimental Eye Research, 169/April, 2018, Kha, C.X., Son, P.H., Lauper, J., and Tseng K.A.-S., A model for investigating developmental eye repair in Xenopus laevis , 38–47.

Quantification of Eye Regrowth Quality

1. To enable the comparison of the quality of eye regrowth between different groups of embryos, a Regrowth Index (RI) was established (Kha et al., 2018) (Fig. 2.3b). The RI is based on four phenotype categories: (1) Full, a fully regrown eye with lens that is comparable in size and external morphology to an unoperated age-matched sibling; (2) Partial, a regrown eye with minor abnormalities and a visible reduction in eye size; (3) Weak, a regrown eye with no lens and severely reduced in size or a malformed regrown eye with most normal structures missing; (4) and None, no visible tissue regrowth of the ablated eye.

2. The phenotypic scoring for each regrown eye is normally performed at 5 days post surgery (dps), when embryos have reached the tadpole stage (the *Xenopus* eye is considered to be mature by st. 42 as it contains all the structures found in an adult eye). Anesthetize tadpoles in 0.1× MMR containing 0.02% tricaine. Examine each regrown eye, and assign the appropriate phenotype category.

3. To calculate the RI for a group of tadpoles, the following formula is used:

100×{[3×(number of full regrown eyes)]+[2×(number of partial regrown eyes)]+[1×(number of weak regrown eyes)]}/(total number of tadpoles)

The RI is a value ranging from 0 to 300. A value of 0 denotes no eye tissue regrowth in any individual, and a value of 300 denotes full regrowth of eye tissues in 100% of individuals in a group. Following this protocol, eye regrowth in st. 27 tailbud embryos consistently generates RI values between 280 and 290.

Molecular and Cellular Approaches to Understand RPC Proliferation

1. Retinal progenitor proliferation is required for functional eye regrowth (Kha et al., 2018). The RI can be used to assess regrowth outcomes from loss- or gain-of-function molecular studies. We utilized this method to discover a required role for apoptosis during embryonic eye regrowth (Kha et al., 2018).

2. Operated embryos can be treated with specific chemical inhibitors dissolved in 0.1× MMR to assess the effects of inhibition on eye regrowth (Fig. 2.4: compare 2.4d (DMSO control) to 2.4e (treatment with MG132)) (Moody, 1987; Tomlinson et al., 2005). Molecular inhibition using gene-specific morpholinos can be achieved with targeted injections during early embryonic stages to restrict expression of the morpholino to the eye (Fig. 2.4c shows GFP mRNA injection as an example) (Moody, 1987; 2018). When used in combination, chemical and molecular inhibition approaches represent a robust platform to identify and define mechanisms that are required for retinal progenitor proliferation in vivo during productive eye repair.

3. Gain-of-function molecular studies can be performed by microinjections of target mRNAs into embryonic cells fated to become eye tissues to induce gene overexpression (Fig. 2.4c).

4. To assess gene expression during eye regrowth, follow published protocols using either RNA in situ hybridization of immunohistochemistry (Monsoro-Burq, 2007; see Note 12).

2.6 Notes

1. It is common to culture Xenopus laevis embryos in the antibiotic gentamicin (Elsner et al., 2000). However, we have found that embryos can develop healthily without gentamicin. In this case, the embryos are monitored daily, and the culture medium is changed as needed.

2. Xenopus laevis developmental stage series is available online at Xenbase (http://www.xenbase.org/anatomy/alldev.do) (James-Zorn et al., 2018). The developmental time

periods listed are approximate. Embryo density also affects developmental timing. Embryo crowding (>100 embryos per 100 × 15 mm dish) tends to delay development.

3. It is recommended to perform eye surgeries using a Petri dish lined with 1% agarose, especially for beginners. The indentations created in the agarose help to hold embryos in place and restrict movement during the procedures. Please note that the tips of No. 5 surgical forceps are sharp, delicate, and easily dented/broken, especially if they make contact with the plastic bottom of Petri dishes. The agarose plate also acts as a soft surface that protects the fine tips of surgical forceps. In general, specific care should be taken to prevent damage to the tips of the forceps. Damaged tips can be re-sharpened using sharpening tools.

4. To anesthetize embryos, use 0.01–0.03% tricaine (final concentration) in 0.1× MMR. A drop of liquid using a transfer pipet is ~50 μL. Avoid incubating embryos in tricaine for >10 min.

5. Within an individual culture plate, natural differences in growth rates will result in embryos that are in a range of developmental stages. Embryos at st. 27 can be identified by the formation of a translucent fin along the dorsal and posterior of the embryo (Nieuwkoop and Faber, 1994). For surgery, make sure to only select healthy tailbud embryos without developmental defects.

6. Our results indicate that there are no observable differences in the eye regrowth process between the right and left eyes (unpublished data). However, eye surgery should be performed on the same side for all embryos in an experiment to maintain experimental consistency. Anesthetized tailbud embryos are mostly stationary. However, tailbud embryos are capable of lateral movement due to the presence of epidermal cilia on the body of the embryo. Tricaine does not inhibit ciliary movement; therefore, occasional embryonic lateral movement may occur.

7. All surgical procedures should be performed with clean surgical forceps. Remaining tissues on the forceps should be cleaned off in-between surgeries to avoid contamination.

8. The initial incision will result in a protrusion of the eye tissues. Do not dig deep into the wound site when continuing the cut around the outline of the eye cup. This may result in damage to the optic stalk and underlying brain structures.

9. Always keep the embryos submerged in the MMR solution. If the open wound of an embryo is exposed to the air-water interface, it will break open the embryo.

10. Quantitative analyses of our surgeries showed that on average, ~83% of eye tissues were consistently removed [28]. About 40% of operated embryos have less than 10% of eye tissues remaining in the embryo.

11. If embryo sections show that surrounding tissues (especially the neural tissues) are damaged during surgery, then adjust surgical excision technique by decreasing the depth of the forceps incision in the eye.

12. Xenbase (xenbase.org) contains information on commercial antibodies that have been successfully used in *Xenopus laevis*.

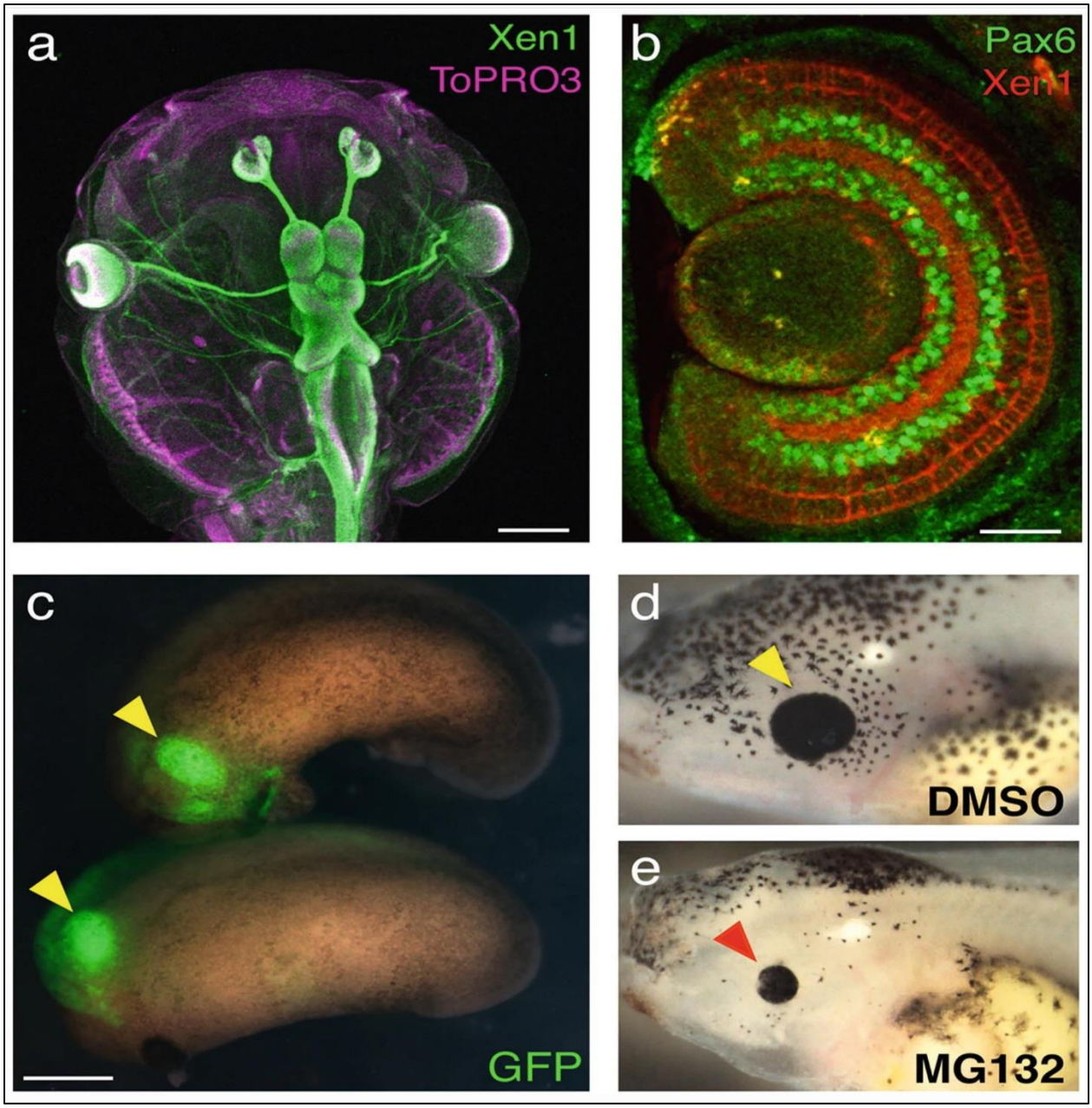


Figure 2.4. Methods to study eye regrowth and RPC proliferation. (a) Whole-mount immunostain of a st. 46 tadpole. Green color indicates neural tissues (Xen1). Magenta color indicates nuclear signal (TO-PRO-3). (b) Vibratome-generated transverse eye section showing a st. 40–41 eye immunostained with anti-Pax6 and Xen1 antibodies. Green color indicates retinal cells in the ganglion and inner nuclear layers (anti-Pax6). Red color indicates neural tissues (Xen1). (c) Targeted microinjection of GFP mRNA into the dorsal blastomere of four-cell embryos resulted in high expression of GFP in the eye region by st. 22. (d, e) Chemical inhibitor treatment of embryos with a (d) DMSO-vehicle control and (e) MG132, a cell-permeable proteasome inhibitor. (a) Up = anterior, down = posterior. (b) Up = dorsal, down = ventral. (c–e) Up = dorsal, down = ventral, left = anterior, right = posterior. Scale bars: (a and c) = 500 μm and (b) = 50 μm

Target	Antigen	Source	Suggested Dilution
Cones	Calbindin	Millipore Sigma, AB1778	1:500
Rods	Rhodopsin	Millipore Sigma, MABN15	1:200
Rods	Rhodopsin	RetP1, Biomedia	1:50
Amacrine, horizontal, bipolar, and ganglion cells	Islet-1	DSHB, AB 528315	1:200
Horizontal cells	Prox1	AB 37128	1:400
Ganglion, neurofilament	3A10	DSHB, AB 531874	1:100
Pan-neural	Xen1	DSHB	1:50
Müller glia	Vimentin	DSHB, AB 528507	1:10
Müller glia	Glutamine Synthetase	Millipore Sigma, G2781	1:500
RPE	RPE65	Millipore Sigma, Q16518	1:250
Pax6	Pax6	BioLegend, 901302	1:500
Basement membrane	Laminin	Millipore Sigma, L9393	1:300
Cleaved caspase-3	Cleaved caspase-3	Cell Signaling Technology, 9661	1:300
H3P	H3P	Millipore Sigma, H0412	1:500

Table 2.1 Published antibodies for *Xenopus* eye

CHAPTER 3

USING THE XENOPUS DEVELOPMENTAL EYE REGROWTH SYSTEM TO DISTINGUISH THE ROLE OF DEVELOPMENTAL VERSUS REGENERATIVE MECHANISMS

3.1 Preface

This chapter was published in Frontiers in Physiology as:

Kha, C. X.*, Guerin, D. J.*, & Tseng, K. A. (2019). Using the Xenopus Developmental Eye Regrowth System to Distinguish the Role of Developmental Versus Regenerative Mechanisms. Frontiers in physiology, 10, 502.

*Authors contributed equally to this work.

Author Contributions: Cindy Kha and I both wrote an equal amount for this paper, with Cindy contributing the Pax6 sections entirely. Sections and staining were performed by me, with Cindy performing the imaging. Cindy and I contributed equally to figure design. Kelly Tseng conceived the project, oversaw the experiments, and provided the main text for the publication.

Editorial note: The manuscript is reproduced here under Frontiers Creative Commons license. Changes were made to the figure numbers to fit with the overall formatting of the dissertation.

3.2 Abstract

A longstanding challenge in regeneration biology is to understand the role of developmental mechanisms in restoring lost or damaged tissues and organs. As these body structures were built during embryogenesis, it is not surprising that a number of developmental mechanisms are also active during regeneration. However, it remains unclear whether

developmental mechanisms act similarly or differently during regeneration as compared to development. Since regeneration is studied in the context of mature, differentiated tissues, it is difficult to evaluate comparative studies with developmental processes due to the latter's highly proliferative environment. We have taken a more direct approach to study regeneration in a developmental context (regrowth). Xenopus laevis, the African clawed frog, is a well-established model for both embryology and regeneration studies, especially for the eye. Xenopus eye development is well-defined. Xenopus is also an established model for retinal and lens regeneration studies. Previously, we demonstrated that Xenopus tailbud embryo can successfully regrow a functional eye that is morphologically indistinguishable from an age-matched control eye. In this study, we assessed the temporal regulation of retinal differentiation and patterning restoration during eye regrowth. Our findings showed that during regrowth, cellular patterning and retinal layer formation was delayed by approximately 1 day but was restored by 3 days when compared to eye development. An assessment of the differentiation of ganglion cells, photoreceptor cells, and Müller glia indicated that the retinal birth order generated during regrowth was consistent with that observed for eye development. Thus, retina differentiation and patterning during regrowth is similar to endogenous eye development. We used this eye regrowth model to assess the role of known mechanisms in development versus regrowth. Loss-of-function studies showed that Pax6 was required for both eye development and regrowth whereas apoptosis was only required for regrowth. Together, these results revealed that the mechanisms required for both development and regrowth can be distinguished from regrowth-specific ones. Our study highlights this developmental model of eye regrowth as a robust platform to systematically and efficiently define the molecular mechanisms that are required for regeneration versus development.

3.3 Introduction

Many animals have the ability to undergo regeneration, the successful restoration of tissues and organs after injury, but some animals lack this ability. Even though there is now considerable knowledge regarding the cellular and molecular pathways that regulate regeneration, the basic question of why the same tissues and organs from diverse (or even closely related) species often respond differently to injury and damage remains largely unanswered. To address this question, an area of focus has been to understand the role of developmental mechanisms in regeneration.

As regeneration requires the restoration of lost body structures generated during development, it is not surprising that a number of pathways involved in development are also active during regeneration (Schaefer et al., 1999; Lin and Slack, 2008; Malloch et al., 2009; Martinez-De Luna et al., 2011; Halasi et al., 2012; Meyers et al., 2012). However, it has been a challenge to effectively identify which developmental mechanisms are required for regeneration and to assess whether the roles of these mechanisms are similar or different during embryogenesis versus regeneration.

A second challenge in understanding the role of developmental mechanisms in regeneration is that existing models largely seek to examine regeneration in adult or mature differentiated tissues. The mature tissues are in contrast to a developmental environment where proliferation is high and cellular differentiation is low or just beginning. Furthermore, recent studies indicate that stem cells may have different functions in developing versus adult tissues (Wang and Conboy, 2010). Thus, it remains difficult to pinpoint the developmental mechanisms that can be successfully manipulated for inducing adult regeneration.

To address these challenges, a model to study regenerative mechanisms in the context of development is needed. This approach can reduce some of the complexities in comparing developmental processes to regenerative processes in mature tissues. For such a model to be valuable, two important characteristics would be needed: a high regenerative ability coupled with well-understood developmental events. *Xenopus laevis*, the South African clawed frog, fulfills these criterion as it is an animal that is an established and well-studied regenerative and developmental model (Beck et al., 2009; Sater and Moody, 2017). In particular, *Xenopus* eye development has been studied extensively (Perron and Harris, 1999; Rapaport, 2006; Henry et al., 2008; Viczian and Zuber, 2015). *Xenopus* can also regenerate mature eye tissues including the retina and lens [reviewed in Araki (2007), Vergara and Del Rio-Tsonis (2009), Henry et al. (2013), Tseng (2017)]. Additional advantages of the *Xenopus* system include: external development of embryos– facilitating developmental eye studies, amenability to molecular and cellular manipulations, and strong genetic similarity to humans. Using *Xenopus*, we established an embryonic model to study developmental eye regrowth (defined here as the ability of an embryo to compensate for missing tissues by restoring normal organ structures and function) (Kha and Tseng, 2018; Kha et al., 2018).

Our recent study showed that the *Xenopus* tailbud embryo at developmental stage (st.) 27 successfully regrew its eye after significant tissue loss (Kha et al., 2018). The completion of eye regrowth occurred by 4–5 days as overall development progressed without delay. Importantly, the regrown eye was age and size-appropriate with the expected complement of structures including the lens, retina, and pigmented epithelium. It was connected to the brain via the optic nerve and functional, displaying visual preference. Furthermore, the function of the regrown eye was dependent upon successful growth of new tissues since remnant eye cells in the regrowth-

inhibited eyes lacked the ability to restore visual function (Kha and Tseng, 2018). To facilitate the use of this model to understand the role of developmental mechanisms in regrowth, we sought to determine whether eye formation during regrowth is comparable to endogenous eye development. Here, we show that while induction of regrowth delayed retinal differentiation and patterning, the overall retinogenesis process was consistent with a recapitulation of normal eye development. Furthermore, loss-of-function studies using our model showed that Pax6, a gene that is required for eye development, is also required for regrowth. In contrast, apoptosis is not required for eye development but is required for regrowth.

3.4 Results

Restoration of Cellular Patterning During Regrowth

In our previous study, histological analyses showed that retinal layer formation in a regrowing eye was delayed during the first 2 days post surgery (dps) even though overall development proceeded normally (Kha et al., 2018). The cellular patterning of the regrowing eye during this period was more similar to embryos at younger developmental stages. Notably, the regrowing eye regained overall size and cellular patterning comparable to an uninjured age-matched eye within 3–5 days post surgery (Kha et al., 2018). To better understand eye regrowth and assess this process as compared to normal eye development, we examined the temporal regulation of eye formation during regrowth at three successive 24-h timepoints. First, we assessed the overall cellular structure and patterning of the regrowing eye as compared to its uninjured contralateral eye. Here, we used the contralateral eye as the control to ensure that the comparative studies were made at the same developmental stages. Our previous work confirmed that the uninjured contralateral control was equivalent to the eye of age-matched sibling embryos [(Kha et al., 2018) and data not shown]. The lens and retina of the developing eye are surrounded

by the basement membrane found in the extracellular matrix. To examine the basement membrane structure of the embryonic eye, a marker recognizing the basement membrane (an anti-Laminin antibody) was used (Kha et al., 2018). At st. 34/35 in the control embryonic eye, the basement membrane outlined the eye cup and the lens vesicle as it proceeds through development (Fig 3.1A4–6,A4'–6'). Induction of eye regrowth required tissue removal surgery, which also disrupted the basement membrane and showed lack of laminin expression (Kha et al., 2018). At 1 dps (st. 34/35), the basement membrane structure was restored as it surrounded the regrowing eye entirely. Similar to the control eye, the basement membrane surrounding the regrowing eye was maintained through to st. 42/43 as normal size is restored (Fig 3.1B4–6,B4–6').

The Xen1 antibody recognizes neural tissues in the Xenopus embryo and is a reliable marker for visualizing retinal layers in the developing eye (Ruiz i Altaba, 1992; Kha et al., 2018). During *Xenopus* eye development, retinal layer formation begins at st. 33/34 and is completed by st. 41 (Holt et al., 1988). Consistent with previous studies, Xen1 expression showed that at st. 34/35, retinal layering was visible in the developing eye but not fully organized. Proper patterned retinal layers are seen by st. 40/41 (Fig 3.1A8, A8'). In contrast, a delay is observed during regrowth as Xen1 expression in the regrowing eye at st. 34/35 (1 dps) showed a lack of organization (Fig 3.1B7,B7'). By st. 40/41, the patterning in the regrowing eye is more similar to that of a younger control eye at st. 34/35 (compare Fig 3.1B8,B8' with Fig 3.1A7, A7'). The retinal layer patterning in the regrowing eye was restored by 3 dps (st. 42/43) (Fig 3.1B9, B9'). Together, our data indicated that the basement membrane of the regrowing eye was fully restored by 1 dps, whereas retinal layer formation was delayed and then restored by 3 dps.

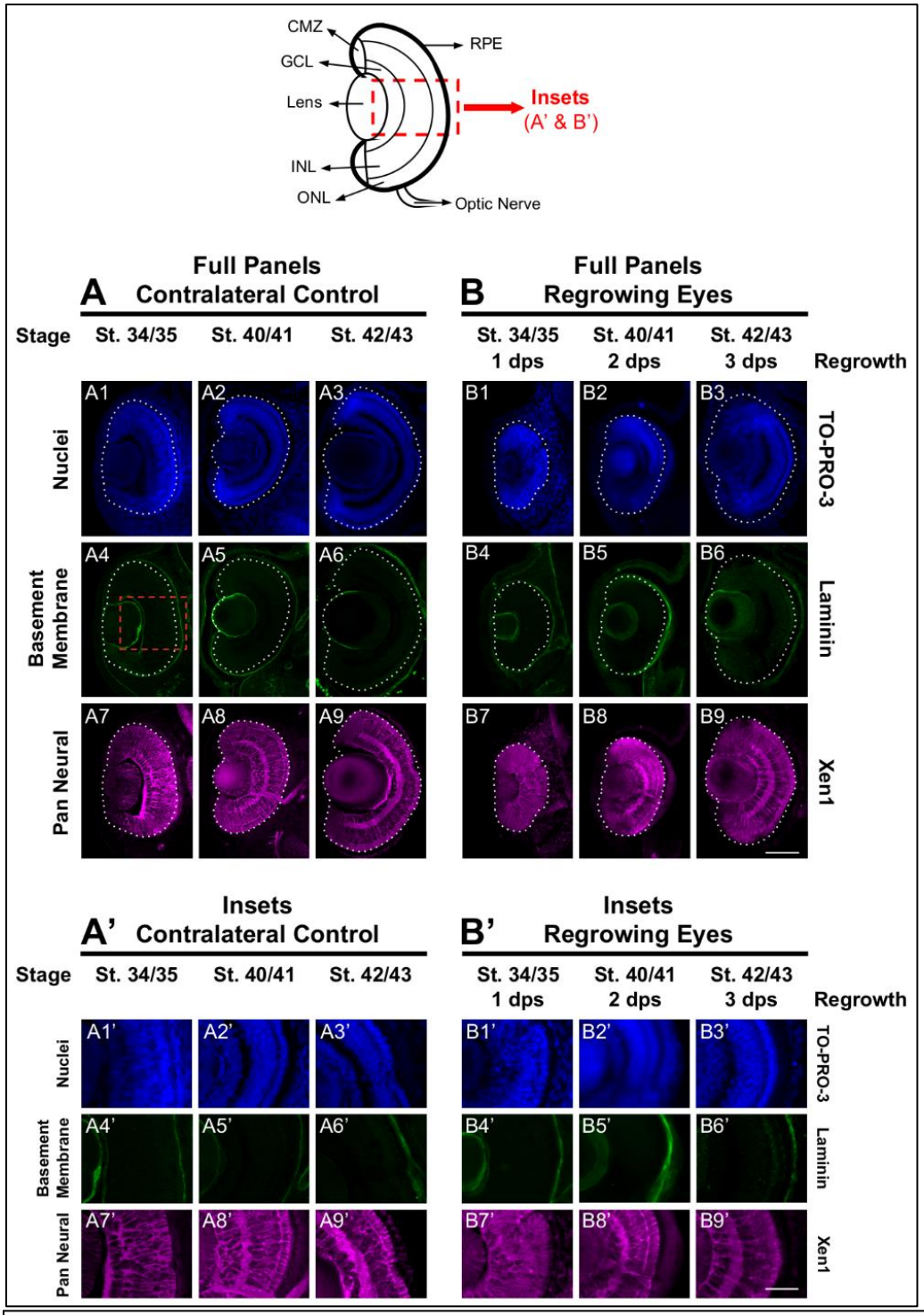


Figure 3.1 Regrown eyes regain cellular patterning by 3 dps. Images shown are immunostained, transverse sections at three developmental timepoints corresponding to 1, 2, and 3 days post surgery (dps). The top schematic is a diagram of a section through a mature, differentiated tadpole eye. (A,B) Regrowing eyes display retinal patterning comparable to the contralateral control eyes (unoperated) by 3 days. White dashed lines delineate each eye. (A',B') Representative images shown in panels A' and B' correspond to the region shown in the inset box in panel A4 for the corresponding A or B panel at high magnification. Blue color indicates nuclear staining (TO-PRO-3). Green color indicates the basal lamina (anti-Laminin), which is expressed in all basement membranes and outlines the optic vesicle. Magenta color indicates neural tissues (Xen1). Sample sizes: 1 day, n = 6; 2 days, n = 5; and 3 days, n = 5. (A,B, A',B') Up = dorsal, down = ventral, lens is on the left. Scale bar: A,B = 100 μm and A',B' = 50 μm.

Restoration of Retinal Differentiation During Regrowth

The mature vertebrate retina is composed of the retinal pigmented epithelium (RPE) and the neural retina. For *Xenopus* eye development, retinal differentiation (retinogenesis) begins at st. 24 at the ventral midline and increasingly spreads toward the periphery along the presumptive retina (Holt et al., 1988). The process is completed by st. 41, when the differentiated structures found in a mature eye are present (Holt et al., 1988). This is a short window representing an overall period of approximately 2 days. The Xen1 expression patterns during regrowth indicated an initial delay in differentiation (Fig 3.1). We thus assessed the formation of the RPE and neural retina during regrowth. To assess RPE differentiation, we used an antibody against RPE65, a protein that is expressed in the mature RPE (Yoshii et al., 2007; Vergara and Del Rio-Tsonis, 2009). During eye development at st. 34/35, RPE65 was first expressed in a short segment extending from the ventral midline (Fig 3.2A4,A4', white dashed lines demarcate the neural retina and lens). It was previously shown that retinal differentiation demonstrated a dorsal bias in maturity – dorsal cells in the central region differentiate slightly earlier than ventral ones (Holt et al., 1988). Indeed, RPE65 expression also showed a dorsal bias (Fig 3.2A4). By st. 40/41, RPE65 expression reached both the dorsal and ventral peripheries and remained the same at st. 42/43 (Fig 3.2A5–6,A5'–6'). During regrowth, RPE65 showed a similar expression pattern at 1 dps as the control (albeit larger) eye at the same stage (compare Fig 3.2B4,B4' with Fig 3.2A4,A4'). This observation is consistent with our earlier finding that the black pigment of the RPE is morphologically visible by 1 dps in a regrowing eye (Kha et al., 2018). Unlike a control eye, RPE65 expression in the 2 dps regrowing eye failed to reach the periphery by st. 40/41 (compare Fig 3.2A5 with Fig 3.2B5). An additional day is required for the RPE65 expression to reach the periphery (Fig 3.2B6). Together, the data indicate that RPE differentiation was delayed as

compared to the control eye. However, RPE differentiation was restored by 3 days as the embryo reached the mature eye stage (st. 42/43).

Next, we examined retinal differentiation during regrowth. The neural retina consists of three nuclear layers and two plexiform layers (Fig 3.1: schematic shows the 3 nuclear layers). Photoreceptor cells (rods and cones) are located in the outer nuclear layer (ONL). Bipolar, horizontal, and amacrine cells are found in the inner nuclear layer (INL). The retinal ganglion cells are located in the ganglion cell layer (GCL). The birth order of retinal cell types occur in a consistent yet overlapping temporal order with the retinal ganglion cells (RGCs) being the first to be specified, followed by horizontal cells, cone photoreceptor cells, rod photoreceptor cells, amacrine cells, bipolar cells, and lastly the Müller glial cells (Wong and Rapaport, 2009). Using known antibody markers that identify retinal cell types, we assessed the timing of retinogenesis.

Islet1 is a marker of vertebrate RGCs including *Xenopus* (Dorsky et al., 1997). The Islet1 antibody that we used also identified additional cells in the INL including subsets of amacrine, bipolar, and horizontal cells (Álvarez-Hernán et al., 2013). At st. 34/35, the presumptive GCL was readily apparent and somewhat patterned in the control eye (Fig 3.2A10,A10'). At this stage, a small number of differentiated cells in the presumptive INL showed Islet1 expression. The number of Islet1-positive cells in the INL increased with increasing age (Fig 3.2A10–12,A10'–12'). At 1 dps (st. 34/35) in the regrowing eye, the presumptive RGC layer is apparent but was poorly patterned and remained incomplete at the periphery as compared to the control eye (compare Fig 3.2B10,B10' with Fig 3.2A10,A10'). At 2 dps (st. 40/41), the RGC layer has reached the periphery with some Islet1-positive cells found in the INL but remained less patterned than the same stage control (compare Fig 3.2B11,B11' with Fig 3.2A11,A11'). At 3 dps, the Islet1 expression pattern was largely comparable to the control eye (compare Fig

3.2B12,B12’ with Fig 3.2A12,A12’). Together, the data showed that retinal differentiation was delayed as compared to the control eye. However, retinal differentiation was restored by 3 days as the embryo reached the mature eye stage (st. 42/43).

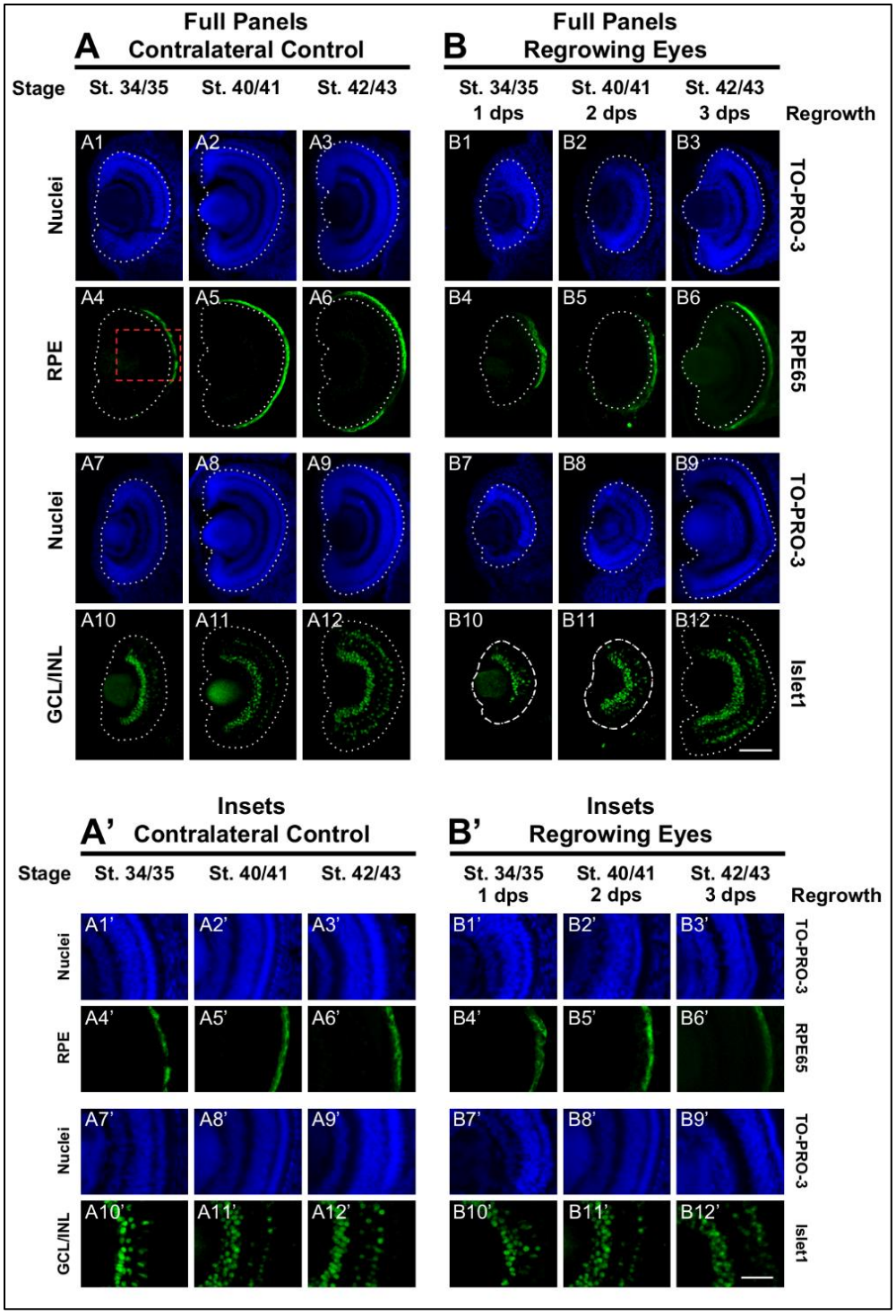


Figure 3.2 Regrown eyes regain retinal differentiation by 3 dps. Images shown are immunostained, transverse sections at three developmental timepoints corresponding to 1, 2, and 3 days post surgery (dps). (A,B) The contralateral control eyes (unoperated) complete retinogenesis by st. 41. By 1 dps, RPE is already visible in the regrowing eye as shown by anti-RPE65 signal (retinal pigmented epithelium; green). By 3 dps, Islet1 expression (identifying subpopulations of retinal ganglion cells and subsets of amacrine cells, bipolar cells, and horizontal cells; green) show expected retinal patterning of a mature eye. White dashed lines delineate each eye. (A',B') Images shown in panels A' and B' correspond to the region shown in the inset box in panel A4 for the corresponding (A or B) panel at high magnification. Blue color indicates nuclear staining (TO-PRO-3). Sample sizes: 1 day, n = 5; 2 days, n = 7; and 3 days, n = 6. (A,B, A',B') Up = dorsal, down = ventral, lens is on the left. Scale bar: (A,B) = 100 μm and (A',B') = 50 μm.

Restoration of Cone Photoreceptor Differentiation

To further define the temporal delay in retinal differentiation during regrowth, we used an anti-Calbindin antibody to assess cone photoreceptor differentiation as we had done previously (Kha et al., 2018). In *Xenopus*, both cone and rod photoreceptors are generated at similar times in the middle of the retinal differentiation sequence. However, a close study of retinogenesis indicated that cone photoreceptors are generated just prior to rod photoreceptors and are the 3rd cell type to be specified (Wong and Rapaport, 2009). During eye development at st. 34/35, a few cone photoreceptor cells were detected by calbindin expression in the central region of the presumptive photoreceptor layer (Fig 3.3A4,A4'). By st. 40/41, cone photoreceptor differentiation reached the retinal periphery and appeared to be restored (Fig 3.3A5,A5'). This pattern was maintained in st. 42/43 (Fig 3.3A6,A6'). In contrast, cone photoreceptors were not observed in the regrowing eye at 1 dps (st. 34/35; Fig 3.3B4,B4'). As regrowth proceeded, cone photoreceptor differentiation was visible by 2 dps and showed patterning that is somewhat comparable to age-matched developing eye (compare Fig 3.3B5,B5' with Fig 3.3A5,A5'). By 3 dps, cone photoreceptor cells have expanded along the retina and showed a comparable pattern to the control eye at st. 42/43 (compare Fig 3.3B6,B6' to Fig 3.3A6,A6'). Our results indicated that in the regrowing eye, cone photoreceptor cell differentiation is delayed by 1 day but is restored by 3 days when the embryo reached the mature eye stage (st. 42/43).

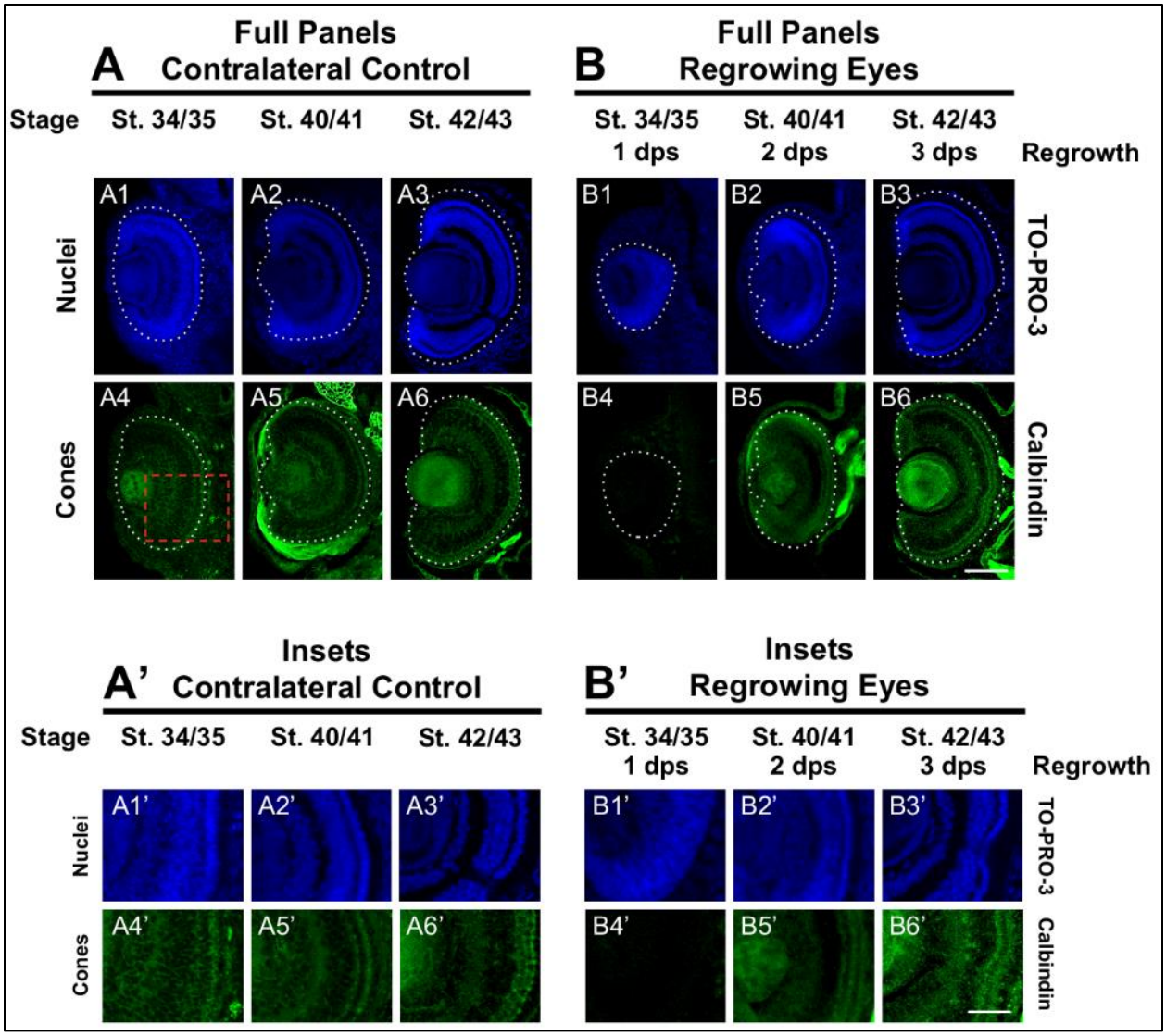


Figure 3.3 Regrown eyes regain cone differentiation by 3 dps. Images shown are immunostained, transverse sections at three developmental timepoints corresponding to 1, 2, and 3 days post surgery (dps). (A,B) Differentiation of cone photoreceptor cells is delayed during 1, 2 dps but regains patterning that is comparable to contralateral control eyes (unoperated) by 3 days. White dashed lines delineate each eye. (A',B') Images shown in panels A' and B' correspond to the region shown in the inset box in panel A4 for the corresponding A or B panel at high magnification. Blue color indicates nuclear staining (TO-PRO-3). Green color indicates anti-Calbindin signal (cone photoreceptors). Sample sizes: 1 day, n = 6; 2 days, n = 5; and 3 days, n = 6. (A,B, A',B') Up = dorsal, down = ventral, lens is on the left. Scale bar: A,B = 100 μm and A',B' = 50 μm.

Restoration of Rod Photoreceptor Differentiation

To further define the temporal delay in retinal differentiation during regrowth, we assessed rod photoreceptor differentiation using anti-Rhodopsin antibody (Kha et al., 2018). Rod

photoreceptor cells are the fourth of seven retinal cell types to be specified (Wong and Rapaport, 2009). At st. 34/35, rod photoreceptor cells were first seen in a short segment extending from the ventral midline (Fig 3.4A4,A4'), reached the periphery by st. 40/41 and maintained at st. 42/43 (Fig 3.4A5,6,A5',6'). In contrast, there were very few rod photoreceptor cells seen in the ventral midline in the regrowing eye at 1 dps (st. 34/35; Fig 3.4B4,B4'). This was in contrast to the formation of GCL, which appeared to be more advanced at the same stage (compare Fig 3.4B4,B4' with Fig 3.2B10,B10'). As regrowth proceeded, rod photoreceptor differentiation expanded along the retina until it showed a similar pattern to the control eye by st. 42/43 (compare Fig 3.4B6,B6' with Fig 3.4A6,A6').

To confirm our observations, we quantitated and compared the number of rod photoreceptor cells during development and regrowth (Fig 3.4C). At 1 dps, there were 28.3 ± 1.8 rod photoreceptor cells in the control eye whereas there were only 4.2 ± 0.8 rod photoreceptor cells in the regrowing eye ($n > 5$ per condition and timepoint, $p < 0.05$). At 2 dps, the number of rod photoreceptor cells in the control eye increased to 67.0 ± 2.7 whereas the number of rod photoreceptor cells in the regrowing eye only reached 31.3 ± 3.8. By 3 dps, there were 69.6 ± 5.4 rod photoreceptor cells in the control eye whereas there was a significant increase in the regrowing eye to 55.5 ± 5.4 rod photoreceptor cells. Measurements of the length of the rod differentiation zone supported the rod photoreceptor cell counts (Fig 3.4D). At 2 dps, the rod differentiation zone was shorter in the regrowing eye as compared to the control eye ($n > 5$ per condition, $p < 0.05$). However, by 3 dps, the rod differentiation zone in the regrowing eye reached comparable length to the uninjured control eye ($n > 6$ per condition, $p = 0.73$). Together, the data showed that rod photoreceptor differentiation and patterning was delayed as compared to the control eye. However, rod photoreceptor differentiation was restored by 3 days as the

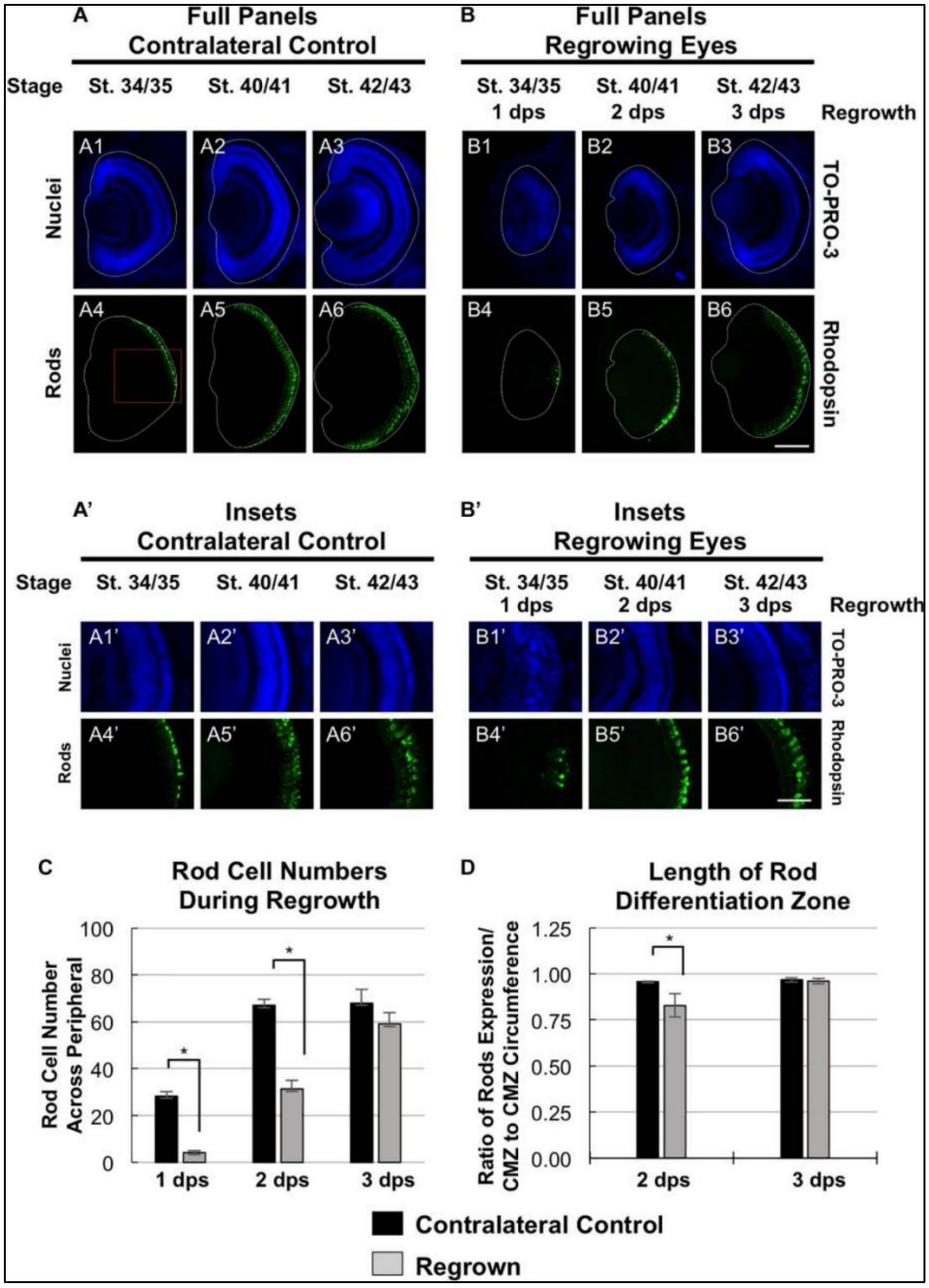


Figure 3.4 Regrown eyes regain rod differentiation by 3 dps. Images shown are immunostained, transverse sections at three developmental timepoints corresponding to 1, 2, and 3 days post surgery (dps). (A,B) Differentiation of rod photoreceptor cells is delayed during 1, 2 dps but regains patterning that is comparable to contralateral control eyes (unoperated) by 3 days. White dashed lines delineate each eye. (A',B') Images shown in panels A' and B' correspond to the region shown in the inset box in panel A4 for the corresponding A or B panel at high magnification. Blue color indicates nuclear staining (TO-PRO-3). Green color indicates anti-Rhodopsin signal (rod photoreceptors). Sample sizes: 1 day, n = 5; 2 days, n = 7; and 3 days, n = 7. (A,B, A',B') Up = dorsal, down = ventral, lens is on the left. Scale bar: A,B = 100 µm and A',B' = 50 µm. (C) Quantification of rod photoreceptor cells in the regrowing eye structure at three developmental timepoints corresponding to 1, 2, and 3 dps. The number of rod photoreceptors per 60 µm section in the regrown eye is comparable to number of rod photoreceptor cells in the contralateral control eyes by 3 dps. ∗denotes $p < 0.05$ ($n > 5$ per timepoint). Data are means ± SEM. (D) Rod photoreceptor cells expression pattern was measured and compared to the overall circumference of the retinal layer from one end of the ciliary margin zone (CMZ) to the end of the opposite CMZ in both regrowing and contralateral eyes. The ratio of rhodopsin expression in the retinal layer over the retinal layer circumference measurements is shown. By 3 dps, the rod photoreceptor cell expression is comparable to the contralateral control eye. ∗denotes $p < 0.05$ ($n > 6$ per timepoint). Data are means ± SEM.

embryo reached the mature eye stage (st. 42/43). Combined, the progress of RGC differentiation at 1 dps as compared to the initial lack of rod photoreceptor differentiation at the same timepoint also suggested that the developmental retinal birth order is maintained during regrowth.

Restoration of Müller Glial Cell Differentiation

In the retina, the Müller glial cells serve as neuronal support cells. They are typically the last retinal cell type to be specified (Holt et al., 1988). Our data on RGC and rod photoreceptor differentiation during regrowth were consistent with the maintenance of the developmental retinal birth order (Fig 3.2, 3.3, 3.4). We hypothesized that if retinal differentiation during regrowth is similar to developmental retinal differentiation, then the cellular patterning of Müller glial cells would be the last to be restored. To test our hypothesis, we used a Müller glial cell marker, an anti-glutamine synthetase antibody, to assess its differentiation pattern as we did previously (Kha et al., 2018). As expected for a cell type that is the last to be specified during retinogenesis, there was no detectable glutamine synthetase expression indicative of Müller glial differentiation in the control eye at st. 34/35 (Fig 3.5A5,A5'). The presence of Müller glial cell patterning was visible by st. 40/41 and full patterning was observed by st. 42/43 (Fig 3.5A6,7,A6',7'). In the regrowing eye, there was also no detectable Müller glial differentiation at st. 34/35 (Fig 3.5B5,B5'). By 2 dps (st. 40/41), only a small number of Müller glial cells were visible – much less when compared to the control eye (compare Fig 3.5B6,B6' with Fig 3.5A6,A6'). By 3 dps (st. 42/43), the pattern in the regrowing eye was similar to that of the pattern observed for st. 40/41 control eye (compare Fig 3.5B7,B7' with Fig 3.5A6,A6'). Müller glial differentiation was restored by st. 45/46 at 4 dps (Fig 3.5B8, B8'). Together, the data showed that Müller glial differentiation was delayed as compared to the control eye. However,

Müller glial differentiation was restored by 4 days – a timepoint that was later than the restoration of patterning observed for other retinal cell types. These findings supported the hypothesis that Müller glial cells are specified later than other retinal cell types in the regrowing eye.

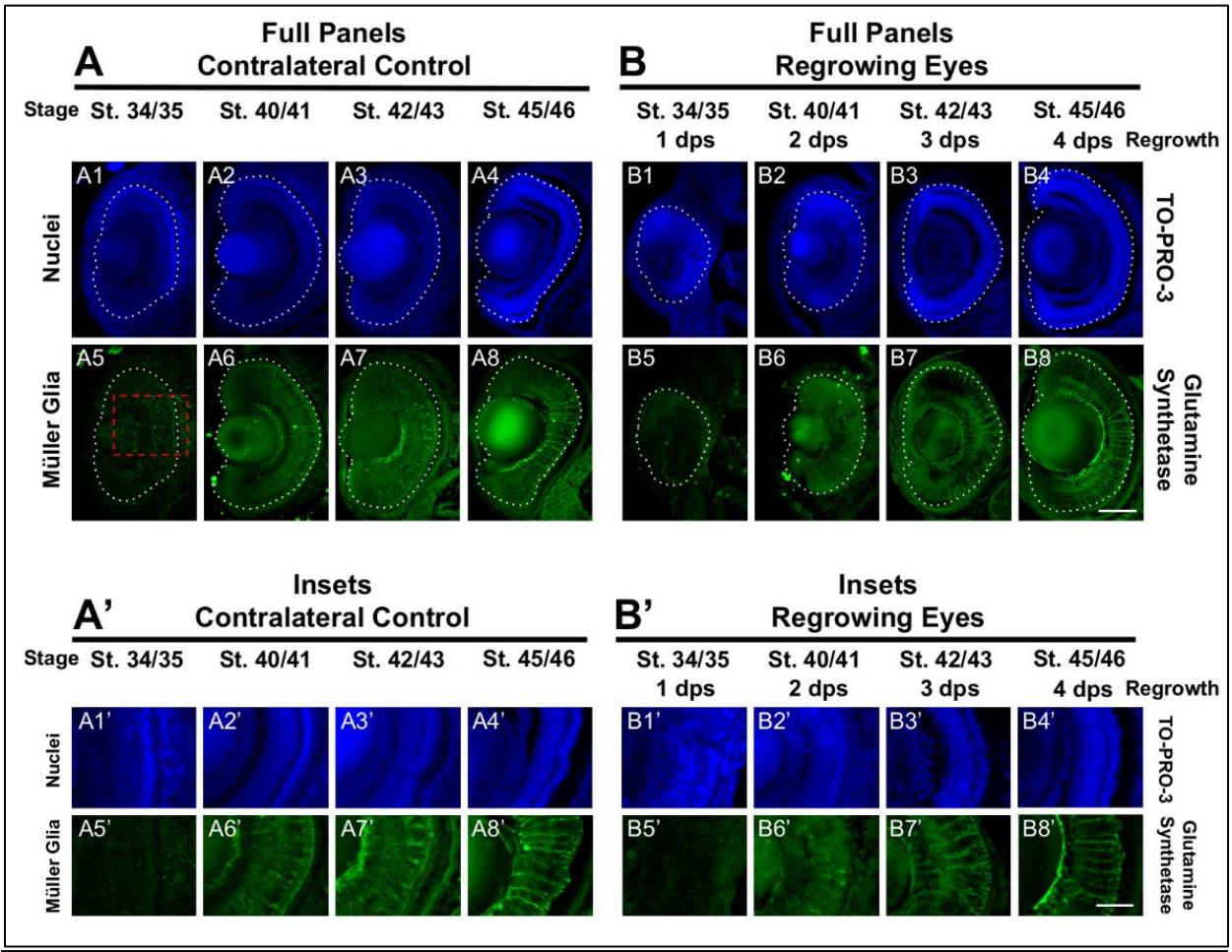


Figure 3.5 Regrown eyes regain Müller glia differentiation by 4 dps. Images shown are immunostained, transverse sections at four developmental timepoints corresponding to 1, 2, 3, and 4 days post surgery (dps). (A,B) Regrown eyes show differentiation of Müller glial cells beginning at 2 dps. However, proper patterning of Müller glial cells is delayed in the regrowing eyes when compared to the contralateral control eyes until 4 dps. White dashed lines delineate each eye. (A',B') Images shown in panels A' and B' correspond to the region shown in the inset box in panel A5 for the corresponding A or B panel at high magnification. Blue color indicates nuclear staining (TO-PRO-3). Green color indicates anti-Glutamine Synthetase (identifies Müller glial). Sample sizes: 1 day, n = 5; 2 days, n = 5; and 3 days, n = 5. (A,B, A',B') Up = dorsal, down = ventral, lens is on the left. Scale bar: A,B = 100 µm and A'-B' = 50 µm.

Changes in Pax6 Expression During Regrowth

Pax6 is an eye field transcription factor that is expressed in the presumptive eye primordium after gastrulation (st. 12.5) and specifies the eye field (Zuber et al., 2003). Prior to st. 33/34, Pax6 mRNA is expressed throughout the neural retina (Hirsch and Harris, 1997). By st. 33/34 and onward, Pax6 mRNA expression becomes more restricted to the presumptive GCL and INL of the retina so that by st. 42, Pax6 expression is observed only in those two layers (Hirsch and Harris, 1997). We used an anti-Pax6 antibody to assess its expression during regrowth (Rungger-Brändle et al., 2010). Consistent with previous reports, we observed that Pax6 expression in the control eye was mostly restricted to the presumptive GCL and INL and extended out to the periphery at st. 34/35 (Fig 3.6A4,A4'). By st. 40/41, Pax6 expression was tightly restricted to the GCL and INL (Fig 3.6A5,A5') and retained this expression pattern through st. 42/43 (Fig 3.6A6,A6'). In the 1 dps regrowing eye, the retinal layers were not apparent (as seen by Xen1 expression, Fig 3.1B7). At this timepoint, Pax6 expression was not localized and remained expanded, with apparent higher expression levels in the central region (Fig 3.6B4,B4'). This pattern was more reminiscent of Pax6 expression in embryos younger than st. 33 (Hirsch and Harris, 1997). By 2 dps (st. 40/41), Pax6 was largely restricted to the GCL and INL in the regrowing eye although expression near the retinal periphery is weaker than those cells located more centrally (Fig 3.6B5,B5'). By 3 dps, Pax6 patterning was restored as its expression became restricted to GCL and INL (Fig 3.5B6,B6'). Together, our data indicated that Pax6 expression was not restricted to the GCL and INL layers 1 dps in the regrowing eye. As regrowth continues, these Pax6-expressing cells changed and became restricted to the GCL and INL of the retina by 3 dps.

Assessment of the Roles of Pax6 and Apoptosis During Development and Regrowth

A key feature of this developmental eye repair model is that it can facilitate a rapid assessment of development and regenerative mechanisms. Our previous work and current data combined suggest that eye formation and differentiation during regrowth is delayed but largely followed the normal developmental process, resulting in an eye that was indistinguishable to a normal one (Fig 2.1) this model now provides the opportunity to use the same developmental context to ask whether specific molecular mechanisms are required in development and/or regeneration for the eye. Therefore, we assessed the roles of Pax6 (which is required for eye development) and apoptosis (which is required for eye regrowth) in both eye development and regrowth.

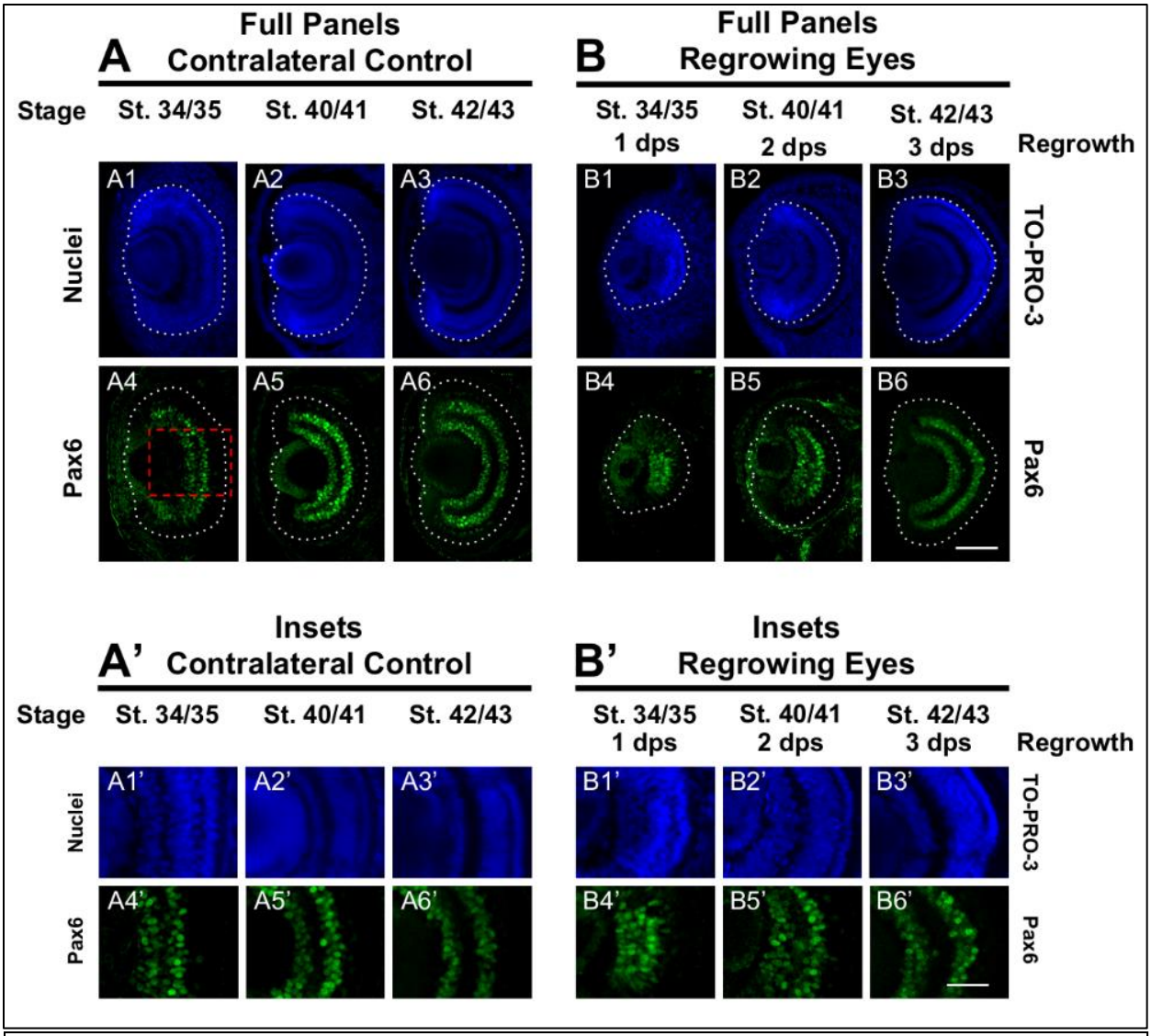


Figure 3.6 Regrown eyes regain Pax6 patterning by 3 dps. Images shown are immunostained, transverse sections at three developmental timepoints corresponding to 1, 2, and 3 days post surgery (dps). (A,B) Pax6 expression in the regrowing eye is less organized at 1 dps but regains patterning similar to contralateral control eyes (unoperated) by 3 dps. White dashed lines delineate each regrowing eye. (A',B') Images shown in panels A' and B' correspond to the region shown in the inset box in panel A4 for the corresponding A or B panel at high magnification. Blue color indicates nuclear staining (TO-PRO-3). Green color indicates anti-Pax6 signal. Sample sizes: 1 day, n = 5; 2 days, n = 7; and 3 days, n = 6. (A,B, A',B') Up = dorsal, down = ventral, lens is on the left. Scale bar: A,B = 100 µm and A',B' = 50 µm.

Pax6 is required for proper vertebrate eye development. In *Xenopus tropicalis*, loss-of-function Pax6 mutations reduced eye size and shows additional eye defects (Nakayama et al., 2015). X. laevis embryos injected with a Pax6 morpholino showed reduced or absent eyes

(Rungger-Brändle et al., 2010). We also examined Pax6 loss-of-function effects on the eye. We injected either a published Pax6 morpholino or a control morpholino into the dorsal blastomere at the 4-cell stage and assessed for eye defects at a tadpole stage (st. 46). Consistent with previous studies, Pax6 morpholino expression resulted in eye defects in the majority of embryos (57.1%, n = 91) as compared to embryos expressing a control morpholino (0%, n = 30, $p < 0.05$) (Fig 3.7A). The eye defects included reduced or absent eyes (Fig 3.7C, compare top panels).

In *X. laevis*, apoptosis can be detected in embryos starting at gastrulation (st. 10.5) and was observed in the anterior region throughout neurulation (Hensey and Gautier, 1998). For apoptosis inhibition during development, we used M50054, a known apoptosis inhibitor that blocks caspase activity and successfully inhibited both Xenopus tadpole tail regeneration and eye regrowth (Tsuda et al., 2001, Tseng et al., 2007; Kha et al., 2018). Embryos were treated with 28 μM of M50054 from st. 10 (gastrulation) to st. 27 (tailbud embryo) and scored at st. 46 (tadpole). Embryos treated with either M50054 (n = 30) or DMSO (vehicle only, n = 30), did not display any morphological eye defects (Fig 3.7A,C, compare bottom panels). Our previous study also showed that M50054 treatment from st. 27 to st. 34/35 did not induce eye defects (Kha et al., 2018). These data were also consistent with a previous study showing that overexpression of the anti-apoptotic gene, BcL-xL, during embryogenesis did not induce eye defects (Johnston et al., 2005). Thus, apoptosis does not appear to be required for eye development.

To assess the role of Pax6 in eye regrowth, the same Pax6 morpholino injection was carried out using a reduced concentration so as to enable normal overall development. This is to ensure that eye tissue removal surgery can be performed on embryos with normal eyes. 81.8% of embryos expressing the control morpholino in the eye region at st. 27 fully regrew eyes (Fig 3.7B, RI = 278, n = 22; and Fig 3.7D, compare top panels). In contrast, only 13.7% of embryos

expressing the Pax6 morpholino in the eye region at st. 27 showed full eye regrowth whereas 86.3% failed (Fig 3.7B, RI = 168, n = 51, $p < 0.01$ when compared to control; and Fig 3.7D compare top panels). Thus, Pax6 morpholino successfully blocked eye regrowth. For apoptosis, we confirmed our previous study showing that inhibition of apoptosis using M50054 blocked eye regrowth [Fig 3.7B, n = 41, $p < 0.01$, and Fig 3.7D; compare bottom panels, and (Kha et al., 2018)]. Our data indicate that Pax6 is required for successful Xenopus eye regrowth. Although this is not an unexpected result, this data showed that at least one key eye development gene is used for eye regrowth.

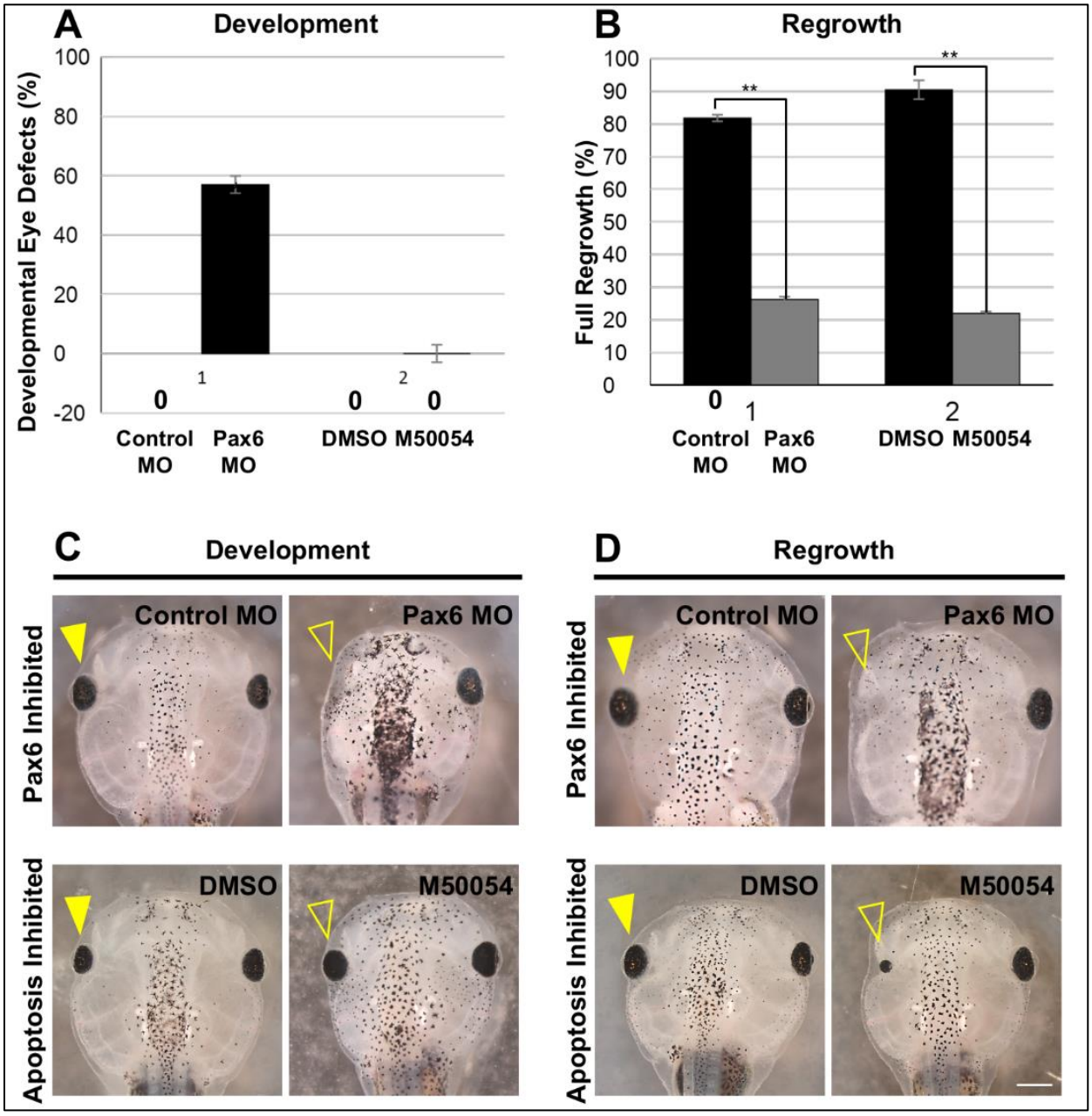
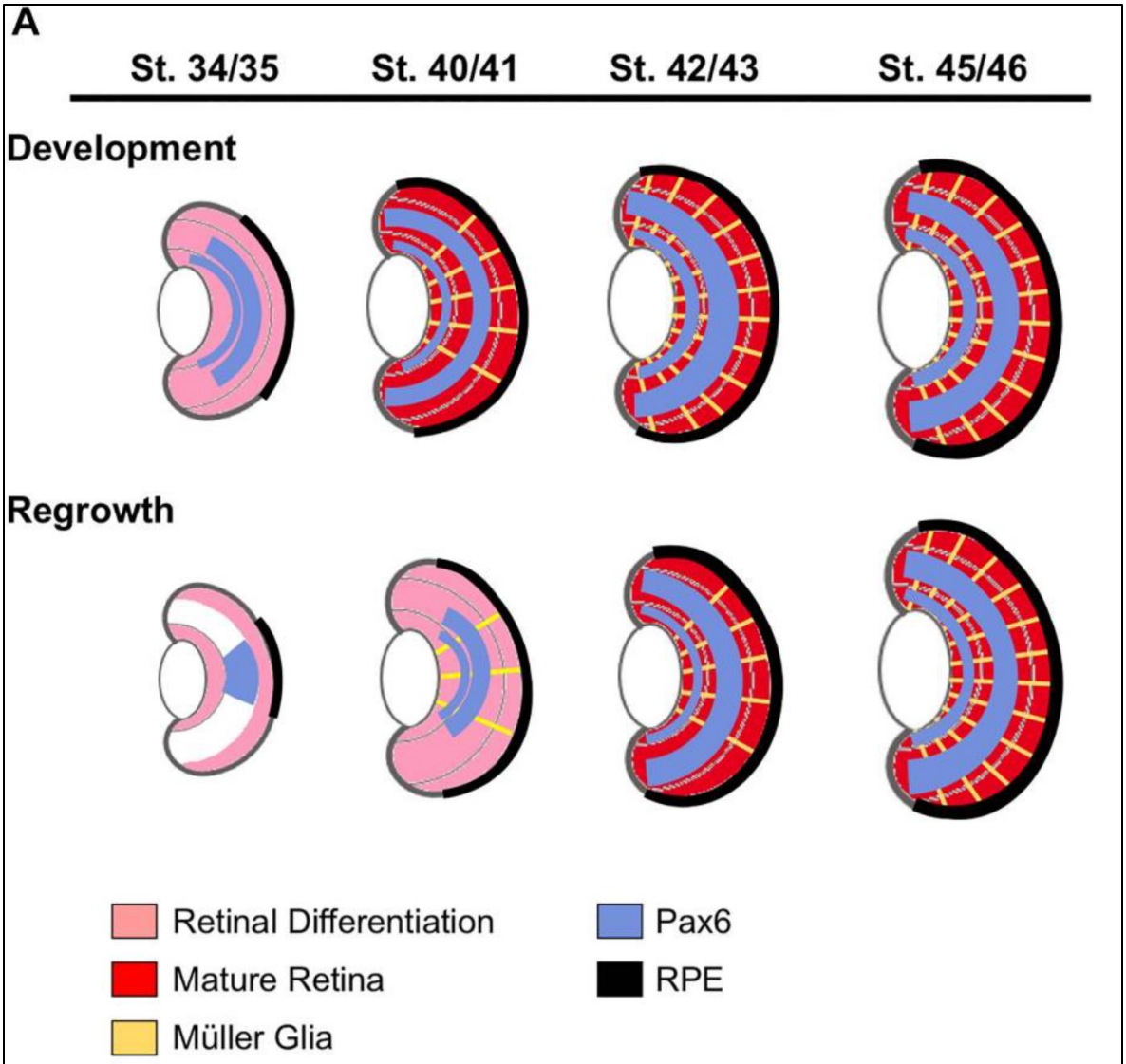


Figure 3.7 Development and regrowth require Pax6, but only regrowth requires apoptosis. (A) Comparison of developmental eye defects percentage from embryos injected at the 4-cell stage (1 blastomere) with either the control or Pax6 morpholino or treated with DMSO control or M50054 at st. 10. A zero denotes no abnormal phenotype in the control by st. 27. ∗denotes $p < 0.05$ ($n > 90$). Data are means ± SEM. (B) Graphical representation of tadpoles achieving full eye regrowth at 5 dps (st. 46) with morpholino injection or apoptosis inhibitor treatment. ∗∗denotes $p < 0.01$ ($n > 20$). Data are means ± SEM. (C,D) Comparison of requirements for eye development and regrowth. (C) Pax6 morpholino injected tadpoles show reduced eyes when compared to the control by st. 46 in development. Apoptosis inhibitor show no effect on eye development ($n > 30$ per condition). Closed yellow arrowhead indicates eye of control, untreated tadpole. Open yellow arrowhead indicates eye of treated tadpole. (D) Pax6 morpholino and apoptosis inhibitor affects eye regrowth ($n > 30$). Closed yellow arrowhead indicates the eye of a control, untreated tadpole. Open yellow arrowhead indicates the eye of an inhibitor treated tadpole. (C,D) Up = anterior, down = posterior. Scale bar: C,D = 500 µm.

3.5 Discussion

In this study, we showed that eye formation during regrowth was delayed but generally followed the endogenous retinal differentiation and cellular patterning process to generate a regrown eye that is age and size appropriate (summarized in Fig 3.8A). Consistent with this data, the formation of the ciliary margin zone (CMZ) was also delayed. The CMZ is located at the periphery of the retina and produces all retinal cell types for eye growth post-embryonically (Hollyfield, 1971). It can be visualized by its distinct spatial cellular organization in eye sections and was formed by st. 34/34 (Fig 3.9A,A'). In the regrowing eye, the formation of the CMZ was delayed until st. 40/41 (2 dps; Fig 3.9B,B').

A



B

Summary of Requirements

	Development	Regrowth
Retinal Differentiation	**YES**	**YES**
Pax6	**YES**	**YES**
Apoptosis	**NO**	**YES**

Figure 3.8 Summary. (A) A summary of retinal cell differentiation during regrowth as compared to development. The stages correspond to 1, 2, 3, and 4 days post surgery (dps), respectively. (B) Summary of required processes identified in eye development and regrowth.

A distinct characteristic of retinogenesis is that it contains an intrinsic timer for initiating differentiation. In *X. laevis,* retinogenesis timing remained the same and began by st. 24 even when there was a significant reduction of retinal progenitors cells by chemical inhibition of proliferation during embryogenesis (Harris and Hartenstein, 1991). In our eye regrowth model, a significant reduction of retinal progenitors (average loss is approximately 83%) is achieved by tissue removal surgery at st. 27 (Kha et al., 2018). Here, we examined the temporal regulation of the regrowth process in more detail. Endogenous retinogenesis is initiated at st. 24 and completes by st. 41, a time period of about 2 days (Holt et al., 1988). Our previous work showed there was a significant increase in proliferation at the injury site during the first 24 h of regrowth (Kha et al., 2018). Here, we report that reparative retinogenesis showed a delay and started at 1 dps (st. 34/35) with completion occurring by 3 dps (st. 42/43). Like the endogenous process, reparative retinogenesis needed a time period of about 2 days. These results suggest that while retinogenesis can be re-induced at a developmental stage later than st. 24, the overall time required to complete the differentiation process was maintained as for development. Even though the eye formation time window can be re-started past the endogenous timeframe, there was no shortening of the eye formation period to catch up as quickly as possible.

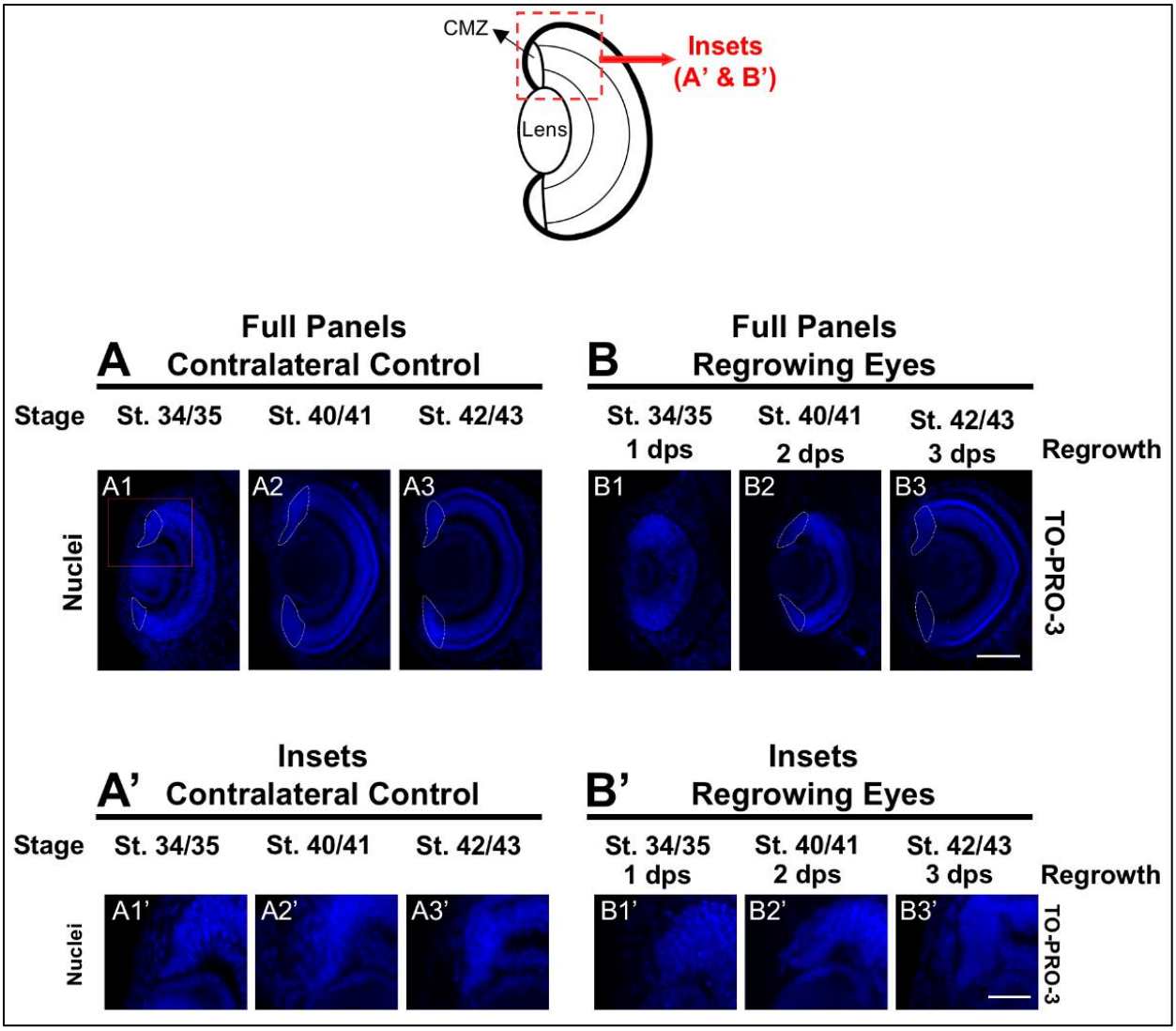


Figure 3.9 Regrown Eyes Develop CMZ by 2 dps. Images shown are immunostained, transverse sections at three developmental timepoints corresponding to 1, 2, and 3 days post surgery (dps). The top schematic is a diagram of a section through a mature, differentiated tadpole eye. (A-B) Regrowing eyes develop ciliary margin zone (CMZ) by 2 dps. White dashed lines delineate the CMZ. (A'-B') High magnification of the boxed region outlined in the dorsal eye region of the eye schematic and corresponds to inset box in A1. Blue color indicates nuclear staining (TO-PRO-3; n > 5 per timepoint). (A-B, A'-B') Up = dorsal, down = ventral, lens is on the left. Scale bar: A-B = 100 µm and A'-B' = 50 µm.

As the first differentiated retinal cells are generated starting at st. 24, there is a continual decrease in the mitotic index of the retinal progenitor cells (RPCs) until most cells have exited the cell cycle by st. 37/38 (Holt et al., 1988). During this time, the estimated cell doubling time increases from 8.6 to 56 h (Rapaport, 2006). In contrast, there is significantly increased mitotic

activity in the first 24 h during eye regrowth that continues until the regrown eye reached the expected age-appropriate size by 3 dps (Kha et al., 2018). The proliferative burst of RPCs in eye regrowth is counter to the endogenous process at the same developmental stages where cells are becoming postmitotic. Moreover, the increase in RPC proliferation, coupled with the delay of retinal differentiation suggests that induction of regrowth temporarily inhibited retinogenesis. There is no specific cell number required for retinogenesis as the initiation of Xenopus retinogenesis is not affected by greatly reduced retinal cell divisions during embryogenesis (Harris and Hartenstein, 1991). One possibility is that the sudden loss of RPCs at st. 27 via tissue removal surgery triggers a signal that extends the stem cell multipotency of RPCs in order to restore normal size. (It is also possible that the source cells may be non-retinal in origin.) Although we used Pax6 as a differentiation marker in this study, it is also required for maintaining the multipotent state of RPCs prior to retinogenesis (Marquardt et al., 2001). The absence of restricted Pax6 expression in the regrowing eye at 1 dps (compare Fig 3.6B4,A4) is reminiscent of its expression at the younger, proliferative, developmental stages (Hirsch and Harris, 1997). It will be highly informative to identify the molecular mechanisms that regulate RPC proliferation during regrowth as this model has the potential to become a useful system to study endogenous RPC expansion.

During eye regrowth (st. 27 to st. 42/43), the embryo is changing rapidly as it proceeds from being a tailbud embryo with unformed organs toward becoming a tadpole with differentiated body structures (Nieuwkoop and Faber, 1994). Once eye regrowth is initiated after tissue loss, it appears to follow the endogenous developmental program and remain unaffected by rapid changes in the surrounding tissues during development. Indeed, the overall retinal birth order that was observed for the cell types examined was consistent with the described order for

Xenopus retinogenesis. Of note, our study of retinal differentiation during regrowth did not specifically examine each individual retinal cell type that is generated during eye formation. It is possible that there may exist some differences in formation of the regrown eye as compared to eye development that was not detected by the retinal markers used in this study.

Our findings revealed that successful eye development during regrowth induced similar cellular events as for eye development. This model now provides the opportunity to directly examine the role of developmental mechanisms in eye regrowth. We used this model to compare the role of two mechanisms, Pax6 and apoptosis, in development and regrowth (summarized in Figure 3.8B). Given the role of Pax6 as a "master regulator" of eye formation, it was not surprising that Pax6 was found to be also required for eye regrowth. In contrast, we found that apoptosis appears to be a regrowth-specific mechanism. Thus we have successfully used this model to define an initial similarity and an initial difference between eye development and regrowth. As there is a wealth of knowledge on the role of Pax6 (and other known regulators) during eye development, it will be highly feasible to distinguish any differences in the function of Pax6 and other genes in regrowth. For further comparison to developmental eye regrowth, follow-up studies can then be performed to examine the role of these genes in tadpole and adult retinal regeneration using established *Xenopus* models (Yoshii et al., 2007; Vergara and Del Rio-Tsonis, 2009; Araki, 2014). Potentially, developmental mechanisms that are not required for eye regrowth can also be identified. In summary, this developmental eye regrowth model will serve as a robust platform for systematically examining the common view that regeneration is a recapitulation of development.

3.6 Materials and Methods

Embryo Culture and Surgery

Embryos were obtained via in vitro fertilization and raised in 0.1× Marc's Modified Ringer (MMR: 1 mM MgSO4, 2.0 mM KCl, 2 mM CaCl2, 0.1 M NaCl, 5 mM HEPES, pH 7.8) medium (Sive et al., 2000). The eye removal surgery and the regrowth assay were performed as described previously (Kha et al., 2018). Embryos at stage (st.) 27 (Nieuwkoop and Faber, 1994) were anesthetized with MS222 (Sigma) prior to surgery. Surgery was performed using fine surgical forceps (Dumont No. 5). An initial cut is first made in the skin surrounding the protruding eye cup and overlying lens placode. The cut is continued around the raised outline of the eye and the protruding tissues are removed. After surgery, embryos were transferred into 0.1× MMR, allowed to recover, and then cultured at 22°C for 1–5 days.

Embryo Sectioning and Immunofluorescence Microscopy

For agarose embedding and sectioning, animals were fixed overnight at 4°C in MEMFA (100 mM MOPS (pH 7.4), 2 mM EGTA, 1 mM MgSO4, 3.7% (v/v) formaldehyde) (Sive et al., 2000) and processed according to Kha et al. (2018). Embryos and tadpoles were embedded in 4–6% low-melt agarose and sectioned into 60 μm slices using a Leica vt1000s vibratome. Sections were stained with primary antibodies including: Xen1 (pan-neural antibody, clone 3B1, 1:50 dilution, Developmental Studies Hybridoma Bank, RRID: AB_531871), anti-Islet1 (retinal ganglion cells and inner nuclear cell layer, clone 40.2D6, 1:200 dilution, Developmental Studies Hybridoma Bank, RRID: AB_528315), anti-Glutamine Synthetase (Müller glia, 1:200 dilution, Sigma-Aldrich, RRID: AB_259853), anti-Laminin (basal lamina, 1:300 dilution, Sigma-Aldrich, RRID: AB_477163), anti-Rhodopsin (rod photoreceptor cells, clone 4D2, 1:200 dilution, EMD Millipore, RRID: AB_10807045), anti-Calbindin-D-28 K (cone photoreceptor cells, 1:500 dilution, Millipore Sigma, RRID: AB_258818), anti-Pax6 (clone Poly19013, 1:500 dilution, BioLegend, RRID: AB_291612), anti-RPE65 (retinal pigment epithelium, 1:500 dilution,

ThermoFisher Scientific, RRID: AB_2181003). Alexa fluor conjugated secondary antibodies were used at 1:1000 dilution (ThermoFisher Scientific). TO-PRO-3 (Molecular Probes) was used for DNA staining. The contralateral eye was used as the control. For each timepoint, at least 5 embryos were analyzed. In all embryos examined, the observed cellular patterns were consistent for each antibody that was used. White dashed lines were determined by the TO-PRO-3 staining. Quantification of rod photoreceptor cell numbers was performed using sections stained with an anti-Rhodopsin antibody. The number of rod photoreceptor cells was counted per 60 μm sections ($n > 5$ per timepoint). Rod photoreceptor cells expression pattern was measured in pixels as a drawn line along the outer nuclear layer and compared to the overall circumference of the retinal layer from one end of the ciliary margin zone (CMZ) to the other end of the CMZ ($n > 5$ per timepoint). The ratio of rhodopsin expression in the retinal layer over corresponding the retinal layer circumference measurement was calculated.

Microscopy

A Nikon A1R confocal laser scanning microscope (UNLV Confocal and Biological Imaging Core) was used to image immunostained tissue sections. Images of whole animals were obtained using a ZEISS SteREO Discovery V20 microscope with an AxioCam MRc camera. ZEN Image Analysis software and/or the open-source FIJI imaging software (Schindelin et al., 2012) were used to analyze and/or process all acquired images.

Chemical Treatments and Morpholino Injections

For apoptosis inhibition, embryos were treated with 28 μM of M50054 (Millipore, EMD Biosciences, Burlington, MA, United States, CAS number 54135-60-3). For vehicle control, dimethyl sulfoxide (DMSO) was used at the same concentration as for M50054 treatment

(0.1%). For the developmental assay, age-matched embryos were raised in 0.1× MMR medium containing the inhibitor starting at st. 10 until st. 27. Eye development was assayed by st. 46. To assay for regrowth, eye surgery was performed on st. 27 tailbud embryos. The embryos were allowed to briefly recover, and then transferred into 0.1× MMR medium containing the inhibitor. After 1 day, embryos were washed with two changes of 0.1× MMR. Eye regrowth was assayed between 1 and 5 days post-surgery.

For morpholino injections, the following morpholinos (MO) were purchased from Gene Tools LLC (Philomath, Oregon): Pax6MO: 5′-GCTGTGACTGTTCTGCATGTCGAG-3′ (Li et al., 1997; Rungger-Brändle et al., 2010); and the non-specific standard control oligomer: 5′-CCTCTTACCTCAGTTACAATTTATA-3′. Each morpholino was modified with 3′ fluorescein. Morpholinos were resuspended in sterile water to a concentration of 1 mM. For both developmental and regrowth studies, morpholinos were injected separately into a dorsal blastomere of a 4-cell embryo using a microinjector (Harvard Apparatus, Holliston, MA)– targeting only one side of the embryo. Embryos with fluorescent signal in the eye region were selected for further analysis. A previously published concentration of 30 ng/embryo (Rungger-Brändle et al., 2010) was used for verification of published phenotypes. The titrated dosages for morpholino injections were: 27 ng/embryo (developmental assay) and 15 ng/embryo (eye regrowth assay). Lethality was observed in st. 27 tailbud embryos that were injected with 35.7 ng of Pax6 morpholino at the 4-cell stage.

Assessment of Eye Regrowth

The regrowth of the operated eyes as compared to unoperated contralateral eyes was assessed using the Regrowth Index (RI) as previously described (Kha et al., 2018). The quality of eye regrowth was scored based on 4 phenotype categories: full, good, weak, and none. Full, RI =

300; Partial, RI = 200; Weak, RI = 100; None, RI = 0. The RI ranges from 0 to 300, where 0 indicates no eye regrowth of all embryos in a given condition, 100 if all embryos achieve weak regrowth, 200 if all embryos achieve good regrowth, and 300 indicates that all embryos achieve full regrowth. Raw data from scoring was used to compare eye regrowth experiments. The unoperated contralateral eyes of embryos showed no difference from unoperated control eye of age-matched sibling embryos.

Statistical Analysis

To compare eye regrowth, raw data from scoring was used. Comparison of two treatments was analyzed with Mann-Whitney U test for ordinal data with tied ranks, using normal approximation for large sample sizes. Multiple treatments were compared using a Kruskal-Wallis test, with Dunn's Q corrected for tied ranks. All other experiments were analyzed using a Student's t-test.

CHAPTER 4

NOTCH IS REQUIRED FOR NEURAL PROGENITOR PROLIFERATION DURING EMBRYONIC EYE REGROWTH

4.1 Preface

This chapter is currently under review.

Author Contributions: Data collection, figure generation, and writing were performed by me, with Belen Gutierrez and Baoyi Zhang contributing some immunostaining images and editing. Kelly Tseng contributed the conception and design of the study, as well as writing and editing.

Editorial note: Changes were made to the figure numbers to fit with the overall formatting of the dissertation.

4.2 Abstract

The ability of an organism to regrow tissues is regulated by various signaling pathways. One such pathway that has been studied widely both in the context of regeneration and development is the Notch signaling pathway. Notch signaling is required for development of the eye and regeneration of tissues in multiple organisms but it is unknown if Notch plays a role in the regulation of Xenopus laevis embryonic eye regrowth. We found that Notch1 is required for eye regrowth and regulates retinal progenitor cell proliferation. Chemical and molecular inhibition of Notch1 significantly decreased eye regrowth through reducing retinal progenitor cell proliferation without affecting retinal differentiation. Temporal inhibition studies showed that Notch function is required during the first day of regrowth. Interestingly, Notch1 loss-of-function phenocopied the effects of the inhibition of the proton pump, V-ATPase, where retinal proliferation but not differentiation was blocked during eye regrowth. Overexpression of a form of activated Notch1, the Notch intracellular domain (NICD) was sufficient to rescue loss of eye

regrowth due to V-ATPase inhibition, suggesting that Notch acts downstream of V-ATPase. These findings highlight the importance of the Notch signaling pathway in eye regeneration and its role in inducing retinal progenitor cell proliferation in response to injury.

4.3 Introduction

The ability of an organism to regrow lost or damaged tissues varies greatly among animals (Illingworth 1974; Michalopoulos and DeFrances, 1997; Slack et al., 2007; Tanaka and Reddien, 2011; Seifert et al., 2012; Frangoginannis, 2016; Tanaka, 2016; Tseng, 2017; Joven and Simon, 2018; Kha et al., 2018) In order to understand why some animals, or even some tissues within otherwise regenerative animals, lack this ability, there is a need to understand the molecular mechanisms that regulate this regrowth. An important model organism to study for regeneration is the African clawed frog, *Xenopus laevis*. *X. laevis* has long been studied as a regenerative model, valuable for their external fertilization and development, large clutch sizes, and relatively rapid development time, the speed of which can be manipulated by ambient temperature regulation (Gurdon and Hopwood, 2000; Sive, et al., 2000; Wlizla et al., 2018)

Xenopus displays age-dependent regeneration. Tadpoles can regrow a number of structures including the tail, limb, retina, and lens (Dent, 1962; Endo et al., 2000; Suzuki et al., 2006; Slack et al., 2007, Vergara and Del Rio-Tsonis; 2009; Mitogawa et al., 2018) with this ability generally decreasing in potency in some tissues as the animal ages (Slack et al., 2004; Beck et al., 2009; Kha and Tseng, 2018). Our previous work showed that *Xenopus* tailbud embryos regrew their eyes following surgical ablation of ~85% of tissues (including the lens placode and most of the optic cup) at developmental stage (st.) 27 (Kha et al., 2018). The eye completes regrowth within 5 days, is functional, and contains the normal complement of cell types (Kha et al., 2018, Kha et al., 2019). The regrowth process requires cell proliferation and

recapitulates retinogenesis. One advantage of this model is that eye regrowth in the embryo occurs concurrently with normal eye development at the uninjured contralateral side. In models where regrowth occurs post development, comparison of regrowth and development can be challenging due to the inherent differences between developing and mature tissues (Higgins and Anderson, 1931; Eguchi and Shingai, 1971; Yamada, 1977; Yoshii et al., 2006; Vergara and Del Rio-Tsonis, 2009). The embryonic eye regrowth model provides the opportunity for a more direct comparison between developmental and regrowth mechanisms in the same animal. This is important as developmental mechanisms are often co-opted into mechanisms regulating regrowth (Del Rio-Tsonis et al., 1997; Slack et al., 2004; Suzuki et al., 2007). Therefore, it is important to understand whether and how developmental mechanisms act as regulators of regrowth.

An important regulator of eye development is the Notch signaling pathway. Notch1 is a transmembrane receptor that upon binding to its ligand undergoes cleavage events resulting in the cleavage of the intracellular domain (NICD), which migrates to the nucleus and acts as a transcription factor to regulate downstream target genes (Bray, 2016). The Notch signaling pathway is a highly conserved, well-characterized developmental pathway that often determines if a cell population will proliferate or differentiate, and in some contexts, can maintain stem cell populations (Coffman et al., 1993; Henrique et al., 1997; Hitoshi et al., 2002; Borggrefe and Oswald, 2009, Reddy et al., 2010).

Notch signaling can also function as a regulator of stem cell proliferation. In the *Drosophila* wing disc, Notch works to upregulate proliferation, and overexpression of the Delta ligand was sufficient to increase proliferation in the wing disc (Baonza and Garcia-Bellido, 2000). In mouse, Notch promotes proliferation and maintains stemness in intestinal crypt base columnar stem cells. Reduction in Notch signaling reduced proliferation and expression of stem

cell specific markers, and promoted differentiation (VanDussen et al., 2012). Similar behavior is found in bone marrow mesenchymal stem cells, where inhibition of Notch1 signaling resulted in reduced proliferation (He and Zou, 2019).

During *Xenopus* tadpole tail regeneration, Notch signaling is required for proper regrowth. Following tail amputation, treatment with MG132 (a proteasome inhibitor that blocks the cleavage of the Notch1 protein) resulted in healing of the tail stump without regeneration (Slack et al., 2004). Additionally, during the refractory period – when the tadpole temporarily loses its tail regenerative ability – activation of Notch signaling stimulated tail regeneration following amputation (Beck et al., 2003; Slack et al., 2004). During *Xenopus* eye development, active Notch serves to maintain a pool of multipotent retinal progenitor cells (RPCs) by regulating cell differentiation (Dorsky et al., 1995). An imbalance of Notch activity during development resulted in eye malformations (Dorsky et al., 1995, Furukawa et al., 2000). Given the roles of Notch in regulating stem cell populations and promoting appendage regeneration, it is likely that Notch acts to regulate RPC proliferation during *Xenopus* eye regrowth.

Here, we investigate the role of Notch signaling in *Xenopus* embryonic eye regrowth. Our study showed that loss of Notch1 function blocked eye regrowth and resulted in small eyes. Notch inhibition reduced RPC proliferation but retinal differentiation remained unaffected. We also determined that activation of Notch1 during regrowth is sufficient to rescue the regrowth-inhibited small eye phenotype caused by V-ATPase inhibition, demonstrating a link between Notch and V-ATPase signaling. Together, our results showed that Notch1 is required for eye regrowth.

4.4 Results

Reduction of Notch following eye ablation inhibits regrowth

Successful eye regrowth requires the complex interaction of multiple cell signaling pathways including bioelectrical signaling and apoptosis (Kha et al., 2018; Kha and Tseng, 2018; Kha et al., 2023). Another candidate pathway is the Notch signaling pathway, which is required for development of the eye (reviewed in Blair 1999, Mills and Goldman, 2017, and Reichrath and Reichrath, 2020). In *Xenopus*, Notch1 is a neural stem cell marker. During development, it is expressed widely in the optic cup and inhibits retinal differentiation (Coffman et al., 1993; Zaghloul and Moody, 2007). However, the role of Notch1 in neural regrowth is unclear. Thus, we investigated whether Notch1 is required for eye regrowth. First, we sought to inhibit Notch signaling during eye regrowth. Previous studies of Notch function have successfully utilized the cysteine protease inhibitor MG132, as well as the γ-secretase inhibitor DAPT (Chapman et al., 2006, Slack et al., 2007, Xu et al., 2021) to inhibit Notch signaling by blocking the cleavage of the intracellular domain of the Notch protein, thereby inhibiting downstream activation. We first titrated each inhibitor to identify dosages that enabled normal development and used these concentrations (10 uM MG132 and 5 µM DAPT) for our experiments. Then we carried out eye regrowth assays to observe the effects of inhibitor exposure. Treatment with either chemical inhibitor following st. 27 ablation surgery caused a noticeable decrease in regrown eye size as compared to age-matched vehicle-treated regrowing controls (Fig. 4.1A-B). We used the Regrowth Index (RI, ranging from 0 to 300; described in Methods) to assess the overall quality of regrowth as judged by eye size and morphology. A 10 uM MG132 treatment resulted in 20.2% of fully regrown eyes (RI=172, n=114) as compared to DMSO (vehicle)-treated regrowing control which resulted in 63% of fully regrown eyes (RI=248, n=100, $p < 0.01$).

Similarly, 5 uM DAPT treatment resulted in 47.9% of fully regrown eyes (RI=218, n=96) as compared to DMSO-treated regrowing control which resulted in 74.47 % of fully regrown eyes (RI=269, n=94, $p < 0.01$) (Fig. 4.1A-B). As chemical inhibitors can potentially have off target effects, these results were confirmed using molecular inhibition.

A verified morpholino against *Xenopus* Notch1 mRNA (Lopez et al., 2003) or a control morpholino was injected into the left dorsal blastomere of four-cell embryos at a concentration that does not affect embryogenesis. The morpholinos were tagged with fluorescein, allowing for the selection of embryos with eye regions that contained high levels of either the control or Notch1 morpholino (Fig. 4.1C). At st. 27, eye ablation surgeries were performed on these embryos and regrowth was assayed at 5 dps. Consistent with the chemical inhibition results, embryos with the Notch1 morpholino had significantly reduced eye regrowth (21.4% full regrowth, RI=165, n=109) as compared to those injected with the control morpholino (79.8% full regrowth, RI=276, n=112, $p < 0.01$) (Fig. 4.1A-B). To confirm that the morpholino effect was due to Notch1 knockdown, we tested if ectopic expression of Notch1 could restore eye regrowth. The Notch Intracellular Domain (NICD) is an activated form of Notch and an established tool to activate Notch signaling in *Xenopus* (Coffman et al., 1993). Dexamethasone-inducible NICD mRNA was co-injected with the Notch1 morpholino during the four-cell stage. To induce NICD activation, 10 µM dexamethasone (Dex) was added after eye ablation surgery. Dexamethasone treatment resulted in 59.1% full eye regrowth (RI=248, n=22) as compared to 9.5% full regrowth in the uninduced control (RI=144, n=21; $p < 0.01$) (Fig. 4.1B). Thus NICD activation was sufficient to rescue Notch1 morpholino-inhibited regrowth (Fig. 4.1D). This result indicated that it was the reduction in Notch1 which caused the inhibition of regrowth. Together, our data demonstrated that Notch is required for successful regrowth of the eye.

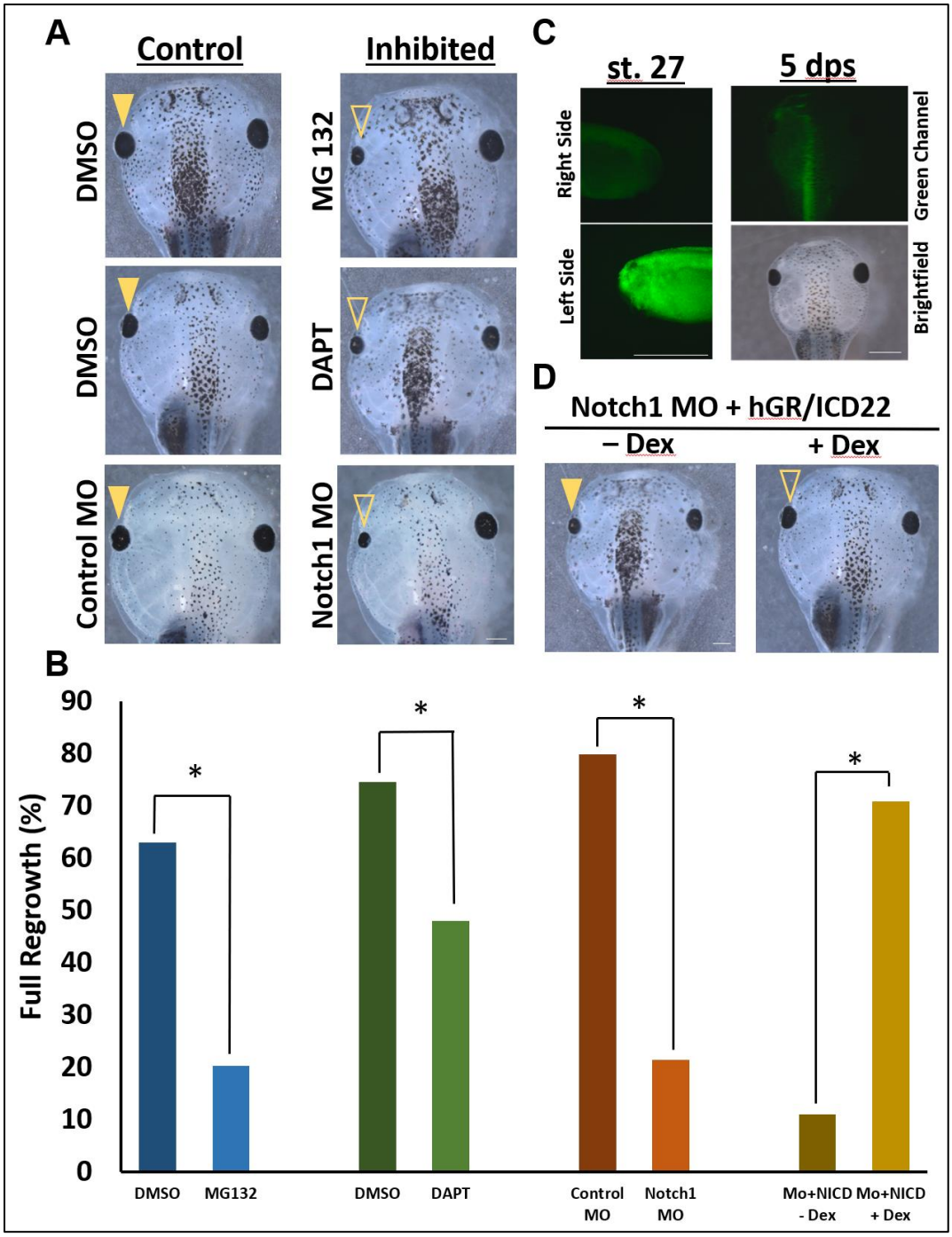


Figure 4.1 Reduced Notch impairs eye regrowth. (A) Comparison of 5 dps tadpoles treated with DMSO (control), Notch inhibitors (MG132 or DAPT), or injected with Notch morpholino (n>30 per condition). Images at 20.5X. Up= anterior. (B) Graph showing the percentage of the population achieving full regrowth at 5 dps under different conditions as determined by eye morphology and size. * = $p<0.01$ (C) Morpholino expression at st. 27 and 5 dps. Cells containing morpholino showed green fluorescence from the fluorescein tagged oligonucleotide. Top left panel shows the right side of the embryo, right= anterior. Bottom left panel shows the left side of the same embryo, right= posterior. For both, up= dorsal. Images at 48X. Tadpole at 5 dps showing green fluorescence still present on the left side of the animal. Top right panel is green channel showing the fluorescein tag on the morpholino at 5 dps showing persistence of morpholino through the regrowth period. Bottom right panel is the corresponding brightfield image. Up= anterior. Image at 25X. Scale bar= 500µm. (D) Comparison of 5 dps tadpoles expressing Notch1 morpholino and the Dex-inducible NICD construct in the left eye region. Left panel shows regrowth without the Dex inducer, right panel shows regrowth with Dex. Closed arrowhead indicates control regrowth-inhibited eye. Open arrowhead indicates rescue of eye regrowth (n>30 per condition). Images at 20.5X. Up= anterior, down= posterior. (A, C, and D) scale bar= 500µm.

Notch is required during the first day of regrowth

Notch signaling is often a regulator of proliferation (Baonza and Garcia-Bellido, 2000; Pallavi et al., 2012; VanDussen et al., 2012). Our previous work showed that approximately 87% increase in eye size during regrowth occurred during the first two days (Kha et al., 2018). We hypothesized that the requirements for Notch function during eye regrowth is during the early time period. To identify the temporal requirement for Notch, the duration of exposure to 10 µM MG132 was varied during the regrowth period. Our data indicated that embryos with reduced Notch function during the first 24 hours post-surgery resulted in similar inhibition (RI=191, n=99) with 25.3% of full regrown eyes as compared to embryos inhibited for the entire five-day period with 24.7% of full regrown eyes (RI=191, n=93, $p > 0.05$) (Fig. 4.2). If the key requirement for Notch activity is during the first day of regrowth, then its inhibition after 1 dps should not affect regrowth. Consistent with this prediction, embryos treated with MG132 from 1 dps through the end of the five-day assay showed no appreciable inhibition of regrowth with 78.8% of full regrown eyes, a level that was comparable to DMSO-treated controls (RI=250, n=92; $p < 0.01$ compared to either 0-5 dps or 0-1 dps treatment) (Fig. 4.2B). Together, our data showed that the first 24 hours is the critical period where Notch function is required to drive eye regrowth.

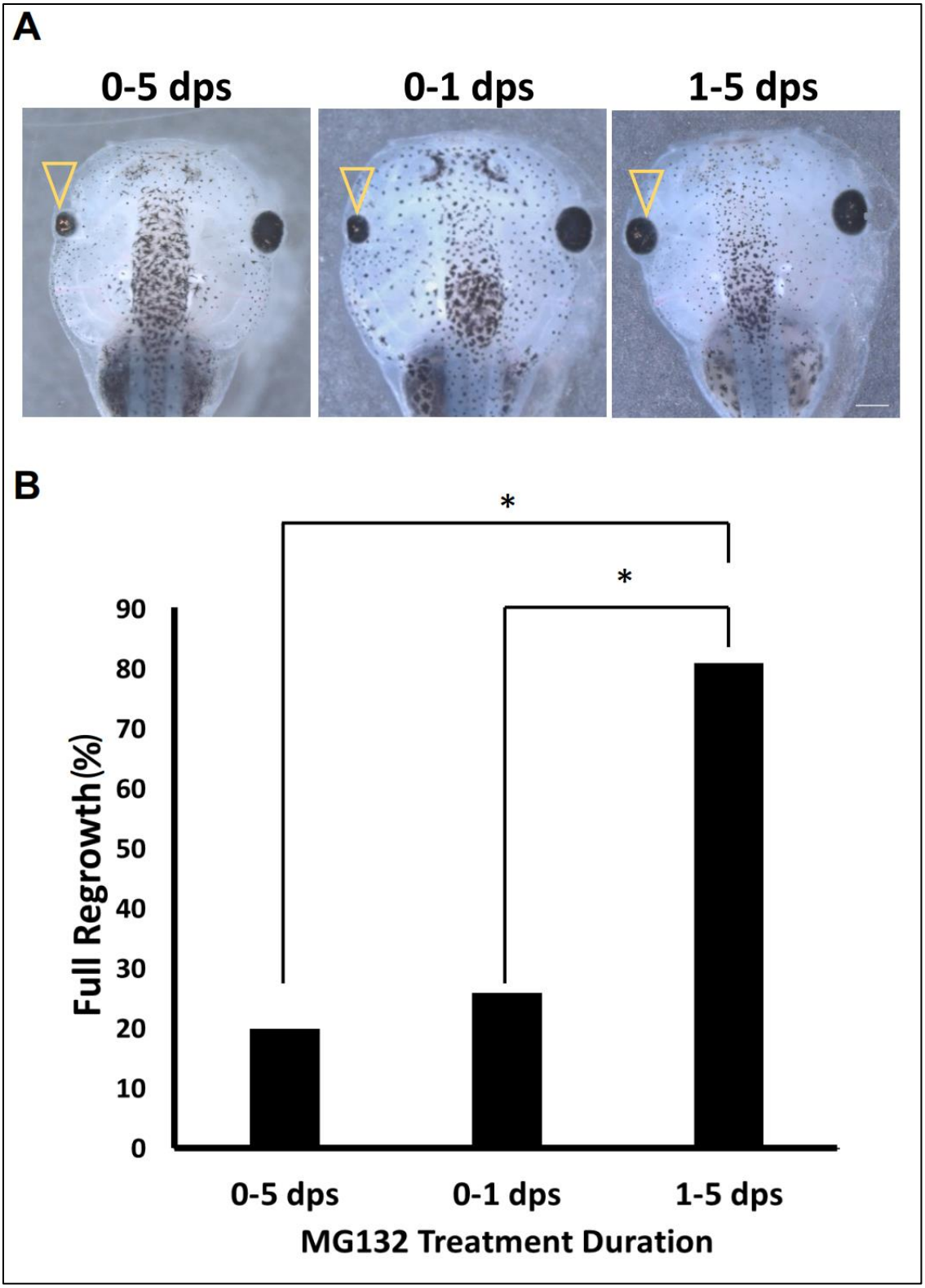


Figure 4.2 Notch is required during the first day of regrowth. (A) Comparison of regrown eyes at 5 dps following treatment of MG132 for variable durations from time of surgery (n>30 per condition). Open arrowheads indicate regrown eye. Up= anterior, images at 20.5X. Scale bar= 500µm. (B) Graphical representation of percent of the population achieving full regrowth at 5 dps, * = p<0.01.

Disruption of Notch does not disrupt differentiation

In order for eye regrowth to occur fully, both cell proliferation and proper retinal differentiation are needed. Previously, we showed that the regrown eye contained the expected complement of retinal cell types including rod and cone photoreceptors, ganglion cells, retinal pigmented epithelium cells, and Müller glia, with the same structure as a normally developing eye (Kha et al., 2018). Defective regrowth could result from disruptions in retinal cell proliferation or differentiation, or both. To characterize the regrowth defects resulting from Notch inhibition, we first examined the effects on retinal differentiation. It is possible that reducing Notch function during regrowth disrupted retinal differentiation, leading to small eyes. In order to test for this, we examined retinal differentiation using known antibody markers. Embryos were fixed at 3 dps following either treatment with MG132 or injection with Notch1 morpholino, as well as control regrowing eyes. Eye sections were obtained and stained with anti-Islet1 antibody as a marker of ganglion cells, or anti-Rhodopsin antibody as a marker of rod photoreceptor cells (Kha et al., 2019). Similar retinal patterns were observed between the Notch-inhibited non-regrowing small eyes and control regrowing eyes in terms of overall morphology and positioning of ganglion and rod photoreceptor cells relative to their eye sizes (Fig. 4.3). Islet1 signal was present within the ganglion cell layer and inner nuclear layer in both the control regrowing and Notch-inhibited non-regrowing eyes (Fig. 4.3A, $n > 5$ for each condition). As expected, the rhodopsin signal spanned the posterior periphery of the eye, in the outer nuclear layer (Fig. 4.3B, $n > 5$ for each condition). These observations are consistent with previous work in *Xenopus* showing that abnormal small eyes can contain largely normal retinal layers (Harris and Hartenstein, 1991; Kha et al., 2019). Additionally, regrowing eyes showed a delay of differentiation as compared to normal developing eyes (Kha et al., 2019). Here, we find that non-

regrowing eyes also showed delayed differentiation, same as the regrowing eyes. Together, these results indicated that perturbation of Notch function during eye regrowth does not disrupt retinal differentiation.

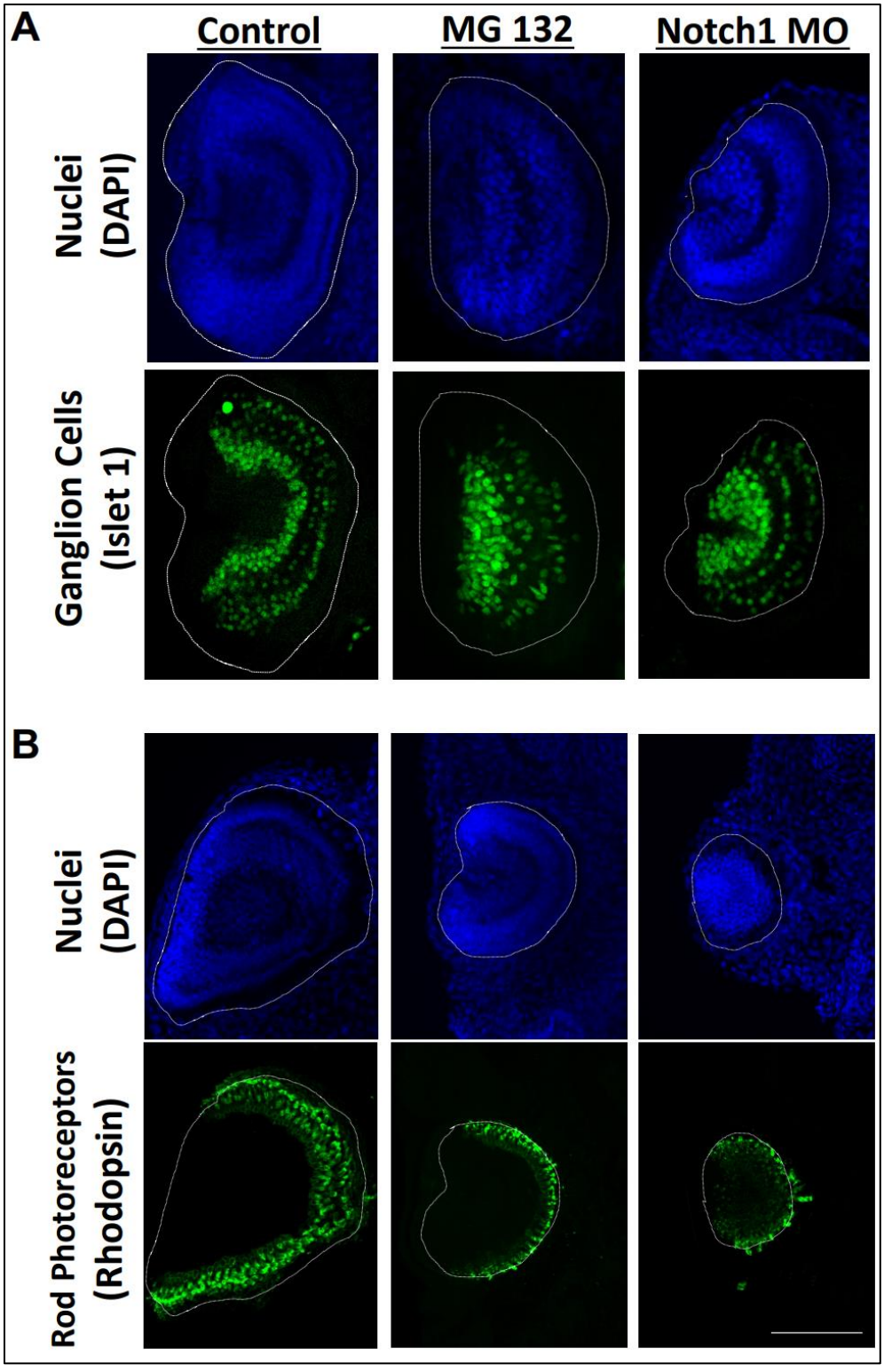


Figure 4.3. Reduction in Notch during eye regrowth does not impair differentiation. (A) Transverse eye sections of regrowing and inhibited eyes at 3 dps. Top panels indicate nuclei. Bottom panels indicate ganglion and inner nuclear layer cells. (B) Transverse eye sections of regrowing eyes at 3 dps. Dotted lines outline the eye region. Top panels indicate nuclei. Bottom panels indicate rod photoreceptor cells. All eyes were sectioned medially and facing left. Images at 20X. Scale bar= 100μm. n>5 per condition. Up= dorsal, Left= anterior.

Inhibition of Notch function downregulates retinal proliferation

In order for the eye to regrow successfully, cellular proliferation must take place to replace the tissues that were lost. There is a significant increase in the number of proliferating cells in the regrowing eye during 0-1 dps relative to normally developing eyes (Kha et al., 2018). This increase is within the same window in which Notch is required for eye regrowth. Notch is known to play a role in regulating proliferation during eye development (Go et al., 1998). As Notch does not appear to regulate retinal differentiation during regrowth, we examined whether Notch regulates cell proliferation during regrowth.

Embryos injected in the left dorsal blastomere at the four-cell stage with Notch1 morpholino or treated with MG132 following surgery were fixed at 1 dps, sectioned, and stained with anti-phospho-Histone H3 (H3P) antibody, an established marker of mitosis (Adams et al., 2007; Kha et al., 2023; Miller et al., 2023). The number of H3P-positive cells was counted and then normalized to the area of the eye to account for the decreased size of the regrowth-inhibited eyes. Consistent with morphological observations (Fig. 4.3A and C), embryos with chemical (n= 13) or molecular (n=9) Notch inhibition showed either 56.7% or 45.8% reduction in the number of mitoses relative to their respective control regrowing eyes (n=10 and n=8; $p < 0.05$) (Fig. 4.4B and D). These data indicated that reduction in Notch during the regrowth period led to a reduction in RPC proliferation within the eye during regrowth.

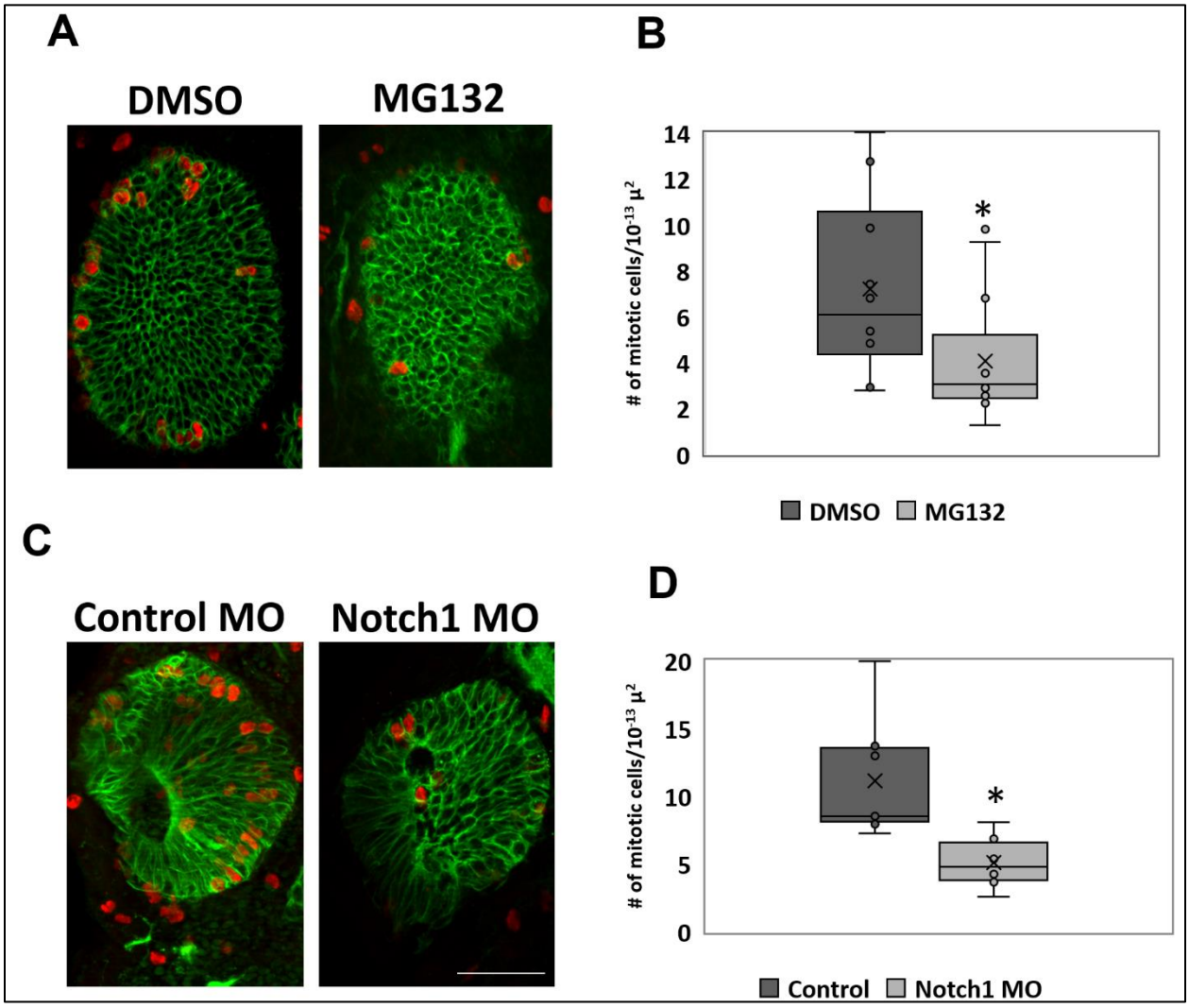


Figure 4.4 Reduction in Notch during eye regrowth reduces proliferation. (A) Transverse eye sections at 1 dps showing mitosis cells in DMSO or MG132 treated eyes (n>5 per condition). Red= phosphorylated Histone 3, Green= Xen1. Images at 20X. (B). Box and whisker plot comparing number of H3P-positive cells normalized to area. (C) Transverse eye sections at 1 dps comparing proliferating cells in control morpholino and Notch1 morpholino injected eyes (n>5 per condition). Red= phosphorylated Histone 3, Green= Xen1. Images at 20X. Scale bar= 100µm. (D) Box and whisker plot comparing number of H3P-positive cells normalized to area. * = $p<0.01$. Up= dorsal, Left= anterior.

Notch1 overexpression restores eye regrowth during V-ATPase inhibition

Although much attention has focused on the roles of well-characterized developmental signaling pathways in regrowth, there are other key mechanisms that also determine regenerative success. The Tseng laboratory recently showed that the proton pump V-ATPase is required for

eye regrowth but did not appear to have a role in eye development (Kha et al., 2023). In mouse, blockage of V-ATPase activity reduced Notch signaling leading to reduced proliferation of neural stem cells (Lange et al., 2011). We observed that the block of eye regrowth caused by Notch inhibition gave the same phenotypes as the effects of V-ATPase inhibition: small eyes and reduced cell proliferation with normal retinal patterning. As such, we asked whether Notch and V-ATPase can interact to regulate eye regrowth.

We hypothesized that V-ATPase acts upstream of Notch signaling during eye regrowth. Thus, we tested whether it was possible to rescue V-ATPase inhibition of regrowth through ectopic activation of Notch signaling. We co-injected the following: GFP mRNA along with mRNA for Dex-inducible NICD into the left dorsal blastomere at the four-cell stage; and later selected for those embryos expressing GFP in the left eye region (Fig. 4.5A). To inhibit V-ATPase activity, the highly specific inhibitor, Concanamycin A (Huss et al., 2002; Adams et al., 2006; Kha et al., 2023) was used. 20 nM Concanamycin A successfully blocked eye regrowth without affecting development (Kha et al., 2023). Following eye ablation, embryos were treated with Concanamycin A and 10 µM dexamethasone, allowing for the ectopic activation of Notch signaling in the eye region after surgery. Controls were treated with Concanamycin A only. Regrowth was assessed at 5 dps (Fig 4.5B-C). Control non-induced embryos treated with Concanamycin A resulted in 22.6% of full regrown eyes with a low RI of 131 (n=110). In contrast, embryos expressing NICD in the presence of Concanamycin A resulted in 75.5% of full regrown eyes with an RI of 260 (n=106, $p < 0.01$), representing a comparable quality of regrowth as untreated regrown eyes. Our data showed a significant rescue of V-ATPase inhibition in the embryos overexpressing NICD as compared to the control. This result showed that activation of

Notch signaling is sufficient to rescue regrowth following V-ATPase inhibition. Moreover, Notch acts downstream of V-ATPase to regulate regrowth.

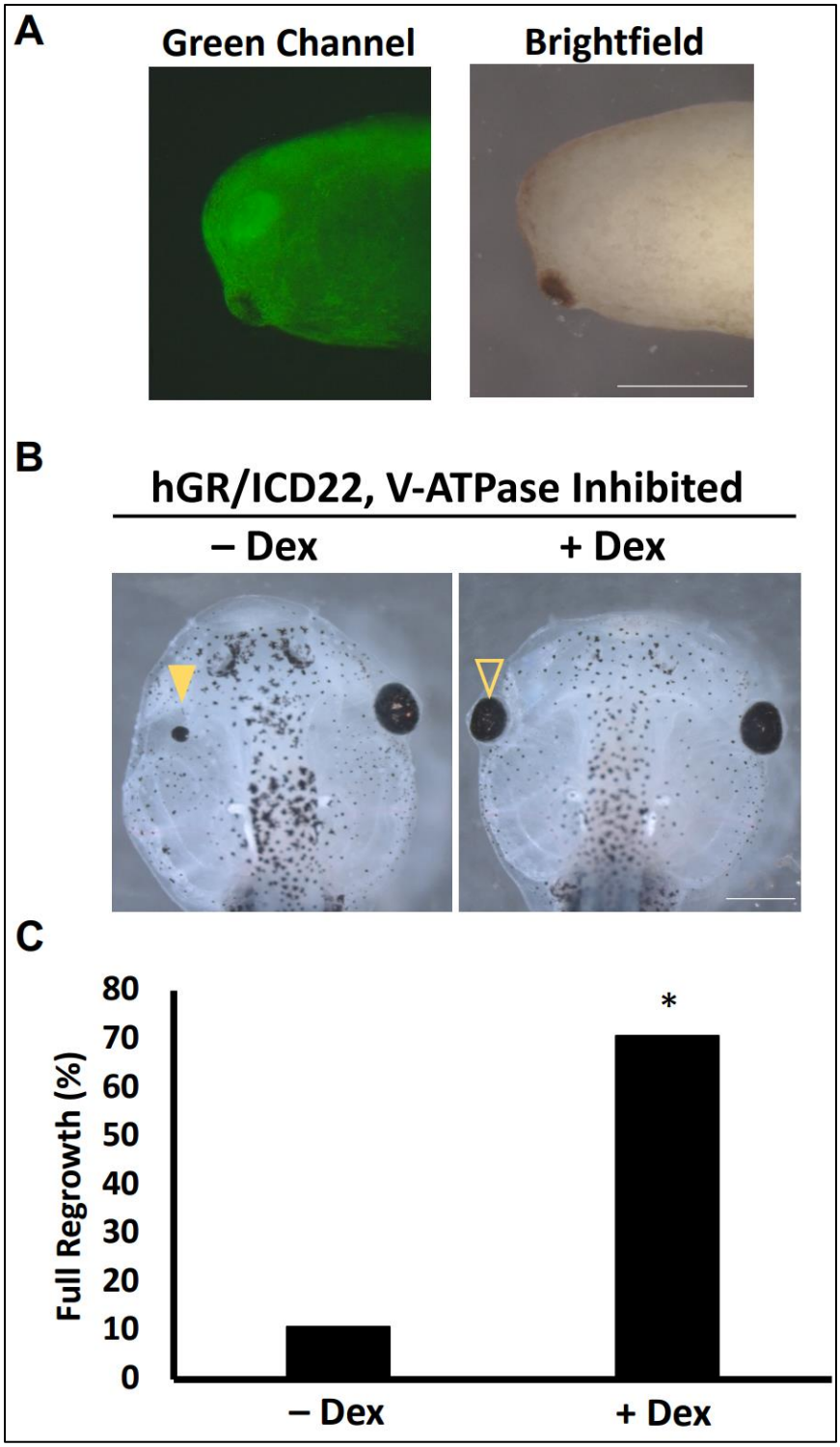


Figure 4.5. Notch1 overexpression during regrowth rescues V-ATPase inhibition. (A) Notch1 RNA is present in the eye at time of surgery. Left side of a st. 27 embryo injected at st. 3 with GR-NICD RNA and GFP RNA. Right= posterior, left= anterior, up= dorsal, down= ventral. Images at 48X. (B) Comparison of tadpoles at 5 dps of concanamycin treatment, injected with Dex-inducible NICD at st. 3 and treated with or without inducer immediately following ablation. Closed arrowhead indicates control regrowth-inhibited eye. Open arrowhead indicates rescued regrown eye though NICD overexpression. n>30 per condition. Images at 25X. Scale bar= 500µm. (C) Graphs showing percent of the population achieving full regrowth at 5 dps with or without NICD activation. * = $p<0.01$. Up= dorsal, Left= anterior.

4.5 Materials and methods

Embryo culture and surgery:

This study was carried out in accordance with the recommendations of the University of Nevada, Las Vegas Institutional Animal Care and Use Committee (IACUC). Embryos were obtained via *in vitro* fertilization and raised in 0.1X Marc's Modified Ringer (1 mM MgSO4, 2.0 mM KCl, 2 mM CaCl2, 0.1 M NaCl, 5 mM HEPES, pH 7.8) (Sive et al., 2000). Eye ablation surgery was performed as described in Kha et al., 2020. Following surgery, embryos were cultured at 22^0C.

Assessment of Eye Regrowth:

The regrowth quality of eyes treated with chemical or molecular inhibitors after surgery were compared to age-matched regrown eyes from the same batch of embryos by measuring the percent of the population achieving full regrowth and calculating the Regrowth Index (RI), a quantitative measurement where the percentage of embryos achieving each category of regrowth is assigned a numerical value, with values for the group ranging from 0-300, with 0 indicating all embryos failed to regrow eyes and 300 being all embryos fully regrew eyes, as described in (Kha et al., 2020).

Chemical Treatments, and Morpholino and RNA Injections:

Inhibitors were dissolved in DMSO. For knockdown of Notch, embryos were cultured in medium containing DAPT (Cayman Chemical, Ann Arbor Michigan, CAS number 208255-80-5) or MG132 (Cayman Chemical, Ann Arbor Michigan, CAS number 1211877-36-9) immediately following surgery and cultured in the medium for five days. Control embryos were immersed in medium containing an equivalent concentration of DMSO. To determine the temporal requirement for Notch, embryos were cultured in MG132 with varying time periods. As

needed, embryos were removed from chemical-containing media, washed in 0.1X MMR, and transferred to 0.1x MMR, or taken from 0.1x MMR at the 1 dps time point and transferred into media containing MG132 for the remainder of the regrowth period (4 days). At 5 dps, embryos were washed with fresh media, anesthetized, and regrowth was assayed.

For morpholino injections, the following published morpholinos were purchased from Gene Tools LLC (Philomath, Oregon): Notch1 5'-GCACAGCCAGCCCTATCCGATCCAT-3' (Lopez et al., 2003) and the non-specific standard control oligomer: 5' -CCTCTTACCTCAGTTACAATTTATA-3'. Each morpholino contained a 3' fluorescein addition. 2.5 ng of morpholinos were injected into the left dorsal blastomere at the four-cell stage. Embryos with fluorescent signal in the eye region at st. 27 were selected for further analysis. hGR/ICD22 and GFP were transcribed *in vitro* from linearized plasmid constructs using the mMESSAGE Transcription Kit (Thermofisher). For injections, GFP and 0.25 ng hGR/ICD22 (Coffman et al., 1993) mRNA were co-injected into the left dorsal blastomere at the four-cell stage. Embryos with fluorescent signal in the eye region at st. 27 were selected for further analysis. Dexamethasone was added to media as an inducer at a final concentration of 10 μM.

Embryo Sectioning and Immunofluorescence Microscopy:

For agarose embedding and sectioning, animals were fixed overnight at 4˚C in MEMFA (100 mM MOPS (pH 7.4), 2 mM EGTA, 1 mM MgSO4, 3.7% (v/v) formaldehyde) (Sive et al., 2000) and dehydrated in methanol. After rehydration, embryos and tadpoles were embedded in 4–6% low-melt agarose and sectioned into 60 μm slices using a Leica vt1000s vibratome. Sections were stained with primary antibodies including: Xen1 (pan-neural antibody, clone 3B1, 1:50 dilution, Developmental Studies Hybridoma Bank, RRID: AB_531871), anti-Islet1 (retinal ganglion cells and inner nuclear cell layer, clone 40.2D6, 1:200 dilution, Developmental Studies

Hybridoma Bank, RRID: AB_528315), anti-Rhodopsin (rod photoreceptor cells, clone 4D2, 1:200 dilution, EMD Millipore, RRID: AB_10807045), and anti-phospho Histone H3 (mitosis marker, 1:500 dilution, EMD Millipore, RRID:AB_310177). Alexa fluor conjugated secondary antibodies were used at 1:1000 dilution (ThermoFisher Scientific). n > 5 was used for each antibody.

Microscopy:

A Nikon A1R confocal laser scanning microscope (UNLV Confocal and Biological Imaging Core) was used to image Islet1 immunostained tissue sections. All other immunostained tissue sections were visualized via Zeiss Axio Upright Imager M2 microscope with a Hamamatsu ORCA flash 4.0 monochromatic digital CMOS camera. Images of whole animals were obtained using a ZEISS SteREO Discovery V20 microscope with an AxioCam MRc camera. ZEN Image Analysis software and/or the open-source FIJI imaging software (Schindelin et al., 2012) were used to analyze and/or process all acquired images.

Statistical Analysis:

To compare eye regrowth, raw data from scoring was used. Comparison of two treatments was analyzed with Mann-Whitney U test for ordinal data with tied ranks, using normal approximation for large sample sizes. All other experiments were analyzed using a Student's t-test.

4.6 Discussion

In this study, we show that Notch1 is a required component of successful eye regrowth in *Xenopus laevis*. This finding is consistent with previous studies linking well-known eye developmental pathways such as FGF, Pax6, retinoic acid, Wnt, and JAK/STAT, as necessary

for successful retinal regeneration (Kaneko et al., 1999; Osakada et al., 2007; Spence et al., 2008; Hochmann et al., 2012; Todd et al., 2016; Tseng, 2017; Todd et al., 2018; Kha et al., 2019; Gao et al., 2021). During *Xenopus* eye formation, Notch promotes RPC proliferation by inhibiting differentiation (Dorsky et al., 1995). Notch is also active in the ciliary margin zone (a self-renewing proliferative region located at the periphery) of the mature tadpole retina and acts to maintain retinal progenitor cells (Perron et al., 1998). We determined that Notch signaling increased retinal progenitor proliferation during regrowth. Although Notch can display pleiotropic effects, its function was not needed for retinal differentiation during regrowth. This is consistent with the observation that Notch is required during the first day of eye regrowth but not later on, when delayed retinogenesis becomes active.

In *Xenopus*, retinal differentiation starts at st. 24 and is completed by st. 42, over a period of two days. At st. 27, RPC cell division time is 8.6 hrs and it increases to 56 hrs by st. 37/38, when most cells have exited the cell cycle (Rapaport, 2006). In other types of neural stem cells, inhibition of Notch signaling has been shown to lengthen the cell cycle time (Borghese et al., 2010; Alhashem et al., 2022). These results suggest that reducing Notch during regrowth may lead to a lengthening of RPC doubling time with the consequence of a smaller RPC pool causing a failure to restore the eye to the appropriate size. Notably, not all eyes within the various Notch inhibited conditions failed to regrow, which is likely due to the fact that these are wild type animals and due to variations in injected volume as well as amount of tissue removed, which is accounted for with the large sample size. In this case, the likely role of Notch in regrowth would be to maintain the short RPC doubling time (as in st. 27) to allow for the restoration of the retinal progenitor population after eye ablation. While functionality of these smaller eyes was not tested,

previous work from the Tseng laboratory indicates that the eye is nonfunctional (Kha et al., 2018)

Multiple studies suggest a dynamic role for Notch signaling in retinal regeneration. After zebrafish retinal injury, the Müller glia responds by asymmetrically dividing to provide a neural progenitor cell population capable of regeneration (Fausett and Goldman 2006; Bernardos et al., 2007, Fimbel et al., 2007, Thummel et al., 2008). Notch signaling is upregulated during regeneration but normally acts to maintain quiescence in adult Müller glia populations by downregulating proliferation when there is no damage (Wan et al., 2012). Chick and rodent retinas undergo limited retinal regeneration. Inhibition of Notch signaling reduced progenitor cell proliferation (Hayes et al., 2007, Karl et al., 2008; Del Debbio et al., 2010). However, continued Notch signaling subsequently prevented neuronal differentiation in the chick retina. Nevertheless, Notch signaling is consistently supportive of increasing progenitor pools during retinal regeneration as is the case for *Xenopus* eye regrowth.

The mechanisms that regulate eye regrowth are beginning to be identified (Kha et al., 2018; Kha et al., 2019; Kha et al., 2023). Here, we found that ectopic expression of NICD rescued eye regrowth failure resulting from inhibition of V-ATPase. Other studies have observed similar interactions between V-ATPase and Notch. V-ATPase is expressed on cellular membranes as an essential H^+ pump. In *Drosophila*, reduction in V-ATPase activity caused disruptions in endocytic acidity, leading to defective trafficking and processing of the Notch protein (Yan et al., 2009). V-ATPase inhibition in mouse led to a reduction of Notch signaling which could be rescued by the expression of NICD but not a plasma membrane-bound form of activated Notch, suggesting that V-ATPase acted upstream of γ-secretase-dependent cleavage of the NICD (Lange et al., 2011). The regulation of Notch processing by V-ATPase could be the

mechanism that is used during eye regrowth. However, other findings suggest alternative mechanisms. The ectopic expression of a yeast plasma membrane H^+ pump, PMA, was sufficient to rescue tadpole tail and eye regrowth failures induced by V-ATPase inhibition (Adams et al., 2007; Kha 2023). As PMA is located on the cell surface, this finding suggests that it is the plasma membrane functions of V-ATPase that is essential for eye regrowth rather than its vesicular membrane roles. During tadpole tail regeneration, Notch1 RNA expression in the regeneration bud is absent when bioelectrical signaling was inhibited (Tseng et al., 2010). Additionally, transcriptional profiling of *Drosophila* neuroblasts suggested that Notch and V-ATPase also interacted in a regulatory loop (Wissel et al., 2018). Further studies will be needed to explore the specific interactions between Notch and V-ATPase in promoting eye regrowth.

Many developmental signaling pathways play an essential role in regrowth of tissues after injury. However, the specific interactions may not be the same during tissue restoration. As in development, we found that Notch promotes neural proliferation during eye regrowth (Dorsky et al., 1995; Wall et al., 2009; Schouwey et al., 2011). We also uncovered an eye regrowth pathway where Notch acts downstream of V-ATPase. Although Notch plays a key role in *Xenopus* eye development, there is no known role for V-ATPase in the same process. Here, activation of V-ATPase appears to be a regrowth-specific signal. Therefore, it would be informative to individually determine which of the common developmental pathways are active within eye regrowth and how they interact with regeneration-specific mechanisms. Furthermore, this knowledge may help to inform potential strategies for ocular repair.

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

We have demonstrated that *Xenopus laevis* embryonic eye regrowth generally recapitulates eye development, with the general differentiation order of the retinal cells: ganglion, horizontal, cone, rod, amacrine, bipolar, and finally Müller glia (Wong and Rapaport, 2009), being maintained in eye regrowth (Kha et al., 2019). The delay in development in the regrowing eye relative to the contralateral control is interesting in that there must exist a way for the eye to "catch up" to its normally developing counterpart. Additionally, we have determined that some common developmental mechanisms are utilized in eye regrowth as well. In particular, the eye-field transcription factor, Pax6 is required for regrowth (Kha et al., 2019), and this finding is consistent with the role of Pax6 in specifying the eye field during early development (Zuber et al., 2003). We have also shown that Notch signaling is required for eye regrowth. Although not an eye-specific gene, Notch is a highly conserved developmental pathway, utilized in many different developmental systems (Coffman et al., 1993; Henrique et al., 1997; Hitoshi et al., 2002; Borggrefe and Oswald, 2009, Reddy et al., 2010). We have determined that in this context Notch regulates proliferation during the highly proliferative first day of regeneration (Kha et al., 2018). It is logical, from an organism's perspective, to utilize such existing mechanisms. Why reinvent the wheel when the are already mechanisms in place to make the eye the first time? Utilizing this framework, we can extrapolate from the known regenerative mechanisms to potentially build a larger pathway of regeneration from wound healing to completion. We must not get ahead of ourselves however, as these mechanisms, though potentially following a similar signaling pathway, need to be tested on a tissue-by-tissue basis.

Perhaps the most obvious signaling mechanism shared by eye development and regeneration is Pax6. As one of the eye field transcription factors, Pax6 is required specifically for eye development (Zuber et al., 2003), and inhibition of Pax6 signaling in both *Xenopus laevis* and *tropicalis* resulted in small, malformed eyes (Rungger-Brändle et al., 2010, Nakayama et al., 2015). Pax6 is required for specification of the eye field. Ectopic injection of the eye field transcription factors in a *Xenopus* embryo is sufficient to produce ectopic eyes, complete with retina, lens, and pigmentation. The efficacy of this cocktail drops upon removal of one eye field transcription factor, but the most significant drop is from the removal of Pax6 (Zuber et al., 2003). It is no surprise then that Pax6 is required for eye regrowth in *Xenopus laevis* as well. This suggests that in order to reinitiate developmental mechanisms during regrowth, the eye field must be respecified, and it is not sufficient for the remaining eye cells alone to proliferate to "refill" the eye, which is congruous with our observations of Pax6 expression in the regrowing eye, which has a spatiotemporal pattern similar to that of an earlier stage eye until 3 dps, reinforcing the idea that regeneration is recapitulating development.

Similarly, it may come as no surprise that Notch signaling is required for *Xenopus laevis* embryonic eye regrowth. Notch signaling is required for development generally, and eye development specifically, as well as regeneration of *Xenopus* tadpole tails, zebrafish fins and spinal cord, and rat liver (Beck and Slack, 1999; Beck, Christen, and Slack, 2003; Dias et al., 2012; Kamei et al., 2012; Grotek, Wehner, and Weidinger, 2013; Zhang et al., 2018). We have shown that Notch signaling is also required for *Xenopus laevis* embryonic eye regrowth. In the context of *Xenopus* embryonic eye regrowth, it seems Notch is working within the first day of the regrowth process to regulate the proliferation of the eye progenitors. Though Notch signaling is often an inducer of proliferation (Schroeder and Just, 2000; Collesi et al., 2008; Baonza and

Garcia-Bellido, 2000), it is not always the case. In zebrafish eye regeneration, Notch signaling inhibits regeneration by maintaining the Müller glia as mature retinal cells, disallowing their dedifferentiation (Wan et al., 2012). The reason for this difference between zebrafish and *Xenopus* may be that the Müller glia are not the source of the regenerative cells in the embryonic frog, though that remains to be seen. In the adult *Xenopus*, needle poke injury to the retina triggered Müller glia cell cycle reactivation (Langhe et al., 2017) and following retinal degeneration via $CoCl_2$ injection the Müller glia proliferation increased (Parain et al., 2024). In both cases the Müller glia were not solely responsible for regeneration of the retina, but they do play a role. Thus it is important to distinguish between the different types of damage as well as timings of regeneration.

Interestingly, it seems that in *Xenopus laevis*, that Notch signaling is regulating eye regrowth only through proliferation, and perturbation does not affect differentiation. Inhibition of Notch signaling produces on average smaller eyes as compared to control, uninhibited eyes, but structurally the inhibited eyes still contain the correct retinal cell types in their correct places. It would be interesting to see the effects of Notch overexpression during regrowth on the number and location of the retinal cells. Animals in which Notch is overexpressed during regrowth appear to have physiologically "normal" eyes as judged by gross morphology, but there may be an organizational aberration that is not detected just by measuring physiology of the exterior eye. In zebrafish fin regeneration overexpression of Notch during the regrowth period causes a lack of regeneration by increasing the size of the proliferative zone in the regrowing fin, while inhibiting osteoblast differentiation (Grotek, Wehner, and Weidinger, 2013). Though osteoblasts are not present in the eye, it is possible that more Notch signaling than usual is changing the number, type, or location of the eye cells, though general eye shape and the RPE seem to be unaffected.

As many of the genes up and downstream of canonical Notch signaling are known, we could extrapolate from the requirement of Notch in *Xenopus* embryonic eye regrowth to potentially identify other genes of interest. The Tseng laboratory has recently determined that the proton pump, V-ATPase is required for embryonic eye regrowth in *Xenopus* (Kha et al., 2023). Not only do *Xenopus* fail to regrow their eyes when V-ATPase is inhibited following surgery, but inhibition of V-ATPase results in reduced proliferation within the regrowing eye (Kha et al., 2023), the same phenotype seen upon Notch inhibition. As V-ATPase is upstream of Notch signaling in a developmental context (Yan et al., 2009; Lange et al., 2011), it is likely playing a similar role in *Xenopus* embryonic eye regrowth. Additionally, we have demonstrated that overexpression of Notch signaling during regrowth is sufficient to rescue V-ATPase inhibition, strengthening the link between the two. Determining the specific interaction between V-ATPase and Notch in *Xenopus* would be an interesting next step in mapping out the regrowth pathway.

An interesting potential area of interest would be the role of reactive oxygen species (ROS) in regeneration, which have recently been gaining interest as a regenerative mechanism and potential linker between wound healing and regenerative initiation (Love et al., 2013; Ferreira et al., 2018; Kakebeen and Wills, 2019). ROS are generated during wounding and are required for regeneration of the *Xenopus* tail (Love et al., 2013; Schieber and Chandel, 2014; Ferreira et al., 2018). A fundamental protein regulating ROS following wounding is Hypoxia-Inducible Factor 1 alpha (HIF1a) (de Beaucort and Coumailleau, 2007), which is also required for *Xenopus* and gecko tail regeneration (Ferreria et al., 2018; Novianti et al., 2019). HIF1a is a regulator of Notch signaling in angiogenesis and maintenance of neural progenitors (Pistollato et al., 2010; Chen et al., 2019), and may be playing a similar role as a Notch regulator in the context of regeneration. ROS may be the bridge linking wound healing to regeneration.

Research on the role of Notch signaling in regeneration has focused either on Notch signaling broadly, or on Notch1 in particular. Mammals have four Notch protein isoforms, while *Xenopus laevis* has three. Broad chemical inhibitors usually function to inhibit cleavage of the ICD from the protein, usually by inhibiting γ-secretase. Since γ-secretase is required for all isoforms, this is sufficient to inhibit downstream signaling, but gives no indication of which Notch protein is responsible for the regenerative phenotype. Use of morpholinos or transgenics are capable of homing in on which Notch protein is responsible for regeneration, but often the focus of the study is Notch signaling broadly, and there is less interest in which of the isoforms is functioning, so Notch1 is targeted as the most well studied isoform. In the future it would be enlightening to determine what role the other isoforms have in the regenerative response, and if they potentially work together.

The general trend is that regenerative mechanisms are developmental mechanisms, but that is not true for all regenerative mechanisms. Though apoptosis is present in the *Xenopus* eye at st. 26 and remains through at least st. 35 (Hensey and Gautier, 1998), inhibition of apoptosis during eye development does not cause eye defects (Johnston et al., 2005). Apoptosis is often used during development as a modeling mechanism, most apparent in the destruction of the webbing between our fingers during our development. In the regenerating eye however, apoptosis may be necessary to remodel the eye in order for regeneration to succeed, or may be playing some other role, such as acting as a signal for proliferation. In other regenerative systems such as hydra, zebrafish tail fin, and Xenopus tadpole tail apoptosis not only acts to sculpt the new tissue, but also as an initiator of proliferation (Tseng et al., 2007; Chera et al., 2009; Beane et al., 2013; Gauron et al., 2013). We do not yet know the source population of the new eye cells, apoptosis may be required to create an environment where the eye progenitors can create a

new eye. Potentially by clearing out retinal cells that have already been specified as a particular retinal cell type, apoptosis is creating an environment more reminiscent of previous developmental stages, restarting the eye regrowth process, and inhibition of apoptosis following ~85% optic cup ablation would keep the more "mature" cells intact, presenting the eye progenitors with an environment that is not conducive to regeneration.

An important question that still remains is where are the cells for the regrown eye coming from? Of the animals that can regrow their eyes, or even just their retina, the source population of cells varies. In newt lens regeneration the pigment epithelial cells, located in the iris, dedifferentiate in order to become lens cells (Eguchi and Shingai, 1971). In zebrafish retinal regeneration the Müller glia dedifferentiate, proliferate, and reprogram to give rise to the new retinal cells (Ramachandran et al., 2010, Powell et al., 2013). In *Xenopus* lens regeneration the cornea that transdifferentiates, receiving signals from the vitreous humor upon damage, to form a new lens (Freeman, 1963; Henry and Elkins, 2001). In chick retinal regeneration, the RPE transdifferentiates in order to give rise to the removed retina, but only if some retina is left over (Coulombre and Coulombre, 1965). Just these examples demonstrate that regeneration of components of the eye can come from various sources. In the case of whole embryonic eye regrowth in our model, there are not fully differentiated tissues remaining that can transdifferentiate to give rise to a missing tissue, as in the systems listed above. We do however leave some of the optic cup behind after surgery (approximately 15%). It is possible that this remnant population contains sufficient eye stem cells to give rise to the regrown eye. This is somewhat supported by anecdotal evidence from learning the surgical techniques required, where I noticed embryos that had taken severe damage from a botched ablation failed to regrow their eyes, but this is not to be taken as a full experiment. It is also possible that removing too

much surrounding tissue removed a vital component to eye regrowth. The source population of cells will need to be determined before we can conclusively say.

As is common with regenerative and other cellular studies, the effects of genetic manipulations can vary. For example, reprogramming of differentiated cells to induced pluripotent cells by overexpression of the pluripotency factors occur at a very low rate and significant efforts have been focused on increasing the efficacy. For *Xenopus* experiments, there could be due to multiple factors. Firstly, all animals are wild type, and therefore have genetic variance between each other, possibly contributing to a lessened reaction to the inhibitor as compared to their siblings. Secondly, as the processes that are being perturbed in the studies are required for development, full inhibition is lethal. Regeneration is more sensitive to small perturbations allowing for the study of regeneration during development, utilizing concentrations that leave development unaffected. This does mean that even small variations in inhibitor amount between animals represents a relatively large difference as a percentage of the inhibitor they are "supposed to" be receiving. Thirdly, natural variance between surgeries allows for variable amounts of remnant tissue. We do not know the source of the new eye tissue, but it may be from the remnant tissue, and more remnant tissue may contribute to an increase in regrowth percentage. These caveats are accounted for in the plan of the study, with the natural variance covered with large n's.

Beyond understanding where the source of the new cells in the regrown eye, we must also ask what is the trigger for those cells to make the new eye? Assuming that the remnant optic cup cells do give rise to the new eye, what made them deviate from their path, enter a proliferative state, and reorganize themselves into the new eye? We have demonstrated that Notch is required for that proliferative state, what was the trigger for Notch to activate? It is

possible that the change in mechanical tension on the cells themselves is the catalyst for initiating regrowth. Cells within tissue pull upon each other and disruption to those forces can trigger cell signals. In *Xenopus* development, mechanical forces regulate cell migration, signal initiation, and protein sequestering (reviewed in Petridou et al., 2017). Determining the role of cell tension during eye regrowth would be an interesting future direction.

In summary, regrowth of the embryonic eye in *Xenopus laevis* recapitulates at least some of the mechanisms involved in eye development. The general order of retinal cell differentiation is recapitulated following a delay of one day, where the eye progenitors proliferate. Both Pax6 and Notch signaling are required for both eye regrowth and development, with Notch signaling playing an important role in regulating progenitor proliferation following removal. Though not all developmental mechanisms are utilized for regeneration, as is the case with apoptosis, broadly we have shown that eye regrowth is following developmental mechanisms. This research acts as a springboard to investigating other eye developmental mechanisms as potential regenerative mechanisms, to someday build a cohesive map of eye regeneration from injury to completion.

APPENDIX

FROM CELL DEATH TO REGENERATION: REBUILDING AFTER INJURY

A.1 Preface

This chapter was published in Frontiers in Cell and Developmental Biology as:

Guerin, D. J., Kha, C. X., & Tseng, K. A. (2021). From Cell Death to Regeneration: Rebuilding After Injury. Frontiers in cell and developmental biology, 9, 655048.

Author Contributions: I contributed the majority of the information collection and writing, as well as the figure. Cindy Kha and Kelly Tseng contributed writing and editing.

Editorial note: The manuscript is reproduced here under Frontiers Creative Commons license. Changes were made to the figure numbers to fit with the overall formatting of the dissertation.

A.2 Introduction

The ability to regenerate lost or damaged tissues is an impressive ability that is not common to all animals. How this feat is achieved by those that can is an ongoing question. Helpfully, a number of species with regenerative capacity are available as model organisms. Some invertebrates, such as the cnidarian *Hydra* and the planarian flatworm, display remarkable regenerative capacity. Both animals regenerate whole organisms from small body fragments (Trembley, 1744; Pallas, 1766). *Hydra* can even regenerate its entire body plan from reaggregated cells (Noda, 1971; Gierer et al., 1972). This regenerative capacity depends on reserves of active adult stem cells: pluripotent stem cells (neoblasts) in planaria and multipotent interstitial stem cells in Hydra (Wagner et al., 2011; Hobmayer et al., 2012; Scimone et al., 2014). Successful regeneration depends on these stem cells to respond to injury by proliferating

and their progeny differentiating to return the organism to full structural and functional integrity (Baguñà, 1976; Saló and Baguñà, 1984; Reddien and Sanchez Alvarado, 2004).

Although vertebrates cannot regenerate an entire animal from small body fragments, some are remarkable for their ability to regrow substantial and complex body parts. One well-studied model is the zebrafish, *Danio rerio*, which regenerates several structures including the epidermis, retina, heart, and appendages (Marques et al., 2019). Another model with high regenerative capacity is the axolotl *Ambystoma mexicanum*, which can regenerate severed limbs and damaged hearts (Joven et al., 2019). Furthermore, some amphibians, such as the clawed frog *Xenopus laevis*, display age-dependent regeneration of larval tails, limbs, and embryonic eyes (Dent, 1962; Morgan and Davis, 1902; Beck et al., 2003; Kha and Tseng, 2018; Kha et al., 2018). This age-dependent feature of *Xenopus* facilitates an examination of the mechanisms that regulate endogenous changes in regenerative capacity and to test strategies for stimulating regeneration in non-regenerative states. Even though mammalian regenerative capacity is more restricted than in these other models, mammals can regenerate some tissues, including the liver and digit tips in mice and humans (Higgins and Anderson, 1931; Illingworth, 1974; Han et al., 2008), and the entire epidermis in the African spiny mouse (Seifert et al., 2012).

Across species and tissues, the broad steps of regeneration after injury are as follows: a successful wound healing response, the initiation of regeneration, followed by cell proliferation and cellular differentiation to rebuild lost tissues (reviewed in Gurtner et al., 2008; Pfefferli and Jaźwińska, 2015; Kakebeen and Wills, 2019). The source of the contributing cell population varies between systems. Unlike invertebrates, vertebrate regeneration appears to often be achieved through the use of lineage-restricted progenitor cells, such as for limb regeneration in zebrafish (Poss et al., 2000; Stewart and Stankunas, 2012), axolotl (Kragl et al., 2009; Makanae

et al., 2014), and *Xenopus* (Gargioli and Slack, 2004). Other mechanisms such as transdifferentiation to regenerate amphibian lens are also used (Eguchi, 1963; Freeman, 1963). The proliferation and differentiation of these cells to regenerate the lost organ requires the action of complex mechanisms, only some of which have been characterized.

One important mechanism in regeneration is apoptosis. Apoptosis, a type of programmed cell death, is a fundamental and evolutionarily conserved process (Metzstein et al., 1998). Apoptosis is required for organogenesis, tissue remodeling, homeostasis, wound healing, and regeneration (Elmore, 2007; Li et al., 2010; D'Arcy, 2019). Dysregulation of apoptosis can have severe consequences including cancer and autoimmune disorders (Goldar et al., 2015). Apoptosis is initiated by the cleavage of inactive initiator caspase proteins to expose their catalytic domains, which allow them to activate executioner caspases through subsequent cleavage events (Cohen, 1997). The executioner caspases then initiate a cascade of events resulting in the breakup of the cell into smaller apoptotic bodies. These are engulfed by macrophages, completing the process (Budai et al., 2019).

Apoptosis has been widely studied in many contexts and in diverse organisms (reviewed in Brill et al., 1999; Pérez-Garijo and Steller, 2015; Tuzlak et al., 2016). Apoptotic cells have been shown to exert diverse non-autonomous effects on neighboring cells through the release of mitogenic factors, inducing cell proliferation (Morata et al., 2011). The role of apoptosis as a regenerative mechanism was more recently identified. Prior work determined that apoptosis is required for regeneration across multiple organisms and tissues (Tseng et al., 2007; Chera et al., 2009; Li et al., 2010; Sîrbulescu and Zupanc, 2010; Gauron et al., 2013; Kha et al., 2018; Brock et al., 2019). How apoptosis promotes successful regeneration is beginning to be understood. In this review, we discuss the requirement of apoptosis in different regenerative contexts, the

initiators and downstream effects of apoptosis during regeneration, and gaps in the field. We focus on recent advances that highlight the importance of apoptosis as a specific response to stimulate and regulate regeneration and not merely as a consequence of tissue damage.

A.3 Initiation and Regulation of Regenerative Apoptosis

Apoptosis is commonly observed starting early in the regeneration process. Programmed cell death has long been known to contribute to wound healing after injury (Greenhalgh, 1998). However, apoptosis has additional and separable functions specific to regeneration after the initial wound healing phase. Pathways regulating these apoptotic events have been identified in some models.

Apoptosis occurs during the early phases of regeneration in two peaks in some regenerative systems. In *Hydra* and planaria, the first peak of apoptosis occurs from 1 to 4 h after bisection (Chera et al., 2009; Beane et al., 2013). At 3 days, there is a second peak of apoptosis (Pellettieri et al., 2010). A similar pair of apoptotic peaks is seen during adult zebrafish fin regeneration from 1 to 12 h post amputation (hpa) and 15 to 72 hpa. The second peak of apoptosis is specific to regeneration, as simple wounding of the fin that healed quickly failed to induce this second peak (Gauron et al., 2013). In Xenopus laevis tadpole tail regeneration, there is only one sustained increased in apoptosis at the injury site. Apoptosis is absent during the wound healing phase and is first activated during formation of the regeneration bud at 12 hpa and remains active during the entire initial proliferative phase from 12 to 48 hpa (Tseng et al., 2007).

An important regulator of apoptosis in regeneration is BMP signaling (Guimond et al., 2010). BMP signaling regulates anterior-posterior patterning, proliferation, differentiation, and apoptosis in the developing vertebrate limb (Pignatti et al., 2014). Here, BMP activates apoptosis

in the apical ectodermal ridge and the limb mesenchyme (Gañan et al., 1998; Guha et al., 2002). BMP signaling is also required for regeneration of axolotl limbs (Guimond et al., 2010; Vincent et al., 2020). In this system, the regulation of apoptosis by BMP2 appears to function the same for both the developing and regenerating limb (Guimond et al., 2010). The overexpression of BMP2 increased apoptosis in the regenerating limb, while overexpression of the BMP inhibitor Noggin caused a decrease in apoptosis relative to controls (Guimond et al., 2010). A similar mechanism is also seen in mouse digit formation, where inhibition of BMP via Noggin caused a reduction in apoptosis in the inter-digit region, resulting in a flipper like appendage instead of a hand (Wang et al., 2004).

Another well-known signaling pathway active in apoptosis induction during regeneration is the Jun-N terminal Kinase (JNK) signaling pathway (Santabárbara-Ruiz et al., 2015; Diaz-Garcia et al., 2016; Camilleri-Robles et al., 2019). JNK regulates apoptosis in the developing brain. A knockout of Jnk1 and Jnk2 caused both reduced apoptosis in the hindbrain and increased apoptosis in the forebrain (Kuan et al., 1999). JNK signaling is required for both apoptosis and regeneration following bisection in planaria (Almuedo-Castillo et al., 2014). In *Drosophila* wing imaginal disc regeneration, JNK signaling is required to induce apoptosis (Diaz-Garcia et al., 2016). Together, the studies suggest that JNK plays a regulatory role for apoptosis in development that may act similarly in regenerative apoptosis.

There is an interesting link between apoptosis and reactive oxygen species (ROS). ROS are detected early in regeneration and are necessary for regeneration in multiple species (Love et al., 2013; Ferreira et al., 2018; Novianti et al., 2019). Perhaps not coincidentally, ROS are important for apoptosis-dependent regeneration. In zebrafish fin regeneration, ROS levels increased at the injury site immediately following amputation and continued rising for 16 h

before returning to baseline. The high levels of ROS around 15 hpa correlated closely with the beginning of the second round of apoptosis. Additionally, ROS levels peaked at 2 h after wounding injuries to the fin that did not require tissue regeneration, suggesting a specific importance of ROS for apoptosis-dependent regeneration (Gauron et al., 2013). ROS are also required for regeneration of the *Drosophila* imaginal disc, where they regulate JNK signaling (Santabárbara-Ruiz et al., 2015). The intersection of ROS, JNK signaling, and apoptosis is an exciting direction for further investigation.

A.4 Regulation of Regenerative Mechanisms by Apoptosis

Apoptosis can promote proliferation during development, notably in the developing *Drosophila* imaginal discs where apoptotic cells promote compensatory proliferation in neighboring cells (reviewed in Diwanji and Bergmann, 2018). Although the specific roles of apoptosis in regeneration are still being explored, it is known that apoptosis can drive proliferation during regeneration in *Hydra*, planaria, *Xenopus*, and zebrafish. As proliferation is a critical aspect of regeneration (Gargioli and Slack, 2004; Jopling et al., 2010; Kha et al., 2018; Stocum, 2019), apoptosis is therefore an important regulator of regeneration.

Apoptotic cells can act as initiators of cell signaling in development (Morata et al., 2011). This is also true in regenerative contexts, for example the importance of apoptotic cells as a source of Wnt3 during *Hydra* head regeneration (Chera et al., 2009). Wnt/β-catenin signaling is also stimulated by apoptosis in zebrafish epithelium regeneration (Brock et al., 2019). In both systems, the Wnt ligand is found in apoptotic bodies and engulfed by neighboring stem cells to induce proliferation. This Wnt-induced proliferation is required for regeneration, as inhibition of Wnt signaling abolished regeneration (Chera et al., 2009; Brock et al., 2019). The observation of apoptosis-induced Wnt-dependent proliferation in both an invertebrate and a vertebrate is

exciting, suggesting a potential conserved regenerative mechanism that could be tested as a strategy to induce mammalian regeneration.

Apoptotic cells secrete additional mitogenic signals in a number of models (Ryoo and Bergmann, 2012). Apoptotic cells in Drosophila secrete Hedgehog (the ortholog of vertebrate Sonic hedgehog) to induce proliferation during eye development (Fan and Bergmann, 2008). Prostaglandin signaling downstream of Caspase activity induces proliferation in mouse cells (Li et al., 2010), with a similar role proposed in zebrafish hematopoiesis (North et al., 2007). Whether apoptosis induces proliferation through these pathways during regeneration remains unclear.

Apoptosis as a patterning mechanism has been extensively studied in development (reviewed in Pérez-Garijo and Steller, 2015; Lin and Xu, 2019). However, much less is known about the patterning role of apoptosis in a regenerative context. An excellent example of an apoptosis requirement for pattering in regeneration is its asymmetric distribution and differential function following bisection in planaria and *Hydra*. Organismal bisection generates both anterior and posterior segments. Although each fragment undergoes apoptosis after bisection, the number of apoptotic cells is significantly higher in the head-regenerating posterior fragment than the tail-regenerating anterior fragment (Chera et al., 2009; Pellettieri et al., 2010). In *Hydra*, inhibition of apoptosis in the head-regenerating fragment blocked head regeneration. However, apoptotic inhibition of the foot-regenerating fragment did not block foot regeneration. Ectopic induction of apoptosis in the foot-regenerating fragment resulted in animals with heads in the presumptive foot region (Chera et al., 2009). These findings showed that a higher level of apoptosis is needed for head restoration, whereas a lower level is sufficient for induction of proliferation. In planarian regeneration, the second apoptotic peak is regulated by bioelectrical signaling and

required for proper head patterning but not cell proliferation. Inhibition of the ion transporter H+, K+-ATPase caused a reduction of apoptosis and resulted in a shrunken head due to the lack of adjustment in organ size and placement (Beane et al., 2013). These studies indicate that apoptosis plays an instructive role in regenerative patterning of complex tissues.

A.5 The Goldilocks Principle in Regenerative Apoptosis

Apoptosis is a potent mechanism that needs to be tightly controlled in regeneration. As in the tale of Goldilocks, the level of apoptosis needs to be "just the right amount" for successful regeneration. During regeneration, unchecked apoptosis could deplete the tissue of the cellular materials necessary to regenerate if it outpaces proliferation. Although one functional consequence of apoptosis is to promote proliferation, apoptosis itself is not always a marker of regeneration, even in normally regenerative tissues. For example, apoptosis is necessary for regeneration of the *Xenopus* tadpole tail. However, if the tail is amputated during the refractory period when the tadpole temporarily loses its tail regeneration ability, there is increased activated Caspase-3 activity relative to the regenerative tail (Tseng et al., 2007), suggesting that there is likely a limiting mechanism where a specific level of apoptosis is required for regeneration. Similarly, if the salamander limb is denervated during regeneration, the normally regenerative tissues will morphologically regress due to increased apoptosis (Mescher et al., 2000). During mouse liver regeneration, Nitric oxide synthase-deficient animals exhibit both increased apoptosis and decreased regeneration relative to controls (Rai et al., 1998).

IAPs (Inhibitors of apoptosis proteins) are known regulators of apoptosis in development and disease yet their role in regeneration is unclear. Bcl-2 orthologs are expressed during axolotl limb regeneration (Bucan et al., 2018). However, the expression patterns of IAPs in other models have not been defined. Overexpression of p35 (a baculoviral caspase inhibitor) showed only a

minor effect on Drosophila wing disc regeneration (Diaz-Garcia et al., 2016). Similarly, Bcl-2 overexpression did not enhance axonal regeneration of retinal ganglion cells in mice (Inoue et al., 2002). Further molecular and functional studies are needed to assess the potential roles of IAPs in regulating regenerative apoptosis.

A.6 Mammalian Regeneration: Apoptosis in the Regenerating Liver

Mammals, including humans, have limited regenerative ability but are able to regrow lost liver tissues following partial hepatectomy—resection of up to two thirds of the liver (Higgins and Anderson, 1931). Apoptosis in this context also differs somewhat from its conserved role in the models discussed in the earlier sections. An additional complexity in studying the role of apoptosis in liver regeneration is that different regenerative mechanisms can be activated depending on the method of injury (reviewed in Fausto et al., 2006; Mao et al., 2014). Following partial hepatectomy or acute chemical damage to the mammalian liver, TNF is released, stimulating ROS and NF-κB to induce apoptosis. Apoptosis induced neighboring progenitors to proliferate (Czaja et al., 1989; FitzGerald et al., 1995). Consistent with this finding, mice deficient in both Caspase-3 and Caspase-7 showed decreased liver progenitor proliferation, and impaired wound healing and regeneration following partial hepatectomy (Li et al., 2010). The liver is a distinct case from the earlier examples because mammalian liver regeneration results in the regrown liver achieving its former size but not its former shape. These may constitute distinct regenerative responses, which in turn may involve different roles for apoptosis.

A.7 Discussion and Future Directions

Apoptosis is a key mechanism of regeneration. Although apoptosis is demonstrably required for regeneration across diverse organisms and tissues, some important aspects of early

stages of regeneration during which apoptosis is active are unknown. The roles of JNK, BMP and Wnt signaling pathways as inducers of apoptosis provide exciting leads but open questions remain (Figure A.1). It is known that ROS are produced by macrophages following wounding (Bae et al., 2009). Moreover, the immune system is implicated in regulating endogenous regenerative ability (Fukazawa et al., 2009; reviewed in Mescher, 2017). A key aspect of the immune response is efferocytosis, the phagocytic clearance of apoptotic cells. In mice, reduced efferocytosis of apoptotic cardiomyocytes induced by myocardial infarction led to enlarged infarct size (Wan et al., 2013). In *Hydra* head regeneration, an immediate and large wave of efferocytosis by endodermal epithelial cells (immune-like cells) was seen beneath the injury plane (Chera et al., 2009) but whether this process plays an active role in regeneration is unknown. Regardless, these studies suggest that understanding the role of efferocytosis in regenerative apoptosis could be a promising area for investigation.

Another important question is which cell types are targeted for apoptosis and where they are located, which has been answered in only a few systems. Even among more fully characterized systems, such as *Hydra* head regeneration, only a portion of the interstitial stem cells undergo apoptosis, which leads to the question of whether there is a specific induction program or if it is a stochastic process. Additionally, in most systems, regenerative events must coordinate the outgrowth of multiple tissue types (Mochii et al., 2007; Lehoczky et al., 2011). Does each lineage undergo a separate round of apoptosis or is there one global apoptotic event that affects all lineages? One tool for addressing this question is the use of fluorescent reporters for caspase activation, allowing real time imaging of caspase activity (Bardet et al., 2008). Another powerful tool for answering this question is single-cell RNA sequencing, which allows for the analysis of individual cells during regeneration.

It is unknown whether regenerative apoptosis functions in the same manner across the multiple organisms and tissues in which it is found. Wnt ligands found in apoptotic bodies during zebrafish epithelium and *Hydra* head regeneration act to promote proliferation (Chera et al., 2009; Brock et al., 2019). That this mechanism is conserved between an invertebrate and a vertebrate suggests that this potentially can be used to stimulate regeneration in non-capable tissues. Additionally, optogenetic tools can be used for spatiotemporal specificity in inducing apoptosis in the tissues of interest after injury (Jewhurst et al., 2014).

The mechanisms linking apoptosis to regeneration remain largely elusive. An intriguing finding is that apoptosis but not JNK signaling induced expression of a pluripotency marker during zebrafish fin regeneration, suggesting that regenerative apoptosis may influence cellular reprogramming (Gauron et al., 2013). Investigators may also turn to the regulation and functions of apoptosis in development for clues since developmental mechanisms are often used in regeneration (Beck et al., 2003; Lin and Slack, 2008; Taniguchi et al., 2014). However, the developmental role of apoptosis is not always recapitulated in regeneration. In zebrafish fin regeneration, JNK signaling induced proliferation but apoptosis induction is JNK independent (Gauron et al., 2013). In *Xenopus*, tail formation does not involve apoptosis, but apoptosis is required for tail regeneration (Johnston et al., 2005; Tseng et al., 2007). In this context, understanding how regenerative apoptosis is induced may provide strategies for stimulating regeneration in tissues where apoptosis does not normally play a developmental role.

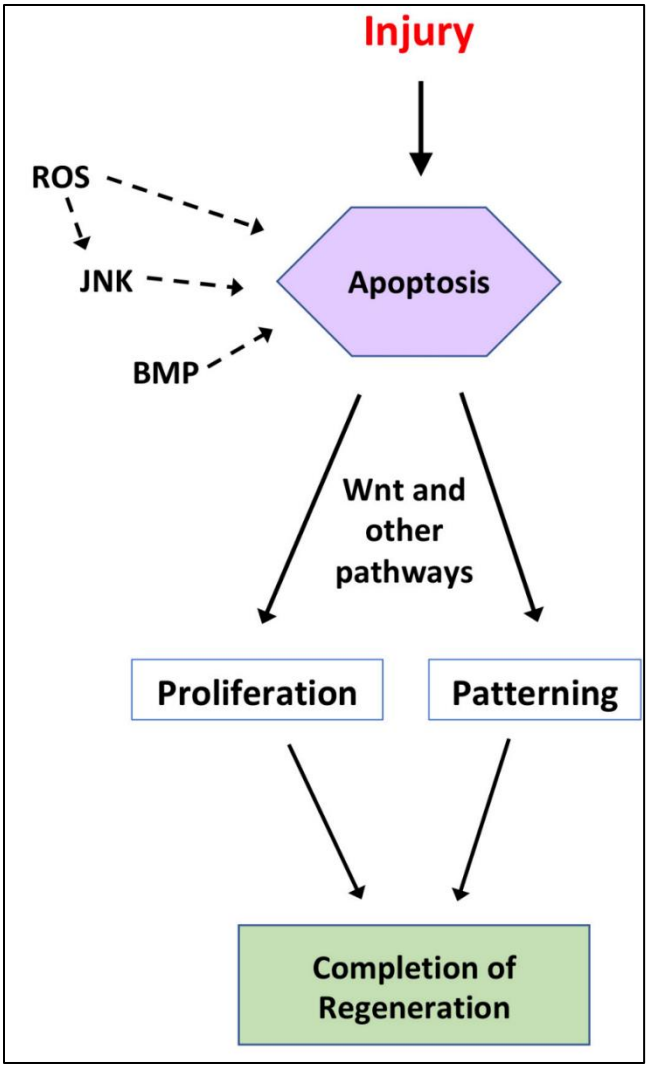


Fig A.1 Summary of the regulation and roles of apoptosis during regeneration. Following injury to a tissue, apoptosis can act as a regenerative mechanism, depending on the organism and tissue in question. In some regenerative systems, apoptosis is regulated by ROS, JNK, and/or BMP signaling. In turn, apoptosis regulates proliferative and/or patterning signals, such as Wnt signaling, to drive initiation and completion of regeneration. This figure is not intended to be comprehensive and represents an amalgamation of studies from several model systems discussed in the text.

The role of apoptosis in regeneration is an important one that merits a detailed investigation. Apoptosis can control the microenvironment in which tumors arise, making the

study of apoptosis important for cancer treatment (Gregory et al., 2016). Similar to cancer, regeneration induces excessive cell proliferation—in this case, to restore a lost structure. In contrast to cancer, regenerative proliferation is tightly controlled such that the process terminates once the missing structure is restored. Comparative studies of apoptosis in cancer and regeneration may help to delineate the differences in controlled vs. dysregulated proliferation. Further investigations into this topic may provide new perspectives in understanding the functions of apoptosis in diseased tissues.

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CURRICULUM VITAE

Dylan Guerin

Dylanguerin1776@gmail.com

Teaching Experience

Laboratory instructor for BIOL 196/190, Introduction to Biology- 2017, 2018, 2020, 2021, 2022, 2023, University of Nevada, Las Vegas

- Instructed first year students in the basics of biology
- Led laboratory discussions about the conceptual material as well as laboratory methods and reagents
- Wrote and graded quizzes as well as graded laboratory reports
- Performed one on one instruction during office hours
- Provided feedback to students both in person and through Canvas
- Proctored exams

Discussion instructor for BIOL 304, Molecular Genetics- 2019, University of Nevada, Las Vegas

- Led a large discussion group of upper level students covering advanced topics in genetics
- Wrote and graded quizzes
- Performed one on one instruction during office hours
- Provided feedback to students both in person and through Canvas
- Proctored exams

Research and laboratory mentorship- 2019-2023

- Mentored two undergraduate students in laboratory techniques including making reagents, use of instruments, tissue care and preparation, and immunohistochemistry
- Trained all incoming members of the lab in proper care of the animals in our aquatics facility

Education

Ph.D. in Biology

University of Nevada Las Vegas, Las Vegas NV

Expected graduation date- May 2024

Bachelor of Science, Biochemistry

University of New England, Biddeford ME

Graduation - May 2013

Graduated *Cum Laude*

Research Experience

Graduate Student, School of Life Sciences, Lab of Dr. Ai-Sun Tseng

September 2017- Present: University of Nevada Las Vegas, Las Vegas NV

The Tseng lab studies the molecular and genetic mechanisms underlying regeneration in vertebrates using the frog *Xenopus laevis* as a model organism. My project in the Tseng lab is focused on characterizing the role of the Notch signaling pathway in *Xenopus laevis* embryonic eye regrowth. This work sheds light on important biological mechanisms that form the foundation for the design of regenerative therapies in the future.

Laboratory Technician, Thayer School of Engineering, Lab of Dr. Margaret Ackerman

October 2016- July 2017: Dartmouth College, Hanover NH

Work in the Ackerman lab is focused on the validations of novel vaccines for a range of diseases including HIV, polio, and influenza. My role in the lab was to run assays to screen for drug-drug interactions in order to verify the safety of treatments

Laboratory Technician, Geisel School of Medicine, Department of Pharmacology and Toxicology, Lab of Dr. Carmen Marsit

December 2013-May 2016: Dartmouth College, Hanover NH

Work in the Marsit lab is focused on elucidating how methylation of target genes affects postnatal health. My role in the lab was to isolate and sequence DNA from mothers and infants to determine target gene methylation.

Undergraduate Researcher in Biochemistry, Department of Chemistry and Physics, Lab of Dr. Stephen Johnson

September 2010- May 2013: University of New England, Biddeford ME

This study focused on the expression of a phytase gene in *E. Coli*. My work involved the incorporation of the phytase gene into *E. Coli* to induce expression.

Work Experience

November 2016- July 2017	**Laboratory Technician, Thayer School of Engineering, Dartmouth College, Hanover NH 03755**
December 2013- May 2016	**Laboratory Technician, Geisel School of Medicine, Dartmouth College, Hanover NH 03755**
May 2010 - December 2013	**Inpatient Pharmacy Technician, Dartmouth Hitchcock Medical Center, Lebanon NH 03756**
May 2009 - August 2009	**Bus Boy, Three Tomatoes Trattoria, Lebanon NH 03766**
May 2007- August 2009	**Line Cook, Mickey's Roadside Café, Enfield NH 03748**

Publications

* Denotes co-first authorship

Guerin, D.J., Gutierrez, B., Zhang, B., and Tseng, K.A. In submission. Notch is Required for Neural Progenitor Proliferation During Embryonic Eye Regrowth.

Guerin, D. J., Kha, C. X., and Tseng, K. A. (2021). From Cell Death to Regeneration: Rebuilding After Injury. Frontiers in cell and developmental biology, 9, 655048.

Kha, C.X.*, **Guerin, D.J***., and Tseng, K.A. (2020). Studying In Vivo Retinal Progenitor Cell Proliferation in Xenopus laevis. Methods in Molecular Biology. 2092, 19-33

Kha, C.X.*, **Guerin, D.J***., and Tseng, K.A. (2019). Using the Xenopus Developmental Eye Regrowth System to Distinguish the Role of Developmental Versus Regenerative Mechanisms. Frontiers in Physiology. 10:502

Conradt, E., Ostlund, B., **Guerin, D**., Armstrong, D.A., Marsit, C.J., Tronick, E., LaGasse, L., and Lester., B.M. (2019). DNA methylation of NR3c1 in infancy: Associations between maternal caregiving and infant sex. Infant Mental Health Journal. 40, 513-522

Green, B.B., Armstrong, D.A., Lesseur, C., Paquette, A.G., **Guerin, D.J**., Kwan, L.E., & Marsit, C.J. (2014). The Role of Placental 11-β Hydroxysteroid Dehydrogenase Type 1 and Type 2 Methylation on Gene Expression and Infant Birth Weight. Biology of Reproduction. 2015 Mar. 18

Conradt, E., Fei, M., LaGasse, L., Tronick, E., **Guerin, D**., Gorman, D., Marsit C.J., & Lester, B.M. (2015). Prenatal Predictors of Infant Self-Regulation: The Contributions of Placental DNA Methylation of NR3C1 and Neuroendocrine Activity. Behavioral Neuroscience. 2015 May. 29

Green, B.B., Kappil, M., Lambertini, L., Armstrong, D.A., **Guerin, D.J**., Sharp, A.J., Lester, B.M., Chen, J., & Marsit, C.J. (2015) Expression of Imprinted Genes in Placenta is Associated with Infant Neurobehavioral Development. Epigenetics. 2015 July. 1

Paquette , A.G., Lester, B.M., Lesseur, C., Armstrong, D.A., **Guerin, D**., Appleton, A.A., & Marsit, C.J. (2014). Placental Epigenetic Patterning of Glucicorticoid Response Genes is Associated with Infant Neurodevelopmental Outcomes. Epigenomics. 2015 Aug. 7

Conradt, E., Hawes, K., **Guerin, D**., Armstrong, D.A., Marsit, C.J., Tronick, E., Lester, B.M., (2016). The Contributions of Maternal Sensitivity and Maternal Depressive Symptoms to Epigenetic Processes and Neuroendocrine Functioning. Child Development. 2016 Feb. 87

Green, B.B., Houseman, E.A., Johnson, K.C., **Guerin, D.J**., Armstrong, D.A., Christenson, B.C., Marsit, C.J., (2016) Hydroxymethylation is uniquely distributed within term placenta, and is associated with gene expression. FASEB. 2016. April. 26.

Armstrong, D.A., Green, B.B., Blair, B.A., **Guerin, D.J**., Litzky, J.F., Chavan, N.R., Pearson, K.J., Marsit, C.J., (2016) Maternal smoking during pregnancy is associated with mitochondrial DNA methylation. Environmental Epigenetics. 2016 Oct. 20

Poster Presentations

2021 South-West Developmental Biology Conference

2021 Nevada Space Grant Fellowship Conference

Awards and Funding

Awarded the Nevada NASA space grant

UNLV GPSA Research Forum, University of Nevada Las Vegas: first place in poster session

HHMI fellowship- Awarded to attend the Xenpus course at Cold Spring Harbor.

UNLV Graduate and Professional Student Association Student Researcher Award- Secured funding from UNLV GPSA during multiple semesters.

Selected as the spotlight student for the Cold Spring Harbor *Cell & Developmental Biology of Xenopus* course. Interviewed and featured in the monthly Cold Spring Harbor newsletter.

Workshops

Cold Spring Harbor *Cell & Developmental Biology of Xenopus* course, Cold Spring Harbor NY, 2019: Selected to attend to meet with other students and experts in the *Xenopus* field at a two-week-long workshop where I learned multiple techniques for working with *Xenopus*.

Community Service

UNLV Science Café co-organizer, 2019-present

- Plan and co-host the event
- Write questions for trivia

Your Brain on Art exhibit, Engine Studios, Biddeford ME, December 2012

- Helped set up an exhibit focusing on artists experience with chronic pain.