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The Role of Intermediate Filament Proteins in Retinal Degeneration and Regeneration

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The Role of Intermediate Filament Proteins in Retinal Degeneration and Regeneration

A Capstone Project Submitted in Partial Fulfillment of the
Requirements of the Renée Crown University Honors Program at
Syracuse University

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and Renée Crown University Honors
May 2016

Honors Capstone Project in Biology

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Stephen Kuusisto, Director

Date: April 27, 2016

Abstract

More than 30 million Americans are blind or visually impaired due to injury or disease. Retinitis pigmentosa is a genetic disease that initially causes death of rod photoreceptor cells and is followed by the progressive degeneration of the retina, the light sensing tissue of the eye, ultimately leading to blindness. Retinitis pigmentosa leads toretinal gliosis, a process in which a specialized neuroglial cell that is specific to the retina, known as Müller glia, undergoes hypertrophy and migration as a reaction to retinal stress or damage. I am investigating the mechanisms that are responsible for this type of degeneration in the African clawed frog (*Xenopus laevis*), as well as those that allow regeneration of the rod photoreceptor cells in this model organism. Intermediate filament proteins (IFPs) are hypothesized to contribute to progressive retinal degeneration and may inhibit retinal regeneration. IFPs are cytoskeletal components of cells responsible for structural and mechanical support, and are thought to be upregulated during a process known as gliosis. Genomic analysis of the *Xenopus* genome has led to the hypothesis that this species does not contain the gene for one of the class III IFPs known as Glial Fibrillary Acidic Protein (GFAP). Therefore, degeneration may be regulated by one of the other class III and IV IFPs including Vimentin (Vim), Peripherin (Prph), Desmin (Des), and α-Internexin (Ina). Using immunohistochemistry and *in situ* hybridization, I examined the expression patterns of IFPs to determine where the proteins and corresponding mRNA localize in the retina and brain. I found that two GFAP antibodies used in previous publications have distinct expression patterns in *Xenopus* retina. Additionally, I found that three of the five class III IFPs are expressed in the retina. Since GFAP is not present in the *Xenopus* genome, the results suggest that the GFAP antibodies must be nonspecific. Given the high level of sequence similarity among the IFPs, and their response to retinal injury, the IFPs Vimentin and Peripherin may be the immunogens detected by the anti-GFAP antibodies used in previous publications. Since gliosis and progressive retinal degeneration are observed in *Xenopus* following retinal injury, future research should investigate the expression patterns of these IFPs in injured retinas using a controllable ablation model.

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Executive Summary

In the United States alone, there are more than 30 million adults that are blind or suffer from visual impairment due to injury or disease. Retinitis pigmentosa is a genetic disease that affects one in 4,000 people worldwide (National Eye Institute 2014). Otherwise known as rod cone dystrophy, retinitis pigmentosa is characterized as a progressive loss of rod photoreceptor cells caused by an improperly made protein (Hartong et al. 2006). This protein is integral to the function of cells that convert photons of light into vision. In the retina, or the light sensing tissue of the eye, there are two types of photoreceptor cells: rods and cones. Rods are responsible for vision in low light environments and are more numerous, while the cones are responsible for color vision. Within the retina is a specialized type of neuroglia known as Müller glial cells. Neuroglia provide mechanical support and protection for neurons. In humans, the loss of rod cells due to diseases such as retinitis pigmentosa result in a reactive process of Müller glia known as retinal gliosis. Following retinal injury, Müller glial cells will grow in size and extend projections that form a gliotic scar.

Progressive retinal degeneration in humans results in increased production of intermediate filament proteins (IFPs), which are responsible for structural and mechanical support in a cell. There are six different classes of IFPs, with class III being of the most importance to retinal gliosis. These include Vimentin (Vim), Desmin (Des), Peripherin (Prph), Internexin-α (Ina-a), and Glial Fibrillary Acidic Protein (GFAP). GFAP is known to be produced in excess during retinal injury and is of special interest in the present study.

African clawed frogs (*Xenopus laevis*) have the ability to regenerate rod cells and essentially regain sight after injury. This makes it a good model system for studying retinal

regeneration. I used a specific line of frogs that is susceptible to the antibiotic Metranidazole. In the presence of the antibiotic, the rod cells in these frogs are selectively destroyed or ablated. This results in a controllable rod ablation model that can also be reversed and allow for a specific time of regeneration.

Previously, GFAP was shown to be produced in excess during retinal injury in *Xenopus*. We believe that the antibodies used in this study may have been recognizing another closely related protein rather than GFAP specifically, because our lab has been unable to find GFAP in the *Xenopus* genome. Antibodies work as tags that attach to proteins that they recognize. They can be labeled with fluorescent markers, so we can visualize where the antibodies attach in the organism. Because closely related proteins can be bound by the same antibody, if GFAP is not part of the genome, then it seems likely that the previous results were caused by binding of the antibodies to proteins other than GFAP. To examine this hypothesis, we tested two GFAP antibodies (DAKO & Sigma G3893) used in a previously published paper on the retinas of *Xenopus* tadpoles to see if they showed identical expression patterns. We found that the GFAP antibodies had distinct expression patterns, suggesting that they either aren't recognizing the same part of a protein or aren't recognizing the same protein at all.

We then performed *in situ* hybridization to detect the pattern of mRNA (protein coding sequence) expression on retinal sections for each of the five IFPs. If we know where mRNA is within an organism, we can tell where that protein is being made. With this test, the mRNA of a specific protein that we are looking for will be stained purple, and we can visualize where the mRNA is located in the organism. Of the five genes we tested (GFAP, Vim, Des, Prph, and Ina-a) we found that Vimentin, Peripherin, and Internexin-α were expressed in the retina. Vimentin

was the only one that was found within the retinal layers and likely to be in the Müller cells, which is the site of the detected antibody expression following retinal injury.

The next step is to investigate how the expression patterns of these proteins change following injury. To accomplish this, the controllable rod ablation *Xenopus* lines will be used, and so we needed to ensure that results would be consistent among the different individuals used in experiments. Using the controllable ablation model described above, I determined if there was a difference in the rates of retinal degeneration of the progeny from different adult females. I found that there was variation within the lines, but there was no significant difference in degeneration rate between tadpoles generated from the three tested females, meaning that embryos any of the three female lines could be used interchangeably in future experiments.

This work provides preliminary data for further experiments investigating the role of IFPs during retinal injury. Gaining knowledge about this specific class of proteins may also provide insight into how a lack of GFAP might in part be responsible for the ability of *Xenopus* to regenerate rod photoreceptors.

Acknowledgements

I have received incredible support and guidance throughout this journey and process. I would like to thank Dr. Zuber for his unwavering belief in my capabilities, and for affording me the opportunity to be a part of the incredible work that he and his lab does. As my research and capstone adviser he has given me direction during every step of the process and has been a role model and leader during my undergraduate studies.

Additionally, I would like to thank Dr. Reyna Martinez-De Luna and Ray Ku for working side by side with me and teaching me with patience and expertise. They both have gone above and beyond to lend a hand or give me advice, not just about lab work but also about life as a female scientist. Thank you Dr. Viczian as well for sharing your wisdom and kindness, teaching me that there's always time for cake between experiments.

I would also like to thank Dr. Segraves for advising me throughout my undergraduate career, and for taking on the task of being my capstone reader. She has guided me through the process of finding who I am as a presenter and helped me to clarify my ideas about the future.

The Reneé Crown Honors Program has been pivotal in shaping my undergraduate education and providing me with the Crown-Wise scholarship that funded and allowed me to continue my research into the summer months.

Lastly, I need to thank my friends and family for their continuing support and relentless encouragement. Without them I never would have began my journey at Syracuse University, and certainly would not have had the confidence to leap, knowing I had someone to catch me if I fell. To my parents- I'll love you forever and like you for always.

Advice to Future Honors Students

Utilize the incredible resources that are your adviser and reader. No matter how much you love your topic, this process will prove to be harder than you think. That being said, do the work ahead of time. Ask questions that are backed by thorough research, not just because you don't want to find the answer yourself.

Write one sentence every day. April creeps up faster than you think it will, and even if what you wrote makes no sense in two months, you'll be glad to have something to work with.

Most importantly, cherish every class you take and professor you work with while in the honors program. The opportunities that are presented to you by the incredible faculty and staff will further your capstone in some way, even if you don't realize it at the time.

<div align="center">**Project Body**</div>

Introduction

Over 30 million adults in America suffer from blindness or visual impairment, and this number is projected to double by the year 2050 (Vision Problems). A number of factors that can lead to blindness, including cataracts, glaucoma, macular degeneration, diabetic retinopathy, and retinitis pigmentosa. Retinitis pigmentosa is a genetic disease that usually starts showing symptoms during middle age. The first symptom is difficulty seeing at night, and as the disease progresses, it eventually results in complete blindness due to the impaired functioning of the retina, the light sensing tissue of the eye (Hartong et al. 2006). In the retina there are two classes of light capturing cells known as rod and cone photoreceptors. In many cases of retinitis pigmentosa a protein within the rods known as rhodopsin is made incorrectly (Hartong et al. 2006). When this protein does not function normally, rod photoreceptors die. As a result of the rod photoreceptors dying, the cones also die by an unknown mechanism. Currently, there is no cure or successful treatment for retinitis pigmentosa (Hartong et al. 2006). Understanding the cellular and biochemical mechanisms that underlie this disease and the process that leads to blindness is key to finding a way to combat retinitis pigmentosa and other blinding diseases. To do this, we must first understand the retina and the key players involved in retinal degeneration.

Within the retina there are six distinct layers of cellular and synaptic layers (Figure 1). Closest to the lens side of the eye is the ganglionic layer composed of ganglion cells and their nerve fibers that eventually join together to form the optic nerve. Next is the inner plexiform layer (IPL) that consists of the synapses between bipolar cells and ganglion cells, and is followed by the inner nuclear layer (INL) that is composed of the nuclei of amacrine and bipolar cells. Next is the outer plexiform layer (OPL) that contains the synapses between the bipolar cells and

the photoreceptor cells, and the outer nuclear layer (ONL) contains the nuclei of photoreceptor cells. The outermost layer of the retina is the photoreceptor layer, which contains the outer segments of two types of photoreceptor cells known as rods and cones. It is here where a photon of light is transformed into an action potential, ultimately causing a nerve impulse to be sent to the brain where it is interpreted as vision.

There are two types of photoreceptors found in the retina. Rod cells are responsible for vision in low light environments, and cone cells detect color and function poorly in low light settings. As these cells die and degeneration increases in severity, a person's sight worsens, and eventually they will become blind. This occurs in any damage or disease that leads to the death of rod photoreceptors.

As light enters the eye, a photon first strikes the back of the retina, where it activates a change in the confirmation of the protein known as rhodopsin within the outer segment of the photoreceptor rod cell. From there, the cell hyperpolarizes and creates a cascade through all the retinal layers until it reaches the ganglion cell. The ganglion cell creates an action potential that travels down the axon that eventually forms a nerve bundle known as the optic nerve. This action potential eventually reaches the brain, where sensory information is interpreted.

Spanning across most of the retinal layers in humans are 8-10 million Müller cells, specialized neuroglial cells found only in the retina. In a general sense, neuroglial cells act to support the function and health of nerve cells. Müller cells provide mechanical support for photoreceptors and regulate the concentration of potassium ions and neurotransmitters in the retina, which are required for normal functioning of photoreceptors and other neural cells (Bringmann & Wiedemann 2012).
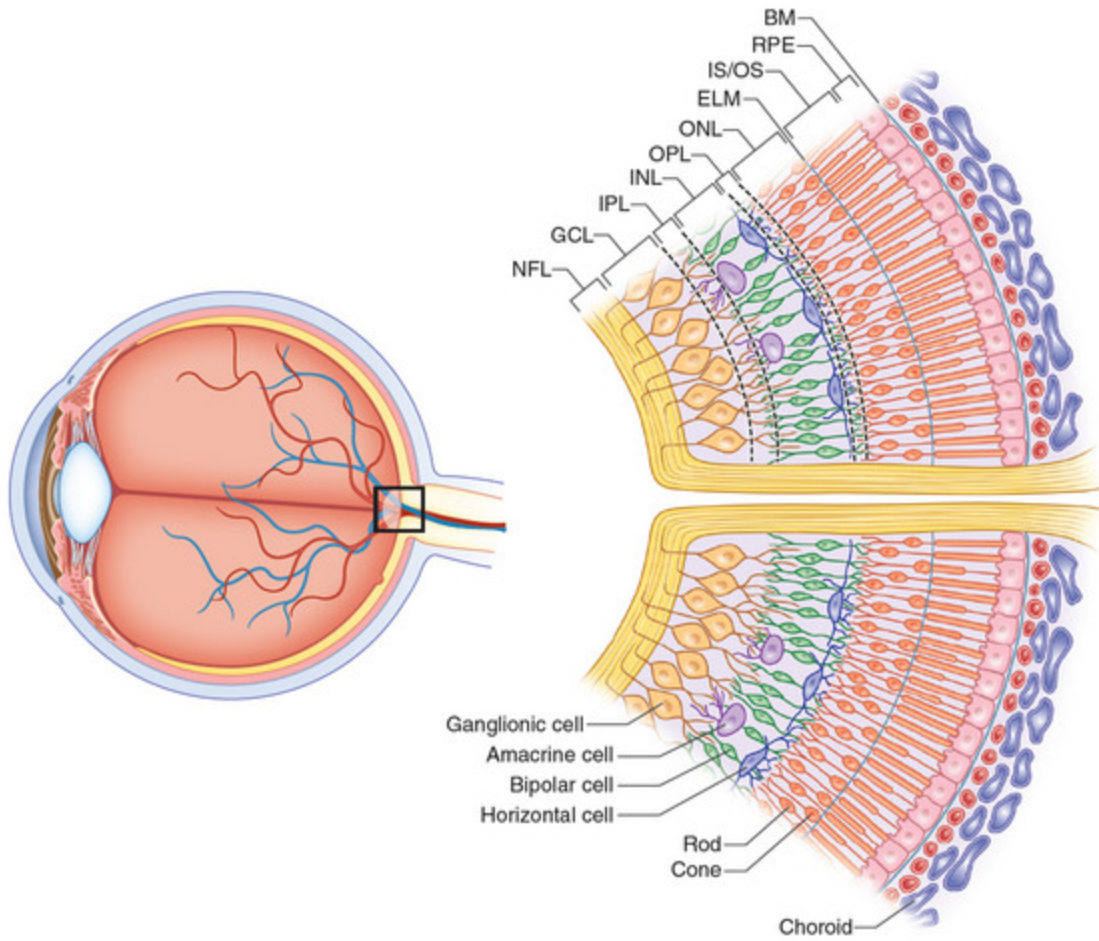
Figure 1. http://www.retinareference.com/anatomy/ Visual of cellular and synaptic layers within retina. Nerve fiber layer (NFL), Ganglion cell layer (GCL), Inner plexiform layer (IPL), Inner nuclear layer (INL), Outer plexiform layer (OPL), Outer nuclear layer (ONL), External limiting membrane (ELM), Rod and cone inner and outer segments (IS/OS), Retinal pigmented epithelium (RPE), Basal membrane (BM)

Retinal gliosis is a condition in which Müller cells react to damage/stress of the retina. Triggered by rod cell death in the human eye, IFPs are produced in excess (Tassoni et al. 2015). This is hypothesized to hinder the regeneration of rod photoreceptors because their induction is correlated with hypertrophy (growth in size) of the Müller cells (Bringmann & Weidemann 2012) along with the sprouting of processes, and when these supportive cells become enlarged, they take up space and form a fibrous layer on the retina known as a gliotic scar that may prevent the regeneration of rod cells (Bringmann & Weidemann 2012) (Figure 2). The death of rod cells leads to the subsequent death of cone cells by an unknown mechanism. Although there are detrimental effects on the retina, the ultimate purpose of gliosis is to seal off the injured portion of the retina to prevent the spread of damage. The effect of Müller gliosis on a retina is both helpful and detrimental; by releasing antioxidants and neurotrophic factors, gliosis can be neuroprotective (Bringmann et al. 2006).

An important characteristic of retinal gliosis is the upregulation of Intermediate Filament proteins (IFPs), the key components in the cytoskeleton of a cell which are responsible for structure and mechanical support. IFPs are organized into six different classes. Class III and IV are of most importance to the nervous system and retina. Class III consists of Glial Fibrillary Acidic Protein (GFAP), Vimentin (Vim), Peripherin (Prph), and Desmin (Des); and class IV consists of the neurofilaments α-Internexin (Ina) and Nestin (Nes). These IFPs are present in the cells that compose the six retinal layers. Although it is known that GFAP is upregulated in Müller cell gliosis in humans (Bringmann et al. 2009), there is still much to be learned about the role of IFPs in response to retinal injury including how and why they are upregulated, and if these mechanisms are conserved across species.
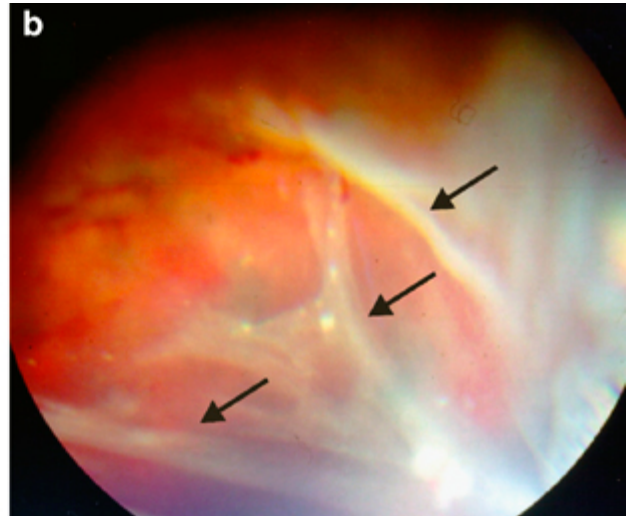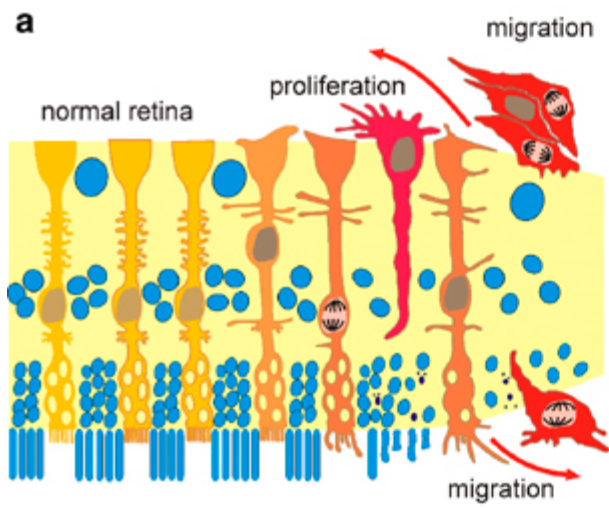
Figure 2. Taken from Giaume et al. 2007; a) cartoon visual of Müller cell undergoing gliosis and forming a scar; b) retinal folding and detachment caused by proliferating and migrating cells

The African clawed frog, *Xenopus laevis*, has the ability to regenerate their rod photoreceptor cells after ablation (Choi et al. 2011), making *Xenopus* an excellent model system in which to study retinal regeneration. Moreover, *Xenopus* eyes are structurally similar to many organisms, including humans, yet develop at a much faster rate, making experimentation on a reasonable timescale feasible.

To investigate the relationship between gliosis, degeneration and retinal regeneration, we used a transgenic strain of frogs as our model system (XOPNTR). XOPNTR frogs express a bacterial gene called nitroreductase (NTR), under the control of the rhodopsin promoter (XOP), resulting in rod photoreceptor-specific expression of NTR. Nitroreductase converts the antibiotic metronidazole (Mtz) into a toxin that causes rod cell-specific ablation. This model mimics rod degeneration in the eyes of human patients with retinitis pigmentosa because of its specificity to the rods. Rod ablation only occurs in the presence of Mtz, thus making a controllable model of rod ablation. This model is also reversible, allowing us to study the ability of retinal regeneration. By altering the length of the Mtz treatment, we can control the degree of degeneration and regeneration that we want to study.

Previously, it has been reported that GFAP has been observed in normal and injured *X. laevis* retina. Based on a genomic and synteny analysis done by others in our lab, we believe that the *X. laevis* genome lacks GFAP, and may have been lost over the course of evolution (Martinez-De Luna et al. 2016). We hypothesized that the anti-GFAP antibodies used in this study may be recognizing one of the other closely related IFPs rather than GFAP.

| Healthy Retina | Retina with Ablated Rods |
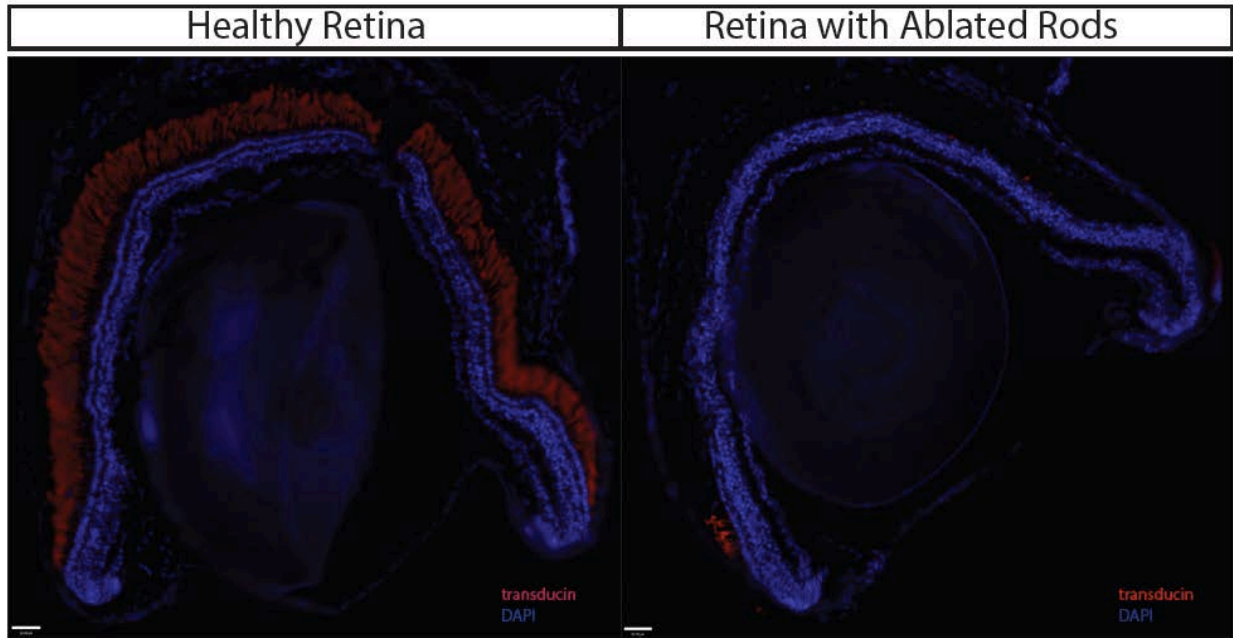
Figure 3. Visual of Health and Injured Retina
Immunolabeled cross section of tadpole retina. The red areas are immunolabeled rod cells, the blue are nuclei of individual cells. On the left panel there are healthy rod cells around the entire retina, this individual has full vision. On the right panel there are few rod cells, only on the progenitor region. This is a XOPNTR animal that has received Mtz treatment and therefore is blind.
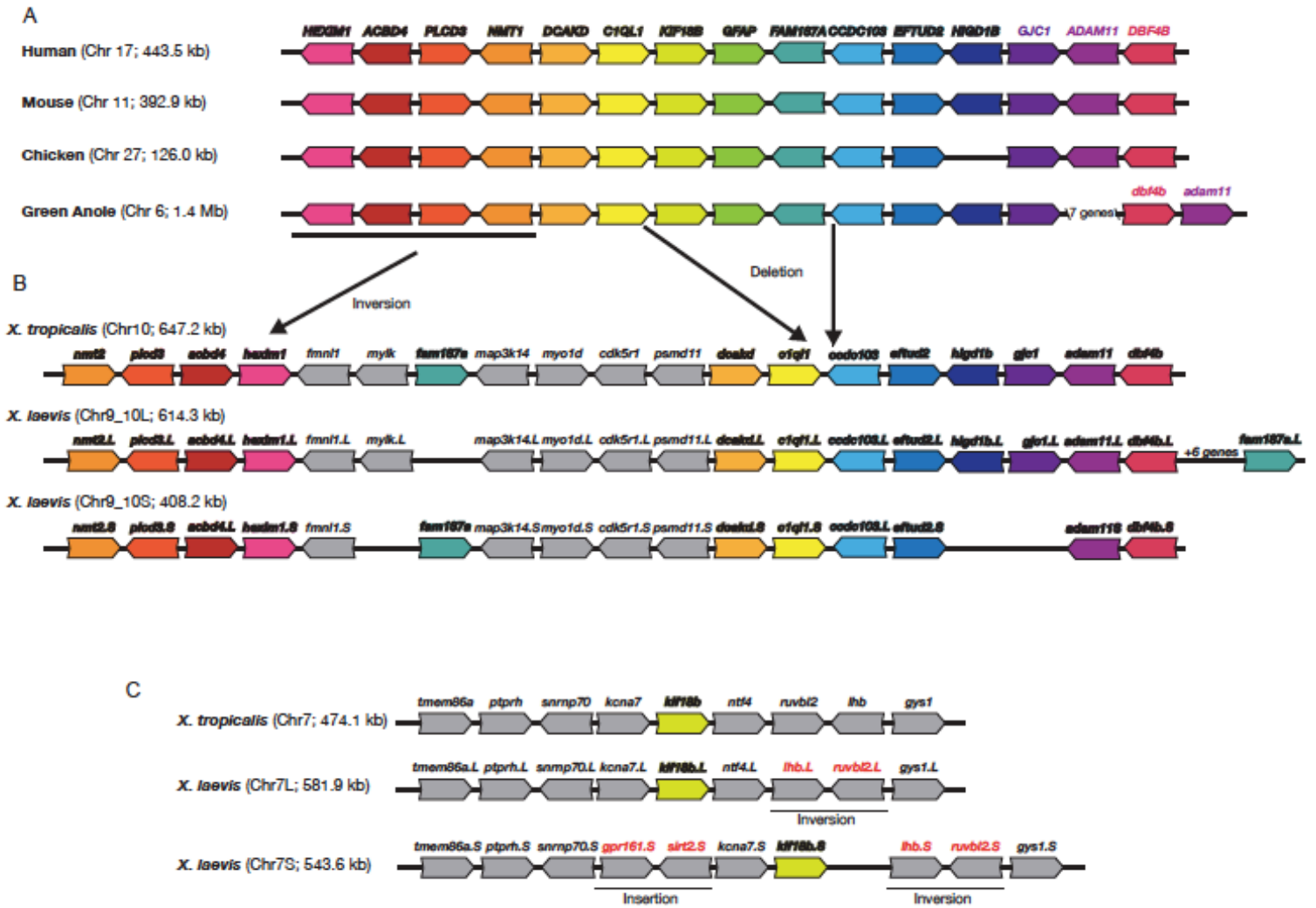
Figure 4. Taken from Martinez-De Luna et al. 2016; Syntenic analysis showing chromosomal rearrangement across species that may have resulted in deletion of *gfap* gene
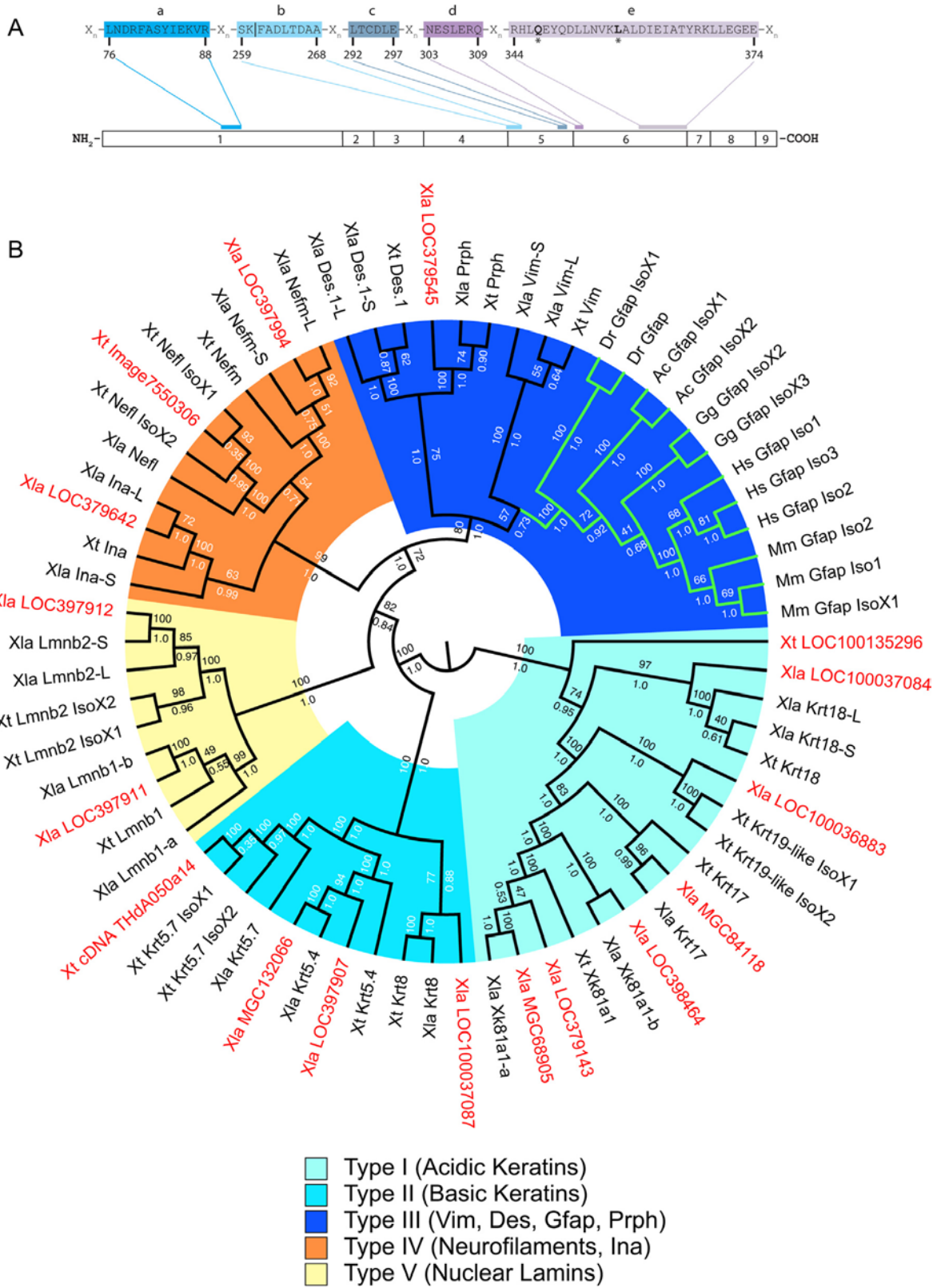
Figure 5. Taken from Martinez-De Luna et al. 2016; Genomic analysis of *Xenopus* A) conserved *gfap* sequences used to distinguish it from other IFPs B) Cladogram showing unknown *Xenopus* sequences in red and known in black, with other GFAP orthologs

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Our overall hypothesis is that the IFPs regulate retinal degeneration and regeneration. The exact mechanisms remain unknown, but if this is true, the absence of GFAP in *X. laevis* may, in part, be the reason *Xenopus* can regenerate their retinal cells whereas mammals cannot. Based on the previously obtained data that suggests that that *Xenopus* lack GFAP, here we investigate whether the immunoreactivity of anti-GFAP antibodies in the *Xenopus* retina is distinct. If there is reactivity in the glial cells and this matches the patterns of other IFP expression, then we hypothesize that the GFAP antibodies are likely recognizing another closely related IFP. Although we hypothesize that IFPs are key players in retinal degeneration, it was still unknown where in the retina these proteins are overexpressed once gliosis was induced. Likewise, it was unknown in what cells and where within those cells these proteins are expressed under normal conditions.

Future studies in my lab will go on to investigate the expression patterns of IFPs in injured retinas, and how this would compare to expression patterns of uninjured retinas. To lay the ground work for these future studies, I compared the rates of degeneration between the progeny of three different adult females to determine which female line should be used for experiments. This was done using the same controllable rod ablation model described above.

Based on this information, three main questions were addressed:

1) Are antibodies that purportedly recognize GFAP in *X. laevis* actually recognizing the GFAP protein?

2) What are the expression patterns of IFPs in uninjured *X. laevis* retina? That is, where in the retina are the class III and IV IFPs being transcribed? Additionally, are these proteins being expressed in the brain tissue, and if so, where?

3) Is there a difference in the rate of rod ablation in the progeny of different female transgenic frogs?

<h1 style="text-align:center">Methods</h1>

Testing GFAP Antibodies on Retinal and Brain Sections

Embryo Fixation, Tissue Isolation, and Sectioning

Untreated tadpoles were kept in frog water (0.5% sea salt; 2mM potassium chloride) in a temperature controlled room set to 22°C. Once they reached stage 50, they were anesthetized using 1% Tricaine and rinsed in 1X PBS. The eyes were removed, and brains and spinal cords were dissected out, fixed in 4% paraformaldehyde (PFA) for 1 h, placed in 20% sucrose overnight at 4°C and then embedded in optimal cutting temperature compound (OCT, Tissue-Tek). Samples were cryostat sectioned at 16μm.

Immunohistochemistry

Slides were thawed at room temperature for 30 min, washed in PBS+0.1% Triton X-100 three times, and blocked for 1 h in 5% heat-inactivated goat serum in 0.1% Triton in PBS. Slides were incubated in primary antibodies overnight at 4°C (Table 1), and in the secondary antibodies for 2 hours at room temperature (Table 2). DAPI (1:1000) was added during the incubation with the secondary antibody.

Table 1. Primary Antibodies

Antibody	Source	Catalog #	Dilution
Polyclondal rabbit Anti-GFAP	Dako	Z0334	1:500
Monoclonal mouse Anti-GFAP	Sigma	G3898	1:200

Table 2. Secondary Antibodies

Antibody	Source	Catalog #	Dilution
Alexa 488 goat anti-mouse IgG	Invitrogen	A-11001	1:500
Alexa 555 goat anti-rabbit	Invitrogen	A21428	1:500

Image Acquisition

Images were acquired using a Leica DM6000 B upright microscope (Leica Microsystems, Bannockburn, IL) fitted with a Retiga SRV camera (Q-Imaging). Auto-exposure was used to determine the exposure time for each filter. The long-pass green filter (wavelength 488) was used for the Sigma antibody and the Cy3 filter (wavelength 555) was used for DAKO.

In Situ Hybridization of Class III IFPs on Retinal and Brain Tissue

Animals, Tissue Isolation, and Sectioning

Tadpoles were grown to stage 50 in frog water. Tadpoles were then anesthetized in 1% methanesulfonate (Tricaine ; Sigma Aldrich, St. Louis, MO). Eyes, brain, and spinal cord were isolated, and fixed in 4% paraformaldehyde (PFA) for one hour, soaked in 20% sucrose for 30 minutes in 4°C, then embedded in optimal cutting temperature compound (OCT, Tissue-Tek) and stored at -80°C. Blocks were cryostat sectioned at 12µm. Slides were dried for 2 h at room temperature then stored at -20°C.

Probe Design

RNA probes used in *in situ* hybridization were designed for each of the five IFPs under study. The probe mix was composed of 2μL of 10X DIG labelling mix, 2μL of 10X transcription buffer, 1μg of linearized template DNA of each respective IFP, 1μL of RNA Polymerase Plus, and filled to a final volume of 20μL with Nuclease-free water. Reactions were held at 37°C for 2 h, followed by adding 1μL of turbo DNase I and incubated in a 37°C water bath for 10 min. 1μL of mixture was tested on a 1% agarose gel run for 10 min to ensure the plasmid was cut. Next, 1μL of 0.5 M EDTA pH 8.0 was added to each mixture, followed by 1.5 μL of LiCl and 66μl of ribonuclease-free 100% ethanol. Mixtures were incubated overnight at -20°C, then centrifuged at 15,600 rcf for 15 min at 4°C. The supernatant was removed and 100μl of ribonuclease-free 75% ethanol was added. The mixture was then centrifuged at 15,600 rcf for five min at 4°C. All excess ethanol was removed and the tubes were dried on ice for 7 min. Pellets were resuspended in 20 μL of ribonuclease-free water. Probes were then resuspended in hybridization buffer (50%: 100% deionized formamide, 15%: 5M NaCl, 10%: 10X PE, 1%: 5% heparin, 5%: 20% SDS, 18%: ddH2O) to the DNA concentration of 20μg/mL. Probes were stored at -20°C.

In Situ Hybridization

Day 1: Slides were thawed at room temperature for 30 min, then washed in 1X PBS for 5 min, washed in 100% methanol for 10 min, and washed in PTw (10%: 10X PBS, 1%: 10% Tween-20, 89%: ddH2O) 3 times for 5 min each. Slides were then treated with 10μg/mL Proteinase K in PTw for 30 s, then washed in 2mg/mL Glycine in PTw twice for 2 min. Next, slides were fixed in 0.2% glutaraldehyde in 4% paraformaldehyde (PFA) for 10 min. Slides were then washed in PTw 3 times for 2 min each. Slides were then washed in 0.1% sodium borohydride in PTw for 10

min, and once again washed in PTw three times for 2 min. Next, slides were washed twice in hybridization buffer for 2 min each, then prehybridized in hybridiztion buffer for 30 min in a 60°C water bath. Next, 100 µL of probe was added to the coverslip, and placed on the slide. Coverslips were sealed with synthetic resin slide mounting medium (DPX; Sigma Aldrich, St. Louis, MO) and dried for 1 h before being placed in a 60°C slide heather overnight.

Day 2: The synthetic resin slide mount medium and coverslips were removed, followed by the slides being washed once in hybridization buffer for 15 min in a 60°C water bath, then washed twice in wash buffer 1 (6%: 5M NaCl, 10%: 10X PE, 5%: 20% SDS, 79%: ddH2O) for 15 min each in a 60°C water bath, then washed twice in wash buffer 1.5 (1%: 5M NaCl, 10%: 10X PE, 0.5%: 20% SDS, 88.5%: ddH2O) for 15 min each in a 50°C water bath. Slides were then rinsed in NTE buffer (10%: 5M NaCl, 1%: 1M Tris-Cl pH 8.0, 0.2%: 0.5 M EDTA, 88.8%: ddH2O) for 2 min, followed by incubating in 100µg/mL RNaseA in NTE for 45 min in a 37°C incubator. Slides were once again rinsed in NTE buffer for 2 min, then washed in wash buffer 2 (50%: 100% Formamide, 6%: 5M NaCl, 10%: 10X PE, 5%: 20% SDS, 29%: ddH2O) for 15 min in a 50°C water bath, washed in wash buffer 3 (50%: 100% Formamide, 3%: 5M NaCl, 10%: 10X PE, 1%: 10% Tween-20, 36%: ddH2O) for 15 min in a 50°C water bath, washed twice in wash buffer 4 (10%: 5M NaCl, 10%: 10X PE, 1%: 10% Tween-20, 79%: ddH2O) for 2 min each at room temperature, then once in wash buffer 4 at 70°C for 10 min. At room temperature, slides were then treated with a MABT and BMBR mixture (10%: 10X MAB, 1%: 10% Tween-20, 20%: 10% BMBR, 69%: ddH2O) for 30 min, followed by treatment with anti-DIG (1:2000) in the MABT and BMBR mixture overnight.

Day 3: Slides were washed 3 times for 10 min each with 1X MABT (10%: 10X MAB, 1%: 10% Tween-20, 89%: ddH2O), then washed twice for 5 min each in AP Development Buffer (0.5%:

1M Levamisole (Tetramisole hydrochloride; alkaline phosphatase inhibitor; Sigma Aldrich, St. Louis, MO), 10%: 1M Tris-Cl pH 9.5, 2%: 5M NaCl, 10%: 0.5 M MgCl2, 1%: 10% Tween-20, 76.5%: ddH2O). Slides were then left overnight in BM Purple Solution (Roche; Basel, Switzerland) mixed with 2mM Levamisole.

Day 4: Slides were washed 3 times for 5 min each in PTw & EDTA (10%: 10X PBS, 1%: 10%Tween-20, 0.2%: 0.5M EDTA, 88.8%: ddH2O), then fixed in 4% paraformaldehyde (PFA) for 30 min, washed in 1X PBS twice for 2 min each, and coverslipped with 50% glycerol. After they dried, slides were stored at 4°C.

Image Acquisition

Images were acquired using a Leica DM6000 B upright microscope (Leica Microsystems, Bannockburn, IL) fitted with a Retiga SRV camera (Q-Imaging). The color filter was used with a bright field light source.

Rates of Degeneration in XOPNTR Progeny

Animals

Embryos were collected from three different XOPNTR transgenic females. 100 tadpoles each were collected from females 13 and 16, and 48 were collected from female 21. Tadpoles from each female were divided into two treatment groups, and half were used for this experiment.

Metronidazole Treatment

Metronidazole at a concentration of 10 mM (Mtz; Sigma Aldrich, St. Louis, MO) was dissolved in frog water, then 0.2% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) was added. Animals were treated for six days in tanks with 20mL of water per tadpole at 22°C in the dark and recovered at 19°C with light exposure for one day. BrdU (1mM; Sigma Aldrich, St. Louis, MO) was added to the Mtz solution starting on day four of treatment.

Embryo Fixation and Sectioning

On day seven, 25 tadpoles from each tank of females 13 and 16, and 12 of female 21 were collected and anesthetized with 1% methanesulfonate (Tricaine ; Sigma Aldrich, St. Louis, MO). After rinsing in 1X PBS, tails were removed and heads fixed in 4% paraformaldehyde (PFA) for one hour, cryoprotected in 20% sucrose overnight at 4°C, embedded in optimal cutting temperature compound (OCT, Tissue-Tek), and cryostat sectioned at 12 μm.

Immunohistochemistry

Slides were thawed at room temperature for 30 mins, washed in PBS+0.1% Triton X-100 three times, and blocked for 1 h in 5% heat inactivated goat serum in 0.1% Triton in PBS. The primary antibodies used were rabbit anti-transducin (1:100), mouse anti-R5 (1:5), and mouse anti-BrdU (1:20) (Table 3). The secondary antibodies used were goat-anti mouse IgM Alexa Fluor 546 (1:500) and goat-anti-rabbit IgG Alexa Fluor 647 (Table 4). Slides were incubated with primary antibodies 4°C overnight, and in secondary antibodies at room temperature for 2 h. DAPI (1:1000) was added during incubation in the econdary antibody.

Table 3. Primary antibodies.

Antibody	Clone	Company	Dilution
Anti-transducin	Sc389	Santa Cruz Biotechnology	1:100
R5	monoclonal	W.A. Harris; Cambridge University	1:5
Anti-BrdU	G3G4	DSHB	1:20

Table 4. Secondary antibodies

Antibody	Product no.	Company	Dilution
Goat anti rabbit IgG Alexa 647	A21236	Invitrogen	1:500
Goat anti mouse IgM Alexa 546	A21045	Invitrogen	1:500
Goat anti mouse IgG Alexa 488	A11001	Invitrogen	1:500

Image Acquisition

Images were acquired using a Leica DM6000 B upright microscope (Leica Microsystems, Bannockburn, IL) fitted with a Retiga SRV camera (Q-Imaging). Images of transducin signal were all acquired using the same exposure time under the filter Cy5 (wavelength 647). The exposure was set to 80% of the transducin fluorescence detected in wild type sibling control animals. Exposure time for R5 was also uniform for all pictures, and was set to 80% of the fluorescence detected in tadpoles carrying the XOPNTR transgene. Images were obtained from three consecutive central retina sections of both eyes.

Measurements

Signal above threshold (SAT) (arbitrary units) and retinal distance measurements were obtained using Volocity (Perkin Elmer). Signal intensity was set between 454 and 1000. Transducin SAT

detection was restricted to the area above the inner nuclear layer excluding any signal observed in the retinal pigment epithelium (RPE). Signal observed in the RPE was excluded because of its phagocytic behavior, suggesting that the transducin was causing fluorescence in dead cells. Retinal distance was measured in retinal sections along the perimeter of the inner nuclear layer to the tip of the ciliary marginal zone. SAT/μm was calculated by dividing SAT value by the retinal distance.

Statistics

A one-way ANOVA test was used to test if there was a difference between the average SAT/μm from each animal. Post-hoc tests were used to analyze whether there was a statistical difference between the SAT/ μm averages from each line.

Results

GFAP Antibody Staining

The DAKO and Sigma anti-GFAP antibodies showed distinct expression patterns in the retina, brain, and spinal cord (fig. 6). In the retina, both antibodies had fibrous recognition patterns, and spanned the multiple layers of the retina (fig. 6 A, B). The DAKO antibody stained more toward the ganglion cell layer (fig. 6 A', B'), while the Sigma antibody didn't stain as strongly in that area and showed more specific recognition down into the OPL and ONL (fig. 6 A", B").

In the brain there was background fluorescence for both antibodies, but there remained portions of the recognition pattern that did not overlap (fig. 6 C, D). DAKO recognition was closer to the outer portions of the brain sections (fig. 6 C', D'), while the Sigma recognition was concentrated more on the inner portions of the brain sections (fig. 6 C", D").

The spinal cord sections also showed significant background fluorescence, but had clear distinct fibrous patterned recognition (fig. 6 E). DAKO had stronger recognition around the distal edges of the spinal cord (fig. 6 E'), and Sigma showed stronger recognition in the center of the spinal cord section (fig. 6 E").
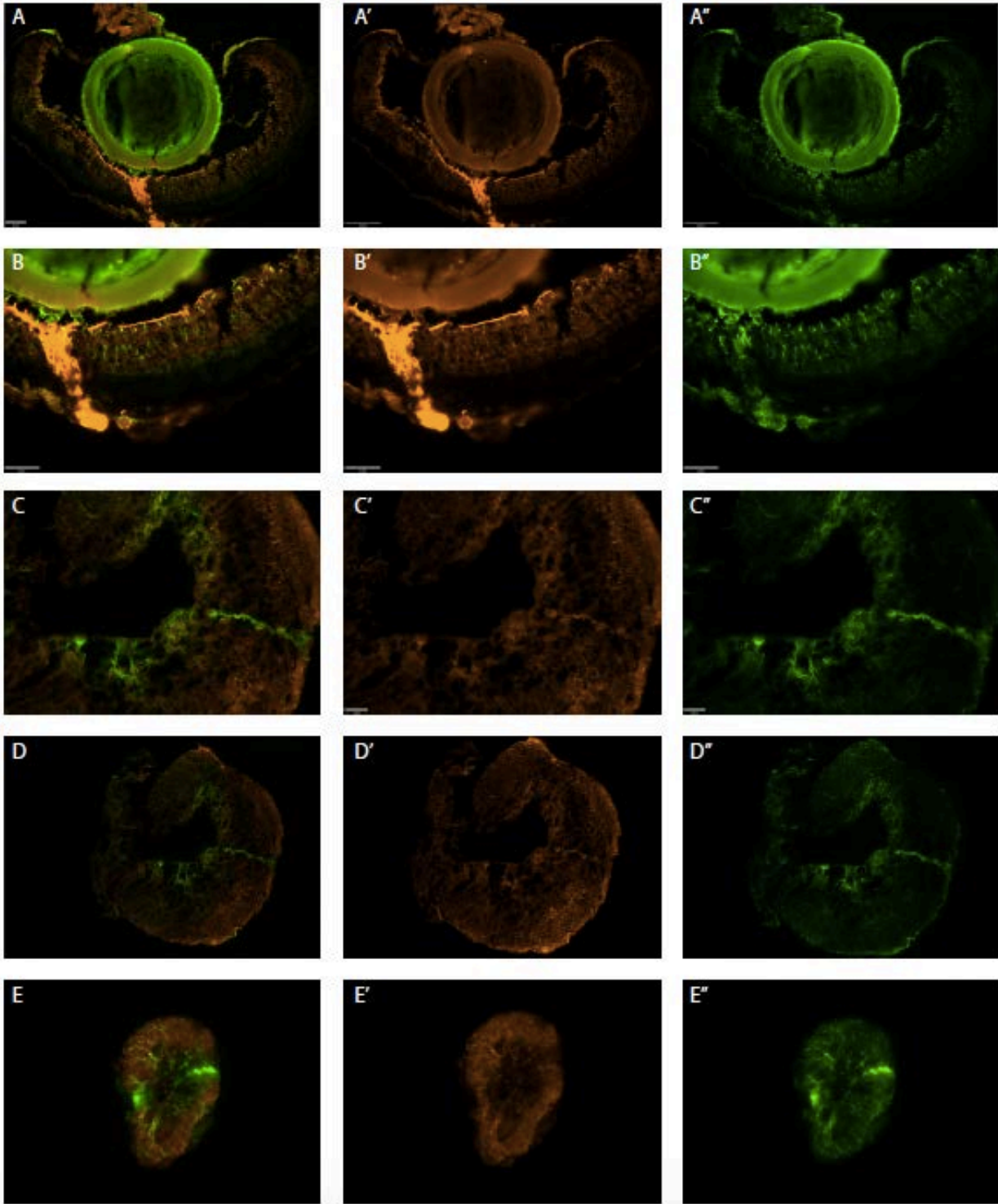
Figure 6. GFAP Antibody Staining. A) Retinal section double stained with anti-GFAP antibodies, A' showing only DAKO and A" showing only Sigma-G3893, all shown at 10X magnification, and the same retinal section stained with the same antibodies shown in B) at 20X. C) Brain double stained with the same anti-GFAP antibodies shown at 20X magnification and D) showing the same brain sections at 10X magnification. E) Spinal cord section stained with the same anti-GFAP antibodies

Intermediate Filament Protein Expression Patterns

The GFAP probe did not stain any tissue within the retina or the brain (fig. 7 A). Likewise, desmin showed no recognition in the retina or brain (fig. 7 B, B'), but did stain muscle tissue quite strongly (fig. 7 B"). Internexin-α showed recognition in the retina along the ganglion layer and possibly into the IPL or INL (fig. 7 C), and showed light recognition in the brain (fig. 7 C'). Peripherin had the strongest staining in the peripheral regions near the proliferating zones at the tip of the retina (fig. 7 D). It also stained the internal portions of the brain, indicating its presence there as well (fig. 7 D'). Vimentin stained the darkest of all the IFPs, with expression in the proliferating zone, the lens, and the inner layers of the retina, possibly extending further into the IPL (fig. 7 E). Staining in the brain was also quite dark and is the most densely concentrated in the interior portions of each lobe (fig. 7 E'). Staining by ISH indicates that there is mRNA of each respective IFP present, and ultimately that is probably where the respective IFP is being transcribed (Table 5).

tissue type	GFAP	Des	Ina-a	Prph	Vim
retina	no	no	yes	yes	yes
brain	no	no	yes	yes	yes
muscle	no	yes	no	no	no

Table 5. IFP *In Situ Hybridization* Expression Summary. For each IFP listed, presence in each named tissue determined by staining by ISH is indicated by yes and no staining is indicated by no.
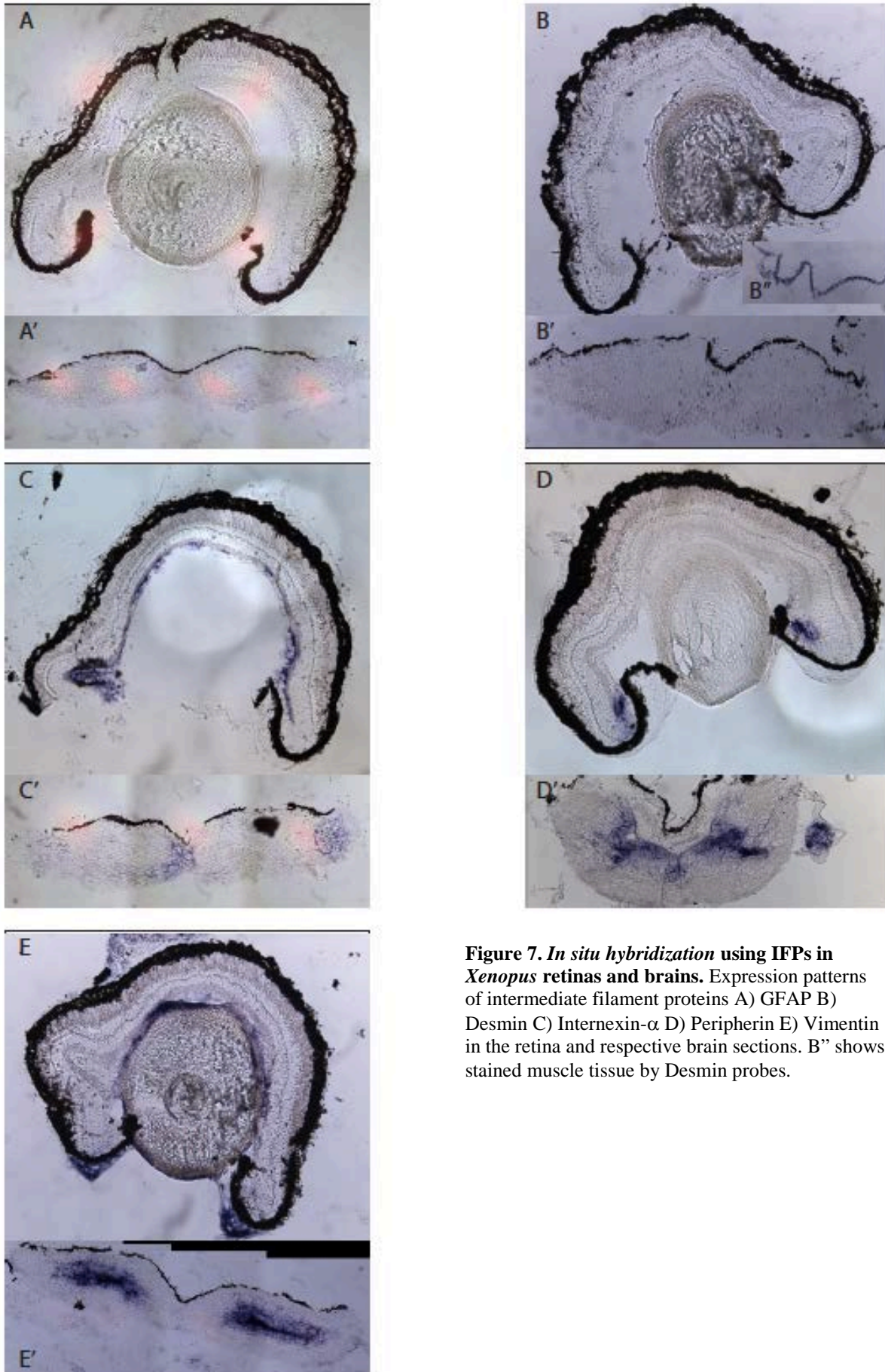
Figure 7. *In situ hybridization* **using IFPs in** *Xenopus* **retinas and brains.** Expression patterns of intermediate filament proteins A) GFAP B) Desmin C) Internexin-α D) Peripherin E) Vimentin in the retina and respective brain sections. B" shows stained muscle tissue by Desmin probes.

Rates of Degeneration Among Female Progeny

After Mtz treatment, tadpoles from the three different XOPNTR females showed no significant difference in rates of rod cell ablation (Fig. 8 A-F). Complete rod ablation was observed in 43% of the tadpoles (n=7) from female 13, 50% (n=8) from female 16 and 50% (n=4) from female 21; p=0.2981 (Figure 8B, D, F, and G). The remaining 57% (female 13; n=7) and 50% (females 16 and 21; n=8 and 4, respectively) still had detectable transducin signal in the retina (Figure 8A, C and E). These results suggest that the rate of rod ablation is comparable in these three XOPNTR transgenic females for this time point, and that any of these females may be used interchangeably in further experiments using an Mtz treatment of six days.
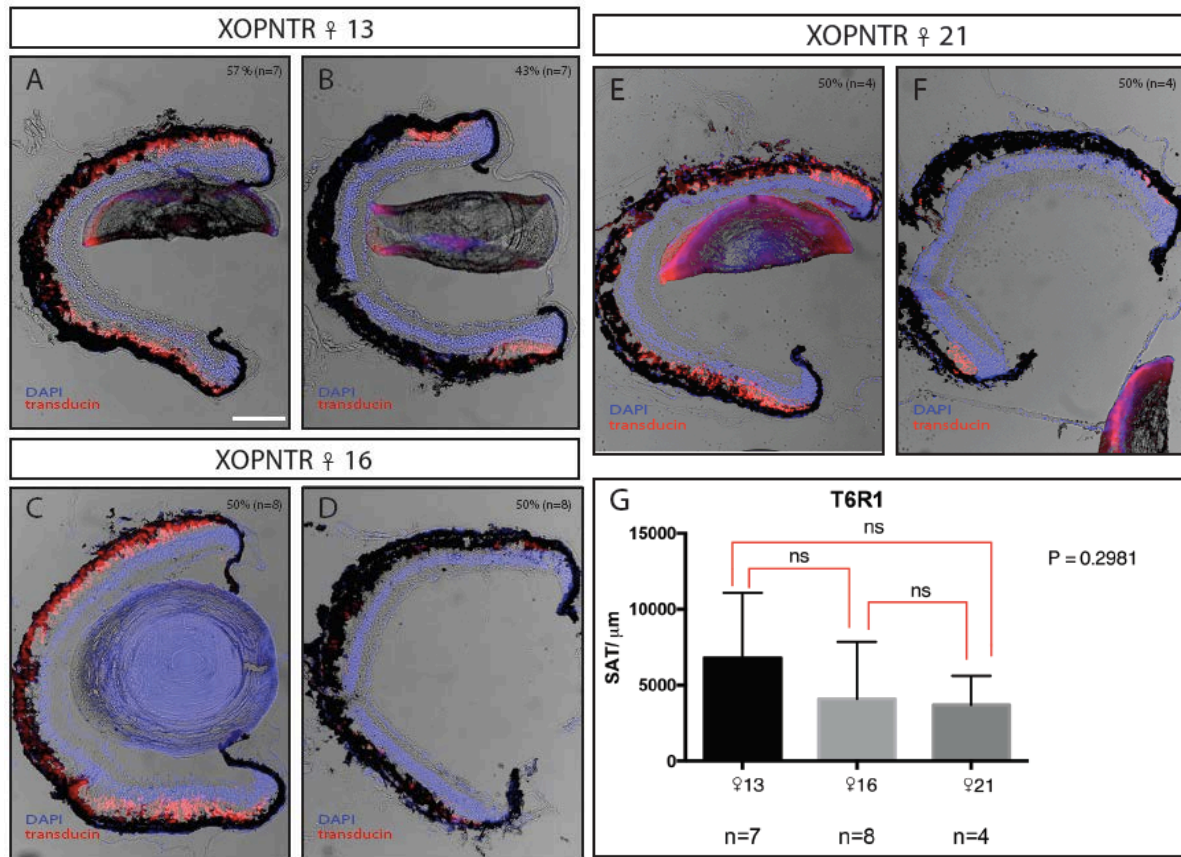
Figure 8. **Rod Ablation in tadpoles treated with Mtz for six days and recovered for one day.** (A-F) Retinal sections stained for transducin and DAPI. Panels A,C, and E show mild rod loss in central retina for all three XOPNTR female progeny. Panels B,D, and F show severe rod loss in central retina for all three XOPNTR females. Results for the transducin SAT/μm results for the progeny of the three XOPNTR females used (G). Scale bar= 100 μm.

Discussion

The number of blind individuals throughout the world is climbing every day. There are currently no effective treatments for retinitis pigmentosa, and one in 4,000 adults will lose their vision entirely because of it. Beginning to understand the role that IFPs play in retinal degeneration and regeneration will bring us closer to understanding and therefore combating retinal diseases such as retinitis pigmentosa. By using immunohistochemistry and *in situ* hybridizations, the present study examined class III IFPs to determine where in the retina and brain they are expressed in *X. laevis*. I also investigated the recognition patterns of two GFAP antibodies in the *Xenopus* retina.

The two tested GFAP antibodies that were purportedly binding to GFAP in the *Xenopus* retina as reported in previous publications are most likely not recognizing the GFAP protein. Because the two anti-GFAP antibodies should both recognize GFAP, their distinct recognition patterns suggest that either one or both of them are not recognizing GFAP. Furthermore, based on the previous genomic and synteny analysis conducted by our lab (fig. 4, 5), we failed to find GFAP in the *Xenopus* genome, suggesting that neither of the tested antibodies are recognizing GFAP. One possibility is that these GFAP antibodies are recognizing one of the other closely related class III IFPs. Based on the expression patterns observed using *in situ* hybridization, it's likely one of the three that are expressed in the retina: Vim, Prph, or Ina-a.

These results are further corroborated by recent work published by members of our lab that suggest that GFAP antibodies are in fact recognizing other IFPs (Martinez-De Luna et al. 2016). Purified proteins (mouse GFAP, Vim, Des, Prph, Ina-a) were myc tagged visualized via western blots with the same antibodies used in the present study, GFAP polyclonal antibody (pAb) DAKO and monoclonal antibody (mAb) Sigma-G3893. The results showed that pAb

recognized all of the proteins, and mAb recognized only the mouse GFAP (Fig. 9). This supportsthe idea that the DAKO antibody used in my study was most likely recognizing one of the other IFPs rather than GFAP.

Vimentin, peripherin, and internexin-a are the most likely to be expressed in the Müller glia based on the results observed in the *in situ* hybridizations. Of the five IFP probes tested, only Vim, Prph, and Ina-a showed localization in the retina. As predicted, GFAP probes were not found in the retina or the brain. Similarly, Desmin did not stain any part of the retina or brain, but did show dark staining in muscle tissue. Based on these results, it is unlikely that Desmin plays any role in the process of retinal degeneration. Because of the presence of Vim, Prph, and Ina-a in the uninjured *Xenopus* retina, it is possible that thse genes may play a role in degeneration. We hypothesize that they are upregulated during gliosis and therefore may be more important for glial scarring than the other related IFPs. In the future, we will go on to test this hypothesis by repeating the experiments presented here, but using injured retinas and the XOPNTR model.

In addition to the results described above, I also found that any of the transgenic females can be used for embryo collection for future experiments because there was no significant difference in the rate of degeneration among progeny originating from different female lines. Previously, it was suspected that degeneration rate might differ among the progeny of different female transgenic frogs. Based on a statistical analysis of the average SAT/ μm score for injured retinas of the progeny in each female line I found that there was no difference between the lines, but there was variation in rate of retinal degeneration within each line.

Moving forward, the Zuber lab will continue to investigate where IFPs are expressed in the injured retina and whether they are in fact upregulated during the process of gliosis in *Xenopus*. The results presented here will form the foundation for future efforts addressing how IFPs are
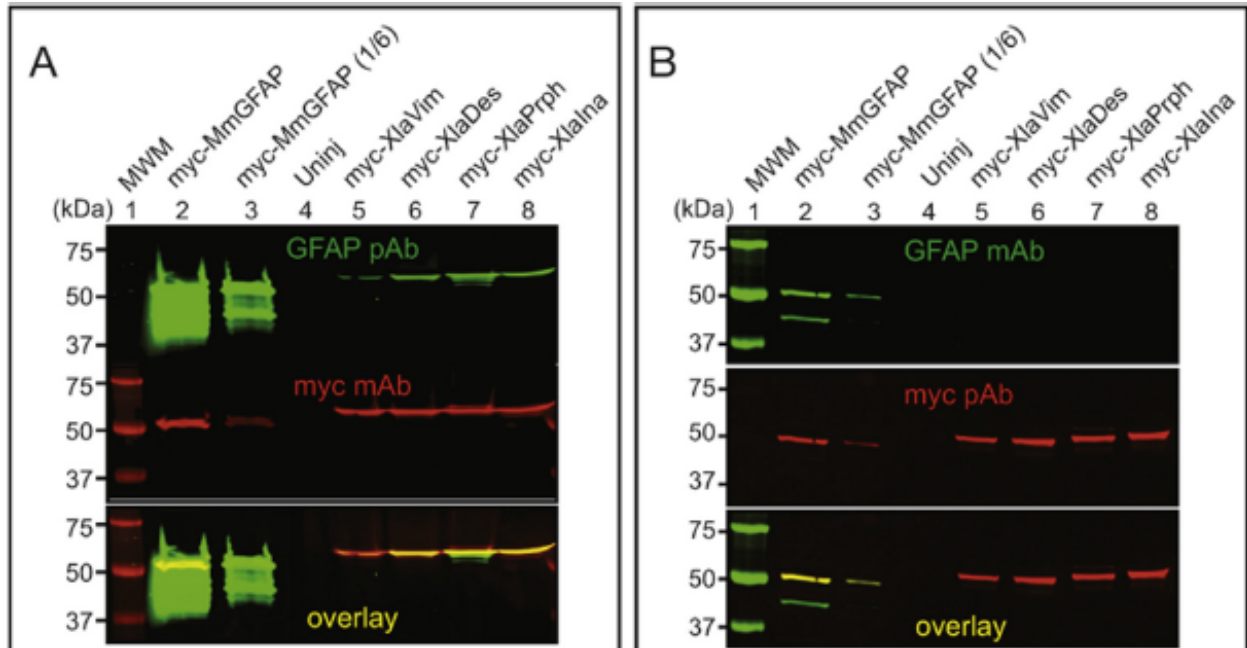
Figure 9. Specificity of GFAP antibodies on western blot. Adapted from Martinez-De Luna et al. 2016. A) GFAP pAb tested against mouse GFAP, uninjected embryos, Vim, Des, Prph, Ina-a in green. In red is myc mAb used as a control for the myc tagged proteins. B) GFAP mAb tested against the same proteins in green, with the myc pAb in red used as a control.

involved in retinal degeneration, and which IFPs to focus on. In particular, Vim, Prph, and Ina-a present a compelling case for future investigations of their role in retinal gliosis and the formation of glial scarring.

IFPs are an important factor in the process of gliosis. Glial cells are present wherever there are neural cells, and neurons and their axons extend throughout the entire body. This means that the process of gliosis can occur almost anywhere within the body. Because IFPs are present in the brain, they may also play a role in the process of gliosis in the brain. The expression patterns of each IFP are unique, and each has a specific purpose within the cell. More importantly, each has a different contribution to the process of retinal gliosis. Discovering the precise function that each IFP has in progressive retinal degeneration would lead to a possible approach to treating blinding diseases such as retinitis pigmentosa.

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