University of Alberta

Using zebrafish to develop a precise model of cone photoreceptor ablation and regeneration

by

Irene Brittany Morgan Fraser

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

Master of Science
in
Molecular Biology and Genetics

Department of Biological Sciences

© Irene Brittany Morgan Fraser
Fall 2011
Edmonton, Alberta

Permission is hereby granted to the University of Alberta Libraries to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only. Where the thesis is converted to, or otherwise made available in digital form, the University of Alberta will advise potential users of the thesis of these terms.

The author reserves all other publication and other rights in association with the copyright in the thesis and, except as herein before provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatsoever without the author's prior written permission.

Abstract

We have engineered a novel model of cone photoreceptor regeneration using transgenic zebrafish to induce cell ablation. Zebrafish were engineered to express the *E. coli* gene *nfsB*, encoding the protein nitroreductase (NTR), within UV-sensitive cones. We have adapted the metronidazole-nitroreductase method and optimized it for ablation of targeted UV cones. The results demonstrated precise cell ablation of the subset of cones expressing NTR, while other cone types continued to persist. Following ablation, proliferation increased in retinal stem cells, indicating that limited cell death was sufficient to induce regeneration. After regeneration, BrdU co-localized with rods, UV cones and at least one other cone type. Analysis of BrdU-positive cones suggests that a bias exists for new UV cones to replace ablated UV cones. In conclusion, this work engineered and began to characterize a model of inducible cone subtype-specific death that will allow researchers to study cone photoreceptor regeneration in a powerful way.

Acknowledgement

I am sincerely thankful to my supervisor, Dr. Ted Allison, whose guidance, support, and encouragement allowed for me to grow as a scientist. Thank you for introducing me to zebrafish genetics and for teaching me all the techniques that were employed in this project. Your help throughout researching and writing my thesis was invaluable. Thank you for believing in me.

Thank you to all the members of the lab for not only supporting me in my research throughout the years, but for being my friends as well. I acknowledge Adina Bujold for being an excellent lab manager, teaching me techniques, and helping me overall with my research. Thank you to Hao Wang for your support with my project and for playing an integral part in the engineering of transgenic fish. Thank you also to Aleah McCorry for doing a great job as fish facility manager and for taking excellent care of our very important fish friends.

I am also very thankful for the feedback and advice of my committee members, Dr. Yves Sauvé and Dr. Andrew Waskiewicz. As my teacher also, thank you to Yves Sauvé for teaching me to think critically and for your belief in my potential.

Thank you to the Department of Biological Sciences at the University of Alberta for the Teaching Assistantship. I also acknowledge NSERC and the FGSR at the University of Alberta for the financial support.

Finally, I am thankful to my parents, siblings, family members, and friends for encouraging me to pursue this degree and supporting me over the years.

Table of Contents

Abstract ....

Acknowledgement ....

Table of Contents ....

List of Tables ....

List of Figures ....

List of Abbreviations ....

Chapter 1 Introduction 1

1.1. Retinal degenerative diseases lead to irreversible vision loss 2

1.2. Advantages for employing zebrafish as a model of the vertebrate retina 2

1.3. Vertebrate retinal development 3

1.3.1. The adult zebrafish retina is organized into a precise photoreceptor mosaic 6

1.4. Proliferation of retinal neurons in the developing and mature zebrafish 7

1.4.1. The CMZ is the source of proliferating cells that contribute to the expanding retina 7

1.4.2. Rod progenitors are continuously derived from Müller glia in the uninjured retina 8

1.4.3. Müller glia are retinal progenitors in the adult zebrafish in response to photoreceptor injury 10

1.5. Methods for inducing retinal neuron death and studying regeneration 13

1.5.1. Surgical lesioning 14

1.5.2. Laser lesioning 14

1.5.3. Light lesioning 15

1.5.4. Chemical ablation 16

1.6. Objectives 17

Chapter 2 Conditional targeted ablation of a photoreceptor subtype and regeneration....20

2.1. Employing the metronidazole-nitroreductase method of cell ablation to induce conditional targeted UV cone photoreceptor death 20

2.1.1. History of nitroreductase and metronidazole use 21

2.1.2. Identifying proliferating cells using BrdU 24

2.1.3. The Gal4-UAS amplification system 24

2.2. Methods 25

2.2.1. Zebrafish maintenance....25

2.2.2. Constructs for transgenesis....25

2.2.3. Generating and characterizing transgenics....27

2.2.4. Optimizing metronidazole-nitroreductase ablation....27

2.2.5. Preparing cryosections....28

2.2.6. Immunohistochemistry on retinal sections....28

2.2.7. BrdU application to fish and antigen retrieval on sections and whole mount retinas 29

2.2.8. *In situ* hybridization and immunocytochemistry of whole retinas 30

2.2.9. Quantification of ablation from retinal sections and assay of cell death 32

2.2.10. Quantification of proliferating cells from retinal sections 33

2.2.11. Analyzing whole mount retinas to determine the identity of BrdU-positive photoreceptors....33

2.2.12. Statistical analysis 37

2.3. Results 37

2.3.1. Characterizing novel transgenic fish and directed cell-specific ablation of UV cone photoreceptors....37

2.3.2. Effects of cone photoreceptor ablation on retinal stem cell proliferation

....39

2.3.3. Regeneration following conditional targeted ablation of UV cone photoreceptors 41

2.3.4. Identity of regenerating cone photoreceptors after the targeted ablation of a subset of UV cone photoreceptors...42

2.4. Discussion 46

Chapter 3 Understanding the ineffectiveness of the metronidazole-nitroreductase ablation system on *Tg(SWS1:nfsB-mCherry)* zebrafish..65

3.1. Metronidazole experimental trials with *Tg(SWS1:nfsB-mCherry)*and *Tg(SWS2:nfsB-mCherry)*..65

3.1.1. Metronidazole treatment failed to produce photoreceptor death in this version of the technology 65

3.2. Potential problems of the metronidazole-nitroreductase ablation method applied to *Tg(SWS1:nfsB-mCherry)*...66

3.2.1. Addressing the construct as a potential problem.................................66

3.2.2. Nitroreductase expression as a potential problem...............................68

3.3. Comparing the *Tg(SWS1:nfsB-mCherry)* line to the *Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264* line.....................................70

3.3.1. Gal4-VP16 Toxicity 70

3.3.2. Investigating VP16 toxicity for a role in metronidazole-nitroreductase ablation 71

Chapter 4 Future Directions...78

4.1.1. Optimizing a zebrafish model for absolute ablation of targeted cone photoreceptors 78

4.1.2. Optimizing a zebrafish model for persistent expression of nitroreductase 79

4.2. Directed cell-specific ablation of photoreceptors in the adult zebrafish retina..80

4.3. Reconstituted caspase: an alternative method for targeted cone photoreceptor ablation..81

4.4. Assessing the functional integration of regenerated photoreceptors...........82

4.4.1. Assessing functional integration using electrophysiology 83

4.4.2. Assessing functional integration using visually-evoked behavioural assays 83

4.4.3. Tracing retinal neural circuits using barley lectin 85

4.5. Characterizing retinal regeneration by manipulating stem cell fate of regenerating photoreceptors 86

Bibliography 88

Appendix 107

List of Tables

Table 1. Transgenic lines used to express nitroreductase (*nfsB*) in various cells ..53

List of Figures

Figure 1. Multiple methods of neuronal ablation as applied to studying regeneration of the zebrafish retina 19

Figure 2. Regeneration investigated via a cell-specific ablation method 54

Figure 3. UV cone photoreceptor cell death by MTZ-NTR mediated cell specific ablation 55

Figure 4. TUNEL detection of apoptotic cells following MTZ-NTR ablation 56

Figure 5. Morphological analysis of photoreceptors indicates that metronidazole does not create a bystander effect 57

Figure 6. Following MTZ-NTR ablation of UV cones expressing the transgene, there is an increase in proliferating cells 58

Figure 7. Early regeneration following prodrug cell ablation 59

Figure 8. Regeneration of morphologically mature UV cones following prodrug ablation 60

Figure 9. BrdU-positive cells were identified as UV cones, BGR cones and rods in *Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264* fish following UV cone ablation and regeneration, with a bias towards a UV cone identity 61

Figure 10. The metronidazole-nitroreductase method failed to induce photoreceptor ablation in *Tg(SWS1:nfsB-mCherry)* fish 73

Figure 11. Retinal regeneration was not observed in *Tg(SWS1:nfsB-mCherry)* fish treated with BrdU following MTZ exposure 74

Figure 12. Following heat shock, *nfsB*-mCherry was mosaically expressed in the muscles, heart and lens of fish transiently expressing the transgene 75

Figure 13. Gal4-VP16 toxicity was not observed to have an influence on the metronidazole-nitroreductase ablation method 76

Supplemental Figure 1. Z-stack analysis for co-localization of BrdU and cone opsin 107

List of Abbreviations

BGR	blue, green and/or red
BL	barley lectin
BrdU	bromodeoxyuridine
BrdU+	bromodeoxyuridine-positive
BSA	bovine serum albumin
CMZ	ciliary marginal zone
CNS	central nervous system
DLR	dorsal light response
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dpf	days post-fertilization
dpl	days post-lesion
E. coli	*Escherichia coli*
ERG	electroretinogram
GFP	green fluorescent protein
HS	heat shock
INL	inner nuclear layer
lor	*lots-of-rods*
LWS	long wavelength sensitive
mRNA	messenger ribonucleic acid
MTZ	metronidazole
MWS	medium wavelength sensitive
NTR	nitroreductase
OKR	optokinetic response
OMR	optomotor response
ONL	outer nuclear layer
PBS	phosphate buffered saline
PTU	phenthiourea
RNA	ribonucleic acid
RPE	retinal pigment epithelium
SWS1	short wavelength sensitive 1
SWS2	short wavelength sensitive 2
Tg	transgenic
UAS	upstream activating sequence
UV	ultra-violet

Chapter 1 Introduction[1]

The adult zebrafish retina possesses the extraordinary capacity to regenerate neurons from retinal stem cells (Cameron and Easter, 1995; Otteson and Hitchcock, 2003; Raymond et al., 1988). This includes the intrinsic ability of photoreceptors, the rods and cones, to regenerate if destroyed, and has been characterized by inflicting retinal neuronal damage from a variety of surgical and toxic chemical lesions (Otteson and Hitchcock, 2003; Easter and Hitchcock, 2000; Hitchcock and Raymond, 2004). Currently, very little is known about the biochemical signalling pathways involved in the functional replacement of damaged retinal cells. In order to begin to answer this question, a better understanding is required of the specific genes and mechanisms involved in both zebrafish retinal development and regeneration.

1.1. Retinal degenerative diseases lead to irreversible vision loss

Human retinal degenerative diseases are extremely common, including macular degeneration, retinitis pigmentosa, choroideremia, cone-rod dystrophy, Best's disease, Leber congenital amaurosis, and Usher syndrome to name a few, each resulting in irreversible vision loss. Macular degeneration in particular is one of the primary causes of legal blindness among older North Americans. This results when the macula, the central retina responsible for our main vision and for seeing fine details, begins to deteriorate, and is usually associated with aging (Jager et al., 2008). Investigations into the therapeutic potential of retinal stem

[1]Portions of this chapter have been published. Fleisch, Fraser & Allison 2011. BBA Molecular Basis of Disease. 1812: 364-380.

cells have begun with the intention of discovering a successful treatment for retinal degenerative diseases (Morris et al., 2008). As more genes are found to be associated with retinal development and regeneration in the zebrafish and their functions are studied, we get closer to the possibility of applying this knowledge towards therapeutics for the treatment of human retinal degenerative diseases. The characterization of the biological mechanisms and signalling pathways involved in these processes is extremely important if we ever hope to induce regeneration in a higher vertebrate organism. Currently, many genes have been described to play roles in vertebrate retinal development, but many others still remain a mystery, especially those involved in photoreceptor cell-fate determination.

1.2. Advantages for employing zebrafish as a model of the vertebrate retina

The zebrafish is an ideal organism to employ for the study of the vertebrate retina, not only because of its intrinsic ability for photoreceptor regeneration, but also because the zebrafish retina is cone photoreceptor-rich (Bilotta et al., 2001). This is an alternative from the other vertebrate model organism available for retinal studies, the mouse, which has a rod-dominated retina (Szel et al., 1992). The human fovea is a cone photoreceptor-rich portion of the retina, approximately 200 000 cones/mm^2, with a complete absence of rods in the foveal centre (Curcio et al., 1990). The fovea is extremely important to

human vision as it is responsible for colour and high acuity vision (Curcio et al., 1990). Although the zebrafish lacks a fovea, the entire retina is composed of a high density of cone photoreceptors which is more relevant and comparable to the human fovea than the rod-dominated murine retina.

As a vertebrate model, zebrafish are easy to maintain and breed. Sexual maturity is reached quickly, around three months, minimizing cost and effort to maintain large populations. Compared to the mouse, generation time is similar, but the number of offspring is much greater for zebrafish. An accommodating characteristic of this vertebrate is that they develop externally to the mother, allowing for easy access to embryos at all stages. The embryos themselves are sturdy and large enough for experimental manipulations such as microinjections. Also significant is that zebrafish develop rapidly and are readily observable, simplifying the task of transgenic and morphant identification and characterization (Meyers, 2007). Another important property is that various drugs and chemicals can be easily administered in the water of both larvae and adult fish, where it gets taken up into the tissue. Overall as a vertebrate model organism, the zebrafish as a tool for research combines this distinctive regenerative capacity with strong genetics.

1.3. Vertebrate retinal development

During development of the zebrafish eye, the first step towards the formation of the retina is the development of the optic cup, where cells make their

first commitment to becoming a neural retinal cell or a retinal pigment epithelium (RPE) cell. This initial differentiation is determined by proximity to specific signals (Vugler et al., 2007). The outer optic cup receives signals from the head mesenchyme, inducing cells in the region to form the RPE. Signals from the surface ectoderm are received by the inner optic vesicle and are induced to become neural retina progenitors (Vugler et al., 2007; Kunz, 2006).

During development, there are four main steps that retinal progenitors must undergo in order to generate the mature retina. First, the progenitors must expand by undergoing mitotic cell divisions. Next, these progenitors must exit the cell cycle, which is followed by their commitment to a specific cell fate (Harada et al., 2007). The homeobox transcription factors *Crx* (cone-rod homeobox) and *Otx2* (orthodenticle homeobox 2) are expressed by photoreceptor precursors, and play an important role in this cell fate decision. It has been shown that in the absence of these transcription factors, the precursors are either not specified or fail to differentiate (Alvarez-Delfin et al., 2009; Furukawa et al., 1997; Nishida et al., 2003). Finally, these precursors must actually differentiate into a specific cell type (Harada et al., 2007).

At approximately 2 days post-fertilization (dpf), the retinal precursors begin to differentiate into photoreceptors. An initial differentiation of both rod and cone photoreceptors appears in a small patch of the ventral retina at this time (Alvarez-Delfin et al., 2009; Easter and Malicki, 2002; Raymond and Barthel, 2004). This is followed by a travelling wave of photoreceptor precursors developing across the retina in the outer nuclear layer (ONL) which differentiate

specifically as cones (Raymond and Barthel, 2004). *Ath5* is a transcription factor expressed only during retinal development and plays a role in regulating this wave-like progression. Currently, the signalling mechanisms for neuronal differentiation by *Ath5* are poorly understood in vertebrates (Ma et al., 2004; Masai et al., 2000).

The retinal photoreceptors of the zebrafish consist of rods and cones, with each subtype expressing specific retinal pigments called opsins, which are sensitive to different wavelengths of light (Rodieck, 1998). Rods are used for vision in dark or dim light conditions and contain the visual pigment rhodopsin. Cones are used for vision in bright light and the opsin detection of the different wavelengths results in the ability to see colour (Rodieck, 1998). Four types of cones exist in the zebrafish retina, two of which absorb short wavelengths, the ultraviolet (UV) - and blue-sensitive cones. The opsin genes that they contain are short wavelength sensitive 1 (SWS1) and short wavelength sensitive 2 (SWS2), respectively. Cones that absorb green light contain medium wavelength-sensitive opsin (MWS) (Raymond and Barthel, 2004). In the zebrafish, four green-sensitive opsin genes exist called *RH2-1*, *RH2-2*, *RH2-3* and *RH2-4*, which can each maximally absorb slightly varying wavelengths (Chinen et al., 2003). Cones that are sensitive to red light hold the long wavelength-sensitive opsins (LWS) (Raymond and Barthel, 2004). Two red-sensitive opsin genes are expressed in the zebrafish, *LWS-1* and *LWS-2* (Chinen et al., 2003). For ease of understanding, I refer to the ultraviolet-, blue-, green- and red-sensitive cone photoreceptors as “UV”, “blue”, “green” and “red” cones.

By 3 to 4 dpf, the retinal pigments start to be expressed in the cones. LWS opsin is expressed first, followed shortly by MWS opsin, and later on, UV cones and blue cones begin to express opsins (SWS1 and SWS2 respectively) (Stenkamp et al., 1997). With the exception of the initial small ventral patch of cells, rods are the final photoreceptors to differentiate in the retina. The rod precursors are generated from a separate lineage from the cone precursors. These rod precursors are associated with the Müller glia in the inner nuclear layer (INL), and migrate along the radial processes of the Müller glia to the ONL. As the precursor cells migrate, they undergo additional cell divisions, and then differentiate into rods, expressing the visual pigment rhodopsin (Otteson and Hitchcock, 2003; Raymond, 1985). The rod photoreceptors do not develop along a gradient such as the cone photoreceptors, but rather accumulate randomly across the retina (Raymond and Barthel, 2004; Schmitt and Dowling, 1999).

1.3.1. The adult zebrafish retina is organized into a precise cone photoreceptor mosaic

A unique feature to the retina of teleost fish which is represented in the zebrafish retina is that the cone photoreceptor subtypes are arranged in precise patterns creating a mosaic (Raymond and Barthel, 2004; Allison et al., 2005; Allison et al., 2010). The pattern of the mosaic varies among different teleost species (Ali and Anctil, 1976; Collin, 2008). The zebrafish mosaic in particular is composed of cones aligned in parallel rows of red and green double-cone pairs that alternate with rows of alternating UV and blue cones in the adult (Raymond

and Barthel, 2004; Allison et al., 2005; Allison et al., 2010). A ratio is thus created of one UV cone and one blue cone for every two red cones and two green cones (Allison et al., 2010).

The retinal mosaic was recently studied in zebrafish larvae to determine the regularity of cone photoreceptor distribution in the developing retina compared to the precision of the adult retina, and it was found that the adult mosaic and larval mosaic do differ (Allison et al., 2010). The larval retina still forms a heterotypic mosaic of cone photoreceptors with spatial relationships among cone subtypes, but the increased intricacy and the precise rows of the mosaic do not form until adulthood (Allison et al., 2010). Due to the regularity of this pattern and the spatial affiliation among different cone subtypes in the adult, there is potential to employ it as a tool for zebrafish retinal studies (Alvarez-Delfin et al., 2009).

1.4. Proliferation of retinal neurons in the developing and mature zebrafish

1.4.1. The CMZ is the source of proliferating cells that contribute to the expanding retina

All vertebrates possess neural stem cells during embryogenesis that are important to the developmental process, but these proliferating cells generally become exhausted by adulthood (Perron and Harris, 2000). Many fish and

amphibians however possess retinas that grow and expand continuously throughout life by the addition of new retinal neurons (Kubota et al., 2002). The eyes of mammals also continue to grow for a time after birth, but the increase in eye size is due to the stretching of the already existing retina and not a result of new cells (Teakle et al., 1993). In fish, the ciliary marginal zone (CMZ) is the source of these stem cells, located at the periphery of the retina, and adult retinal growth occurs by the addition of concentric rings of new retinal cells (Perron and Harris, 2000). Cells at the periphery of the CMZ are the youngest, expressing genes associated with earliest retinal development while the most mature cells, expressing genes related to later stages of development, are located more centrally (Perron et al., 1998). Although it possesses multipotent progenitor cells, the CMZ is the source for stem cells contributing to the growing retina only and does not supply injury-activated progenitors or neuronal stem cells involved in regeneration of the mature retina (Wetts et al., 1989).

1.4.2. Rod progenitors are continuously derived from Müller glia in the uninjured retina

Rod progenitors continuously proliferate in the developing and mature zebrafish retina, migrating radially from the INL to the ONL (Morris et al., 2008; Raymond et al., 2006). During retinal growth, as the optic cup expands, the existing retina is stretched, resulting in a reduction of photoreceptor density (Otteson and Hitchcock, 2003). As the retina grows, visual sensitivity is

maintained by the addition of rod progenitors to the central retina to fill in the consequential gaps (Morris et al., 2008).

The potential for any mitotic activity of Müller glia in an uninjured adult retina was previously investigated (Bernardos et al., 2007). Müller glia are the housekeepers of the retina, regulating ions, pH, and osmosis as well as maintaining the structure of the vertebrate retina (Bringmann et al., 2006; Newman, 1996). In teleost fish, they are also known to act as neuronal guides for rod progenitors to migrate along (Raymond et al., 2006).

Fish 1-2 months post-fertilization were treated with bromodeoxyuridine (BrdU; described in Chapter 2), which incorporates into the DNA of proliferating cells, for a period of 7 days, and found that BrdU incorporated into the nuclei of some Müller glia. Mitotic activity was observed in some differentiated Müller glia, with their migrating progeny spanning from the INL to the ONL, forming radial chains (Bernardos et al., 2007).

Pax6 and *Crx* expression in the proliferating Müller glia were examined. *Pax6* is a homeobox transcription factor required for neurogenic capacity and *Crx* is the cone-rod homeobox protein, expressed in late-stage neuronal progenitors (Bernardos et al., 2007). This includes both rod and cone progenitors in the zebrafish embryo, and expression is known to persist in photoreceptors in the differentiated retina (Shen and Raymond, 2004). Proliferating Müller glia co-labeled with *Pax6* expression in the INL, while *Crx* co-localized with the Müller glia-derived precursors once they had migrated to the ONL (Bernardos et al., 2007). It is known that cone photoreceptors do not generate from the

differentiated retina unless it is in response to injury (Hitchcock and Raymond, 2004; Raymond and Hitchcock, 2000), therefore they hypothesized that the proliferating Müller glia in the uninjured retina were fated to become rod photoreceptors. This was confirmed, as rhodopsin was also observed to co-localize with the Müller-derived precursors in the ONL. From these experiments on the uninjured zebrafish retina, it was concluded that proliferating Müller glia generate progenitors expressing *Pax6* and *Crx*, which can differentiate into new rod photoreceptors (Bernardos et al., 2007).

1.4.3. Müller glia are retinal progenitors in the adult zebrafish in response to photoreceptor injury

The ever-expanding zebrafish retina contains persistently proliferating cells (Perron and Harris, 2000). The majority of these stem cells are located in the peripheral regions of the retina near the boundary between the neural retina and ciliary epithelium called the CMZ, and are supplied to the growing eye throughout the lifecycle (Perron and Harris, 2000). Additionally, in the uninjured retina, select Müller glia continually give rise to proliferating cells that are restricted to a rod-precursor fate which migrate radially to the ONL (Bernardos et al., 2007; Julian et al., 1998; Otteson et al., 2001; Raymond and Rivlin, 1987).

Cone photoreceptors, however, are not generated from the differentiated retina unless in response to injury (Hitchcock and Raymond, 2004; Raymond et al., 2006). An injury response occurs when the retina is damaged, activating Müller cells to re-enter the cell cycle and dedifferentiate, expressing progenitor-

specific genes (Bernardos et al., 2007; Thummel et al., 2008). The progenitors resulting from the injury response are multipotent stem cells, characteristically similar to those found in the CMZ, and possessing the capacity to regenerate into any variety of retinal neurons (Raymond et al., 2006).

Bernardos *et al.* (2007) investigated the effects of retinal injury in the zebrafish by exclusively destroying the photoreceptor layer using intense light (Bernardos et al., 2007). Specifically, they were looking to see if Müller glia trans-differentiated into photoreceptor precursors in response to injury, which then differentiated into rods and cones to replace the destroyed cells. Following the ablation of all photoreceptors, Müller glial stem cells dedifferentiate, showing increased expression of *Pax6* by 20 hours post ablation and were actively proliferating in the INL by 3 days post-lesion (dpl) (Bernardos et al., 2007; Thummel et al., 2008). These progenitors begin to migrate to the ONL and differentiate at 4 days post-lesion as evidenced by *Crx* expression (Bernardos et al., 2007). By examining the transition of specific genetic markers at each stage, Bernardos *et al.* (2007) tracked the progression of the activation of Müller glial cells in the INL to dedifferentiate into proliferating pluripotent stem cells, their migration towards the ONL, and the subsequent differentiation into specific photoreceptor types following retinal lesioning (Bernardos et al., 2007). Transgenic zebrafish in which GFP was expressed in the Müller glia (driven by the glial-specific intermediate filament protein promoter, GFAP) were employed. To determine if the activated Müller glia expressing *Crx* were actually differentiating into new photoreceptors to regenerate the retina, they looked for

co-localization in the ONL of the GFP expressing progenitors with a photoreceptor antibody, zpr1, against double cone pairs. Zpr1 is an antibody that recognizes a surface epitope of the zebrafish red and green double-cone pairs. By 4 dpl, a few cells with zpr1 were observed, and co-labelled with GFP. The strongest co-localization was observed at 5 days post lesion, indicating the critical time point for the conversion of a neuronal precursor to a distinctively-fated regenerating photoreceptor (Bernardos et al., 2007).

Bernardos *et al.* (2007) also observed from their retinal light lesioning experiment that by 6 days post ablation, there was a complete loss of GFAP expression in the neuronal precursors, as inferred by the loss of GFP in ONL cells. Increased zpr1 labelling of photoreceptors was observed, indicating that the cells had fully differentiated into cone photoreceptors at this time (Bernardos et al., 2007).

It was determined that upon the complete ablation of all photoreceptors, Müller glia in the INL were induced to trans-differentiate into photoreceptor precursors in response to injury, which then migrated to the ONL and differentiated into rods and cones, replacing the destroyed cells (Bernardos et al., 2007). This investigation confirmed that Müller glia are retinal stem cells in the adult zebrafish retina (Bernardos et al., 2007).

In a recent study of photoreceptor regeneration in zebrafish using light ablation, the transition from the initial injury response through to the end of regeneration was traced (Thummel et al., 2008). As the end of the regenerative response approached, the transcription factor neurogenin1 (*Ngn1*), involved in

regulating neuronal differentiation in the CNS, persisted in a subset of cells in the INL (Thummel et al., 2008; Blader et al., 2004; Lee et al., 2005). At 17 days post light treatment, *Ngn1* co-localized with a population of re-established Müller glial cells, marking the suggested completion of the regenerative response (Thummel et al., 2008).

1.5. Methods for inducing retinal neuron death and studying regeneration

A variety of lesioning paradigms have been employed for the induction of neuronal cell ablation with goals of learning more about regeneration of the zebrafish retina. Techniques vary in the specificity of lesioning (Fleisch et al., 2011). Whereas surgical excisions of pieces of tissues are not cell-specific, methods such as laser or light lesioning target specific layers of the CNS (Fig.1). These techniques have allowed research groups to observe the regeneration process, but a pitfall is that many cell types are ablated and thus questions concerning specific cell fate determination cannot be readily answered (Braisted et al., 1994; Vihtelic and Hyde, 2000; Wu et al., 2001). Cell ablation using a laser can also be used for targeting only specific neuronal cells of interest in a tissue (Fig.1) (He and Masland, 1997; Liu and Fetcho, 1999). The use of chemical toxins, such as ouabain, injected into the eye also possesses a certain level for controlled cell death (Fig.1). The concentration of the injected toxin dictates the extent of penetration of the toxin into the retinal layers, and therefore the amount

of cell death (Fimbel et al., 2007; Maier and Wolburg, 1979). Novel techniques employing transgenic-chemical ablation of neurons are proving to be the most specific means for targeted cell ablation (Fig.1) (Curado et al., 2008).

1.5.1. Surgical lesioning

Retinal regeneration has been investigated by surgically removing portions of the retina or the retina in its entirety (Cameron and Easter, 1995; Hitchcock et al., 1992). Mensinger & Powers (2007) investigated the effects of removing parts or the entire retina on regeneration in the teleost models goldfish and sunfish (Mensinger and Powers, 2007). While excision of the whole retina completely prevented regeneration, leaving only 5% of retinal tissue intact led to restoration of functional vision, as assayed by electroretinograms (ERGs) and dorsal light response (DLR). Surgical lesioning of the retina has also been used as a means to study gene expression profile changes during retinal regeneration (Cameron et al., 2005). Retinal damage via surgery has been useful for observing neuronal regeneration, but is less powerful for studying only a specific subset of the retinal neurons because all neurons are regenerating at once (Fleisch et al., 2011; Hitchcock et al., 1992).

1.5.2. Laser lesioning

Laser lesioning has been applied to induce ablation of neural cells in various tissues and organisms (Braisted et al., 1994; Wu et al., 2001; Hughes et al., 1992). Regeneration of the retina of teleost fish has been investigated by

ablating photoreceptors with an argon laser. The argon laser promotes accurate placement of the lesions and can be used to ablate photoreceptor cells in the outer nuclear layer of the retina. In this procedure, the lens of the fish is surgically removed in order to gain access to the retina for focusing the laser. The resulting lesions appear as bleached spots on the retina (Fleisch et al., 2011; Braisted et al., 1994; Wu et al., 2001).

1.5.3. Light lesioning

Light-induced lesioning is a popular method of cell ablation because it targets photoreceptor cells while leaving the remainder of the retina relatively unharmed, with less inflammatory response compared to tissue injury (Shahinfar et al., 1991). This system has been shown to be very effective for studies of the teleost retina, and is the most commonly used lesioning paradigm for assessing regeneration in fish (Marotte et al., 1979; Organisciak and Winkler, 1994; Penn, 1985). Mechanisms of cell death in light lesion likely include both generation of reactive oxygen species as well as accumulation of heat leading to photoreceptor death (Organisciak and Winkler, 1994; Wenzel et al., 2005).

Fish are initially dark adapted for a particular period of time, followed by the application of constant light, which induces photoreceptor cell death through apoptosis (Bernardos et al., 2007; Vihtelic and Hyde, 2000; Shahinfar et al., 1991; Abler et al., 1996). Photoreceptor regeneration following such lesions is extensive. The degree of retinal injury to the fish is affected by the history of light exposure, the period, quality and intensity of light exposure, and the

pigmentation and genetic background of the fish (Fleisch et al., 2011; Vihtelic and Hyde, 2000; Organisciak and Winkler, 1994; Allison et al., 2006a).

1.5.4. Chemical ablation

Another technique employed in the study of teleost retinal regeneration is to expose the tissue of interest to toxic chemicals. Ouabain, a metabolic poison inhibiting the Na^+/K^+-ATPase, has specifically been employed to induce degeneration of cells in the retina (Raymond et al., 1988; Fimbel et al., 2007; Maier and Wolburg, 1979). Depolarization of the plasma membrane triggers a cascade of enzymatic events, leading to cell death (Valente et al., 2003). The extent of cell death is proportional to the concentration of intravitreal toxin (Fimbel et al., 2007; Maier and Wolburg, 1979; Sherpa et al., 2008). High concentrations of ouabain have been shown to rapidly cause death to all retinal nuclear layers, while the use of lower concentrations can be useful in biasing ablation towards the inner nuclear layers while leaving the photoreceptor layer relatively unscathed (Fleisch et al., 2011). Fimbel *et al.* (2007) took advantage of this element and demonstrated that selective damage to cells of the INL by injection of low doses of ouabain could stimulate regeneration (Fimbel et al., 2007). A zebrafish model for whole retina regeneration has been presented by Sherpa *et al.* (2008) who showed regeneration of red-green double cone pairs after cytotoxin injection (Sherpa et al., 2008).

1.6. Objectives

My Master's thesis research project employs zebrafish as a vertebrate model in order to detect cone photoreceptor regeneration and acts as a stepping stone towards providing insight into the genes involved is this intrinsic process. This regenerative capacity offers a promising route to understanding how a stem cell can be activated to regenerate specific cone photoreceptors and direct the functional re-wiring to secondary neurons.

The aim of my thesis has been to implement a novel model of potent cone photoreceptor ablation and regeneration using an innovative transgenic conditional cell-specific ablation technique. By specifically targeting the degeneration of a particular cone class, rather than causing simultaneous death to many types of retinal neurons, as has been the standard procedure thus far, alterations and disruptions in relevant gene pathways can be analyzed for their potential roles in cone regeneration and functional integration (Fleisch et al., 2011). Through this study, contributions to the understanding of visual system evolution of teleosts can potentially be made. It has been previously reported that the retina must be damaged, initiating an injury response in order for cone photoreceptors to regenerate from the differentiated retina, but the required extent of the injury is unknown (Hitchcock and Raymond, 2004; Bernardos et al., 2007). Therefore the second aim is to determine if the ablation of a subset of cone photoreceptors is sufficient to induce the activation of retinal stem cell proliferation.

My final aim is to characterize the identity of the regenerated photoreceptors. It is widely accepted that the zebrafish retina regenerates. By narrowing the spectrum of damaged neurons to just one photoreceptor subtype, we can observe if that same subtype regenerates or if the regenerating neurons differentiate into other cell fates.

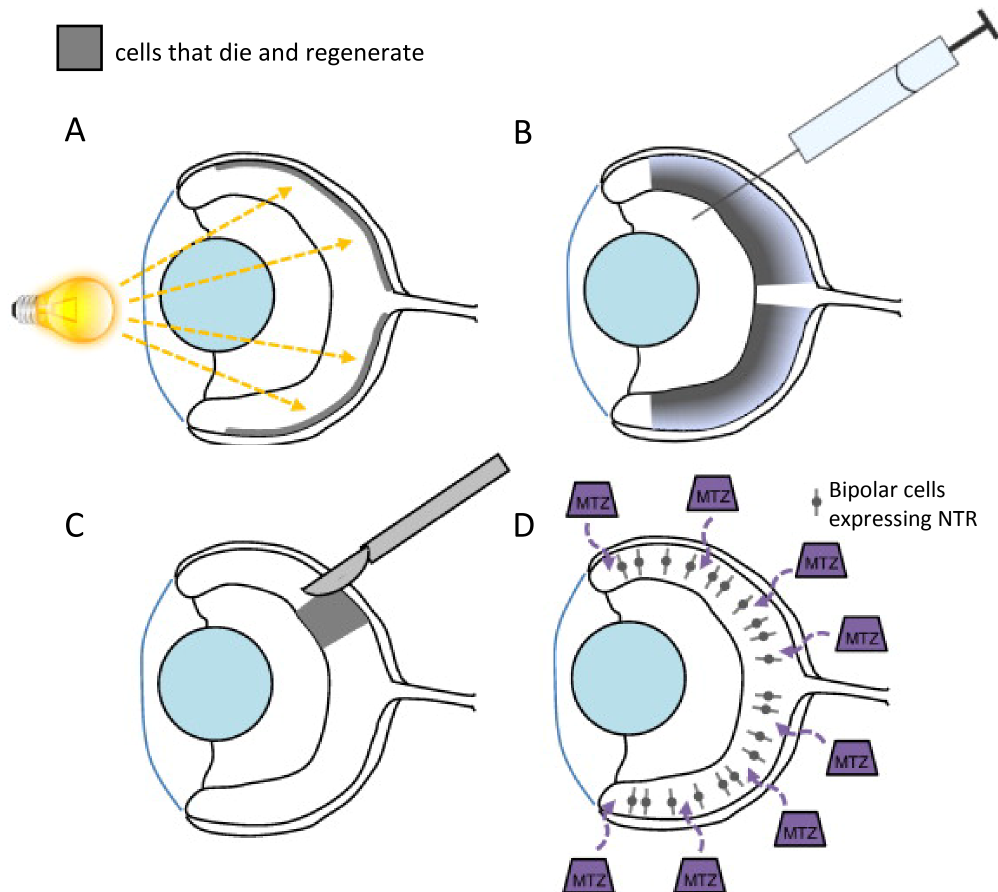


Figure 1. Multiple methods of neuronal ablation as applied to studying regeneration of the zebrafish retina. The specificity of the neuronal lesioning (ablation of only one or some neuronal cell types versus ablation of an entire section of retinal tissue) is dependent on the technique: A) light/laser lesion, B) chemical ablation, C) surgical lesion or D) chemically-inducible genetic (MTZ-NTR) ablation.

Chapter 2 Conditional targeted ablation of a photoreceptor subtype and regeneration

2.1. Employing the metronidazole-nitroreductase method of cell ablation to induce conditional targeted UV cone photoreceptor death

Employing the Gal4-UAS system, transgenic zebrafish have been engineered to express the *E. coli* gene *nfsB* encoding the nitroreductase (NTR) enzyme fused to a fluorescent marker gene, mCherry, to allow for visualization (Fig.2). Nitroreductase is driven by a cone-specific opsin promoter, resulting in its expression in the targeted neuron type (UV-sensitive cones). These cones survive and function normally until the application of a prodrug, metronidazole (MTZ). Metronidazole binds to nitroreductase and is electrochemically reduced, converting it into a DNA cross-linking cytotoxin (Curado et al., 2008). The toxic form of metronidazole remains isolated in the neurons of interest and induces base transitions, transversion mutations, and fragmentation of DNA (Pisharath et al., 2007). This results in precise cell ablation of the subset of cones expressing nitroreductase and can be observed by the reduction or loss of fluorescence, while other neighbouring cells remain unaffected. Ablation is terminated by removing fish from the metronidazole treatment, allowing for regeneration of the ablated cones to occur and resulting in the restoration of fluorescence (Curado et al., 2008).

Transgenic larval zebrafish were treated with a solution of 10mM MTZ for 48 hours to allow for targeted cone ablation. Bromodeoxyuridine (BrdU) was employed to determine if the regenerated retinal neurons had recently undergone proliferation, indicating that photoreceptors regenerated from retinal stem cells (Allison et al., 2006a). Observations and quantification of ablation and regeneration were made at various time points following prodrug exposure.

2.1.1. History of nitroreductase and metronidazole use

Bacterial nitroreductase, originally identified due to its role in nitrofuran antibiotic sensitization, is a flavin mononucleotide-containing flavoprotein homodimer with a molecular weight of 24kDa (Anlezark et al., 1992; McCalla et al., 1978; Sastry and Jayaraman, 1984). The enzyme works via a two-step mechanism, first requiring NADH or NADPH as a cofactor which reduces the bound flavin mononucleotide. The reduced flavin mononucleotide is then available to bind various substrates including nitroimidazole derived drugs (i.e. metronidazole) and CB1954 (Anlezark et al., 1992; Lindmark and Muller, 1976).

A prodrug is a precursor of a drug which is administered in the inactive form. In the presence of a metabolizing agent, the prodrug is converted to the active form. The prodrug CB1954 was examined in mammalian anti-tumour studies, and was found to be cytotoxic to tumours in rats due to the activity of DT diaphorase, an enzyme found in tumours which reduces prodrugs (Anlezark et al., 1992; Venitt and Croftonsleigh, 1987). Toxicity studies of CB1954 on bacteria showed that strains that were nitroreductase-deficient had greatly reduced

sensitivity to the prodrug (Venitt and Croftonsleigh, 1987). This classical nitroreductase, first characterized in *Salmonella typhimurium,* was later isolated from *E. coli* B and was found to share many properties with the mammalian tumour enzyme DT diaphorase (Anlezark et al., 1992; Watanabe et al., 1990). Nitroreductase has since been expressed in the tissues of various vertebrates in order to induce conditionally selective ablation by exposure to the prodrug CB1954 (Bridgewater et al., 1995; Felmer et al., 2002).

An important property of the nitroreductase enzyme is that the transgene is benignant in vertebrates until the association with a prodrug, proving this targeted ablation paradigm to be extremely attractive in studies of regeneration and cancer research (Pisharath et al., 2007; Bridgewater et al., 1995; Felmer et al., 2002; Searle et al., 2004). However, the prodrug CB1954 in particular is not as precise. Bridgewater *et al.* (1995) employed this selective killing system, infecting mammalian cells with a recombinant retrovirus encoding nitroreductase, and found that unmodified cells mixed in with nitroreductase expressing cells could also be killed by the CB1954 cytotoxicity (Bridgewater et al., 1995). CB1954 possesses four diffusible cytotoxic metabolites (2- and 4-hydroxylamines and their corresponding amines) which may contribute to this bystander effect (Helsby et al., 2004). This characteristic has been beneficial for some research concerning anti-tumour treatment in which few cells are required to express nitroreductase and may cause the ablation of many unwanted cancer cells (Helsby et al., 2004). However, for regenerative studies, the death of a specific tissue or cell type is

usually desired while allowing the neighbouring cells to remain intact and functional, therefore a bystander effect is unfavourable.

The solution to the bystander effect produced by CB1954 was the selection of another prodrug, metronidazole. Metronidazole is a derivative of the nitroimidazoles, a family of drugs possessing activity against a vast range of microorganisms and bacteria as well as tumours (Lindmark and Muller, 1976; Edwards, 1993; Grunberg and Tisworth, 1973). Metronidazole was the first of the nitroimidazole drugs to show promising clinical application against infections (Edwards, 1993; Grunberg and Tisworth, 1973). From these applications, it was deduced that certain strains of bacteria expressing nitroreductase converted metronidazole to a cytotoxic compound, suggesting that the bacterial enzyme was involved in the reduction of the prodrug (Lindmark and Muller, 1976).

Pisharath *et al.* (2007) performed a study of pancreas regeneration in the embryonic zebrafish and were the first to employ metronidazole as the prodrug for nitroreductase-ablation in live vertebrates, targeting beta cell death (Pisharath et al., 2007). Metronidazole, which is non-toxic on its own at appropriate concentrations, is reduced by nitroreductase into a cytotoxin, similar to CB1954. In contrast to CB1954, the cytotoxicity of metronidazole has been shown to stay confined to the cells of interest, causing ablation of only cells expressing nitroreductase while neighbouring populations of cells remain unaffected (Curado et al., 2008; Pisharath et al., 2007). This is consistent with other reports that metronidazole, at an equivalent concentration to that employed in this study (10mM), is not toxic to zebrafish embryos (Curado et al., 2008; Edwards, 1993).

2.1.2. Identifying proliferating cells using BrdU

BrdU, 5-bromo-2'-deoxyuridine, is a synthetic nucleoside which can incorporate into newly synthesized DNA of proliferating cells, substituting for thymidine (Kee et al., 2002). BrdU is detectable in the nucleus by the application of BrdU-specific antibodies. This chemical tool allows for the identification of cells that were undergoing proliferation at the time of BrdU exposure.

2.1.3. The Gal4-UAS amplification system

The Gal4-UAS system has been widely used in genetic studies employing *Drosophila*, and the more recent adaptation of the technique to zebrafish has proved to be very effective in amplifying the expression of a targeted transgene (Brand and Perrimon, 1993; Fischer et al., 1988; Scheer and Campos-Ortega, 1999). The system is based on two transgenic lines, an activator line and an effector line. The activator line expresses the tissue-specific yeast transcriptional activator Gal4. The effector line contains the gene of interest under the promotion of upstream activating sequences (UAS), which are Gal4-specific binding sites, and alone, is transcriptionally silent. When the activator and effector lines are crossed, Gal4 is expressed in the targeted tissue and binds exclusively to UAS, activating the expression of the effector (Scheer and Campos-Ortega, 1999). The Gal4-UAS amplification system was adapted in order to amplify the expression of the nitroreductase gene, *nfsB*, in a targeted subset of cone photoreceptors.

2.2. Methods

2.2.1. Zebrafish maintenance

Zebrafish (*Danio rerio*) were raised and maintained in E3 media as embryos and larvae and aquaria system water as juveniles and adults using standard procedures (Westerfield, 1995; Westerfield, 2000). Some larvae were treated with 10-fold PTU (1-phenol-2-thiourea) to block formation of melanin pigment. Fish were maintained at 28°C and were fed twice daily with brine shrimp and flaked food. All protocols were approved by the Animal Care and Use Committee: Biosciences at the University of Alberta with support of the Canadian Council on Animal Care.

2.2.2. Constructs for transgenesis

We engineered zebrafish that express nitroreductase (*nfsb*) fused to mCherry in either UV- or blue-sensitive cone photoreceptors using two different paradigms. In the first strategy, we generated zebrafish that express nitroreductase-mCherry under control of opsin promoters. In the second strategy we generated zebrafish that express the transcriptional activator Gal4-VP16 under control of opsin promoters and bred these to zebrafish expressing nitroreductase-mCherry under control of the UAS promoter. Constructs for transgenesis were generated using published methods of multisite Gateway cloning into vectors amenable to Tol2 recombination (Kwan et al., 2007). Nitroreductase was cloned into pDONR221 to create a middle-entry vector, creating pME-nfsb, using

primers (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3') and (5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-3') to amplify DNA from *E. coli*. Cone photoreceptor-specific promoters were used to create p5E-*sws1* and p5E-*sws2* plasmids, to direct expression in UV- and blue-sensitive cones respectively. These promoters are equivalent to those published previously, denoted *-5.5opn1sw1* [ZDB-GENE-991109-25; previous nomenclature zfSWS1-5.5A (Takechi et al., 2008)] and *-3.5opn1sw2* [ZDB-GENE-990604-40; previous nomenclature zfSWS2-3.5A (Takechi et al., 2008)] and derived from clones kindly provided by Shoji Kawamura. These plasmids were variously combined with p5E-*hsp70l* (ZDB-GENE-050321-1; previous nomenclature p5E-*hsp70*), pME-Gal4VP16, p3E-mCherrypA, p3E-polyA and pDestTol2CG2 generously provided by Chi-Bin Chien (Kwan et al., 2007) to generate the following constructs: i. pDestTol2CG2; *sws1:*Gal4VP16-polyA, ii. pDestTol2pA2; *sws1:*nfsb-mCherrypA, iii. pDestTol2CG2; *sws2:*Gal4VP16-polyA, iv. pDestTol2pA2; *sws2:*nfsb-mCherrypA, v. pDestTol2CG2; *hsp70l:*nfsb-mCherrypA. Final constructs were validated by sequencing.

It is noteworthy that the *nfsb*-mCherry construct we created by Gateway cloning is predicted to produce nitroreductase and mCherry protein domains with amino acid residues identical to those previously published (Davison et al., 2007). However our Gateway cloning strategy generated an *nfsb*-mCherry fusion protein that has an amino acid linker between the protein domains that differs from that previously published (Davison et al., 2007). We tested the function of this new fusion protein (below).

2.2.3. Generating and characterizing transgenics

Constructs i. through iv. above were used to generate stable transgenic zebrafish, whereas the latter construct was expressed transiently (Table 1). In each case, the construct was injected with 25-30 pg of Tol2 mRNA generated as previously described (Kwan et al., 2007) into hemizygous *Tg(-5.5opn1sw1:EGFP)kj9* zebrafish (ZDB-GENO-080227-6) expressing GFP in UV-sensitive cones. Transient expression of injected constructs ii. and iv. was detected by mCherry expression in UV photoreceptors. Transient expression of injected constructs i., iii., and v. was detected via GFP expression in the heart, which is derived from the pDestTol2CG2 vector backbone. These fish were raised if appropriate and screened for stable transgenesis by examining the F1 generation (outcross to UAS:*nfsb*-mCherry for Gal4-VP16 transgenics) for mCherry expression in cone photoreceptors (i. through iv.) and GFP expression in the heart (i., iii., v.).

Characterization of transgene expression in the retinas of stable transgenics was achieved by comparison of mCherry distribution to that of GFP in UV-sensitive cones.

2.2.4. Optimizing metronidazole-nitroreductase ablation

Transgenic zebrafish [*Tg(SWS1:nfsB-mCherry)ua3003*, *Tg(SWS2:nfsB-mCherry)ua3001* and *Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264*] were placed in a metronidazole (Sigma-Aldrich, St. Louis, MO; No. M3761) solution with E3 media or system water to induce metronidazole-

nitroreductase ablation. To optimize the treatment, various metronidazole doses were tested (1mM, 2.5mM, 5mM, 6mM, 6.5mM, 7.5mM and 10mM), with and without DMSO, and incubation time ranged from 24-60 hours. The resulting optimized treatment, 10mM MTZ solution in 0.2% DMSO for 48 hours at 28°C, was applied to all subsequent ablation experiments with larvae, with treatment beginning at 4-6 days post fertilization (dpf). Following treatment, larvae recovered in E3 media for 8-24 hours, with at least 2 changes of fresh E3 to wash away remaining metronidazole (Curado et al., 2008).

2.2.5. Preparing cryosections

Frozen retinal sections were prepared as described (Barthel and Raymond 1990). Larvae were placed in fixative (4% paraformaldehyde/5% sucrose/0.1M phosphate buffer pH 7.4) overnight at 4°C, and were embedded in Tissue-Tek O.C.T. embedding compound (Sakura Finetek; No. 4583). 10µm-thick cryosections were thaw mounted to SuperFrost Plus glass slides (Fisher, Pittsburgh, PA; No. 12-550-15). Sections were air dried at room temperature for 30 minutes and stored at -80°C until use.

2.2.6. Immunohistochemistry on retinal sections

Stored sections were air-dried upon removal from the -80°C freezer. Tissue was blocked for at least 30 minutes at room temperature using 10% normal goat serum in phosphate buffer saline (pH 7.4) with Tween-20 (1xPBS/1%Tween-20). The sections were incubated in a humid chamber with

primary antibody diluted with 2% normal goat serum in 1xPBS/1%Tween-20 overnight at 4°C (anti-BrdU rat 1:20 [Accurate Chemical, Westbury, NY; No. OBT0030S] anti-BrdU mouse 1:50 [BD Pharmingen, San Diego, CA; No. 555627]; anti-DSRed (mCherry) mouse monoclonal 1:200 [Clontech, Mountain View, CA, No. 632393], anti-zpr1 mouse monoclonal 1:200 [ZIRC, Eugene, OR, ZDB-ATB-081002-43]). For double labelling, primary antibodies were diluted and applied simultaneously (Vihtelic et al., 1999). Tissue was quickly rinsed twice and washed in 1% normal goat serum in 1xPBS for at least 30 minutes at room temperature. Primary antibodies were detected by incubating sections in a humid chamber with secondary antibodies anti-mouse or anti-rat conjugated to AlexaFluor fluorochromes 350, 488, 555 or 647 (Invitrogen, Carlsbad, CA), diluted in 2% normal goat serum in 1xPBS/1% Tween-20 (1:1000), at room temperature for at least 1 hour or overnight at 4°C. Tissue was rinsed and washed in 1% normal goat serum following secondary antibody exposure. Nuclei were detected with 1mg/L DAPI (incubated for 1-2 mins; Invitrogen, Carlsbad, CA) or 2μg/ml Hoechst 34580 (incubated for 10-15 mins; Invitrogen, Carlsbad, CA, No H21486).

2.2.7. BrdU application to fish and antigen retrieval on sections and whole mount retinas

Larvae were incubated in 5mM BrdU (5-bromo-2'-deoxyuridine; Sigma-Aldrich, St. Louis, MO; No. B5002) dissolved in E3 for 24-48 hours after recovery from metronidazole. For quantifying proliferation, larvae also received

BrdU treatment for 24 hours prior to metronidazole treatment. Larvae were maintained and fed fry powder until they were selected for analysis.

Tissue (cryosections or whole mount neural retinas) was incubated in 2N HCl with 1xPBS/1% Tween-20 for 30 minutes at room temperature to expose the BrdU antigens. Tissue was washed in 1xPBS/1% Tween-20 for 15 minutes at room temperature, then in 1xPBS for 15 minutes at room temperature. The immunohistochemistry procedure (previously described), beginning with blocking, was resumed at this point.

2.2.8. In situ hybridization and immunocytochemistry of whole retinas

To label UV cones, a fluorescein-labelled riboprobe was prepared against short wavelength sensitive 1 opsin (*sws1*), synthesized as previously described (Barthel and Raymond, 2000). To label all blue, green and red cones of the retina, a cocktail of digoxigenin-labelled riboprobes against the short wavelength sensitive 2 opsin (*opn1sw2*, accession No. AF109372, ZDB-GENE-990604-40), the four medium wavelength sensitive opsins (*opn1mw1*, *opn1mw2*, *opn1mw3*, and *opn1mw4*, accession Nos. AF109369, AB087806, AB087807, and AF109370, ZFin ID: ZDB-GENE-990604-42, ZDB-GENE-030728-5, ZDB-GENE-030728-6, and ZDBGENE-990604-43, respectively) and the two long wavelength sensitive opsins (*opn1lw1* and *opn1lw2*, accession Nos. AF109371 and AB087804, ZDB-GENE-990604-41 and ZDB-GENE-040718-141, respectively) were used. Full-length antisense riboprobes (varying in length; see accession Nos.) were synthesized from linearized plasmid in each case. All 8

riboprobes were mixed into a cocktail and applied in excess to isolated adult retinas as described previously (Barthel and Raymond, 2000), except hybridization temperatures and post-hybridization washes were at 65°C. Riboprobes were detected in sequence using anti-fluorescein (antibody produced by direct immunization of fluoroscein into sheep; then, ion-exchange chromatography and immunoabsorption were used to isolate IgG; Roche Diagnostics; No. 11426346910) then anti-digoxigenin (antibody produced by direct immunization of digoxigenin into sheep; then, ion-exchange chromatography and immunoabsorption were used to isolate IgG; Roche Diagnostics; No. 11207733910) antibodies conjugated to peroxidise (POD). After application of the antibody as previously published (Barthel and Raymond 2000), the tissue was incubated in tyramide-conjugated to AlexaFluor 488 or 555 in the absence of light until the fluorescence was developed and visible (approximately 30-60 minutes) (Invitrogen, Carlsbad, CA; Nos. T20932 and T30955). After development of each fluorescent signal, the antibody was deactivated by incubating the tissue in 1.5% H_2O_2 for 30 minutes at room temperature. After several washes with PBS/1% Tween-20, the tissue was probed with the next antibody and the appropriate tyramide-conjugated fluorochrome.

Following *in situ* hybridization and visualization of all signals, BrdU was retrieved and detected with rat anti-BrdU antibody by immunohistochemistry methods described above except that the blocking solution and antibody dilutions were made with normal goat serum diluted in PBS/BSA/DMSO/Triton X-100.

2.2.9. Quantification of ablation from retinal sections and assay of cell death

Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264 fish, treated with either a metronidazole solution or a control DMSO solution, were quantified for conditional ablation of UV cones. Employing cryosections of retinal tissue, a representative section for each larval fish was chosen for quantification. The length (in µm) of the ONL of the retina was measured and UV cones expressing mCherry in the section were counted. A ratio of the number of UV cones expressing mCherry per length of ONL (µm) was calculated for each retina, and the average was taken for each group. Samples were acquired from fish fixed after a treatment of 10mM MTZ (or DMSO) for 48 hours (control n=10, experimental n=8). Standard error was calculated and is represented as error bars in Figure 3E.

TUNEL detection was performed to identify apoptotic cells on retinal cryosections (Roche, Laval, QC; No. 11684817910). Retinal tissue sections on slides were incubated in blocking solution (3% H_2O_2 in methanol) for 10 minutes at room temperature, then rinsed with 1xPBS. Slides were next incubated in fresh permeabilisation solution (0.1% sodium citrate/0.1% Triton X-100) for 2 minutes at 4°C, then rinsed with 1xPBS. Label solution and Enzyme solution were combined to form the TUNEL reaction mixture and applied to slides. After a 60 minute incubation in a humid chamber at 37°C, slides were rinsed with 1xPBS and analyzed for 515-565nm (green) fluorescence. This green signal was converted if tissues were already expressing GFP in the UV cones. Converter-POD (supplied in the kit) was applied to sections for 30 minutes at 37°C in a

humid chamber. A tyramide conjugated to AlexaFluor 647 (Invitrogen, Carlsbad, CA; No. T20936) was applied as described above.

2.2.10. Quantification of proliferating cells from retinal sections

Larval *Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264* zebrafish were treated with 5mM BrdU for 24 hours at 4 dpf, incubated in 10mM MTZ (or DMSO) for 48hrs from 5-7dpf and then were exposed to 5mM BrdU again for up to 48 hours. Sample fish were fixed at various time points: 24 hours post MTZ treatment (control n=9, experimental n=10), 1 week post MTZ treatment (control n=2, experimental n=4) and 3 weeks post MTZ treatment (control n=2, experimental n=2). Cryosections of retinal tissue were analyzed for BrdU incorporation into cells in the INL and ONL. A representative section from each eye of an individual fish was quantified whenever possible and the average was taken. Quantification of proliferating cells was performed by measuring the area (μm^2) of the retina and counting the number of BrdU-positive cells within the retina, excluding those in the CMZ. A ratio of the number of BrdU-positive cells in the INL or ONL per area of retina (μm^2) was calculated for each fish. Standard deviation was calculated for each group and time point and is represented as error bars in Figure 6E, F.

2.2.11. Analyzing whole mount retinas to determine the identity of BrdU-positive photoreceptors

Juvenile *Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264* zebrafish were subjected to a series of BrdU and metronidazole (or DMSO) treatments, dissolved in system water, over the course of 1 week (7 dpf: 24 hours in 5mM BrdU; 8 dpf: 24 hours in 10mM MTZ [or DMSO], 9-10 dpf: 48 hours in system water, 11 dpf: 24 hours in 10mM MTZ [or DMSO], 12 dpf: 24 hours in system water, 13-14 dpf: 48 hours in 5mM BrdU). Fish were fed fry food and brine shrimp twice daily. Following recovery from treatments, fish were placed in the aquaria system to grow.

At 3 months post-fertilization, 6 fish treated with metronidazole and 6 fish treated with DMSO were dark adapted overnight to prepare for retinal dissection. The fish were deeply anesthetised with MS-222 and euthanized by severing the brain stem. The eyes were removed from the head and the retinas were carefully dissected and separated from the RPE. The whole retinas were placed into fixative (4% paraformaldehyde/5% sucrose/0.1M phosphate buffer pH 7.4) overnight at 4°C.

Following *in situ* hybridization and BrdU detection (as described above), only retinas that had the region of the optic nerve intact were chosen to flat-mount on glass slides (MTZ treated n=4; DMSO treated n=4).

The larval remnants of the whole mount retinas, proximal to the optic nerve, were examined for BrdU incorporation into photoreceptors via confocal microscopy. For each retina, 2 rings of BrdU-containing cells were visualized, representing the cells that had been proliferating in the CMZ at each time of BrdU application. The inner most ring represented the earliest BrdU treatment at 7 dpf

for 24 hours, while the outer ring represented the second BrdU treatment at 13 dpf for 48 hours. Analysis of the larval remnants excluded the cells that contributed to either ring. Only BrdU-positive cells that were clearly distinguishable as in between the 7- and 13-day rings or inner to the 7-day ring were included (Fig.9B).

A series of z-stack confocal images of portions of each larval remnant were taken for the 4 metronidazole treated and the 4 DMSO treated retinas for analysis of BrdU co-localization with photoreceptor subtypes. The control and experimental retina images (41 in total) were combined and randomly assigned numbers such that the researcher was blinded to the treatment. As expected, BrdU incorporation in the nucleus of a photoreceptor was typically in a different plane compared to the *in situ* hybridization labelling of the photoreceptor opsin. Therefore analysis was performed using the ZEN software (2009; Carl Zeiss MicroImaging), employing the Ortho tool which allows for 3-dimensional analysis of a z-stack, making the visualization of co-localizing opsin and BrdU possible (Fig.S1). BrdU-positive cells in the scleral portion of the ONL that did not co-localize with a cone photoreceptor opsin were counted as rods or rod progenitors but herein we make the simplifying assumption that they were rods. The BrdU-containing cells were classified into six categories based on opsin *in situ* hybridization and nuclear position: i) cells that were unambiguously UV cones, ii) cells that were unambiguously BGR (blue, green and red) cones, iii) cells that were unambiguously rods, iv) cells that were possibly UV or BGR cones, v) cells that were possibly UV cones or rods, and vi) cells that were possibly BGR cones or rods. Following classification, only the unambiguous

categories were considered for further analysis (i, ii and iii) because the ambiguous categories included only a small percentage of total cells and appeared to be distributed approximately evenly amongst treatments.

Following quantification of BrdU-incorporated cells and analysis of photoreceptor identity, retinas were excluded from the remainder of the analysis based on the quantity of BrdU-positive cells. Retinas with less than half of the larval remnant lasting and fewer than 20 BrdU-positive cells were excluded. The remaining viable retinas (MTZ treated n=3; DMSO treated n=3) were included.

The percentage of BrdU-positive cells for each of the three categories (UV, BGR, and rods) following metronidazole or DMSO vehicle control treatment was calculated for each retina, and the average was taken. Standard deviation was calculated and is represented as error bars in Figure 9.

For analyzing experimental retinas individually, the ratios of the percent of BrdU-positive UV cones, BrdU-positive BGR cones and BrdU-positive rods, normalized by the mean percent of BrdU-positive cells from each category for DMSO treated retinas, was calculated.

BrdU-positive cone ratios were also analyzed (excluding rods). In an adult zebrafish retina, the predicted ratio of cone subtypes in the mosaic is 1 UV: 1 blue: 2 green: 2 red (Allison et al., 2010). The expected larval remnant in the adult differs because it has less UV cones compared to the adult and the exact value is difficult to predict due to variability in the larval portion of the retina, therefore the predicted ratio is <1 UV: 1 blue: 2 green: 2 red (Fig.9H) (Allison et al., 2010). From the expected ratio, we predicted that in a normal larval remnant,

<17% of the BrdU-positive cells would be UV cones. The actual ratios of BrdU-positive UV cones to BrdU-positive BGR cones were then calculated for MTZ treated and DMSO treated fish, under the assumption that it is equally likely for BrdU to incorporate into blue, green and red cones.

2.2.12. Statistical analysis

Unpaired *t* tests were performed on quantification data to determine significance. The QuickCalcs online calculator was employed to generate t values, degrees of freedom, standard error of difference, and p values (GraphPad Software, 2005, http://www.graphpad.com/quickcalcs/index.cfm).

2.3. Results

2.3.1. Characterizing novel transgenic fish and directed cell-specific ablation of UV cone photoreceptors

I developed transgenic zebrafish wherein UV-sensitive cones could be ablated by addition of a prodrug. I characterized expression of *nfsb*-mCherry, based on mCherry fluorescence in sectioned and wholemount materials, in the transgenic zebrafish *Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264*. The transgenic fish line *Tg(SWS1:Gal4-VP16)ua3016* is a novel line created in the Allison laboratory, and we first asked if the expression of this transgene was limited to UV cones and was consistently present in all UV cones,

as we expected. We crossed this line to *Tg(UAS-E1b:NfsB-mCherry)c264* (Davison et al., 2007; Balciunas et al., 2004) and observed mCherry expression in the photoreceptors of a portion of fish expressing Gal4-VP16 (confirmed by expression of GFP in the heart). Overall, the mCherry was contained exclusively in UV cones (Fig.3A,B), though not in all UV cones, with an apparent decrease in the number of cones expressing it with age. This is consistent with recent reports indicating that the repetitive UAS sequence becomes disabled during development due to gene silencing (Goll et al., 2009; Li et al., 2007).

I next assessed the quality and quantity of UV cone ablation following prodrug application. Following metronidazole treatment for 48 hours, *Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264* zebrafish larvae showed an observable reduction in mCherry fluorescence within the photoreceptor layer of the retina (Fig.3A,B). Sections of individual fish retinas were examined to quantify the reduction of nitroreductase-mCherry expressing UV cones. Cones expressing mCherry were decreased in abundance by 91.3% following metronidazole application compared to fish that did not receive the prodrug, and this difference was significant ($p < 0.0001$, control n=10, experimental n=8) (Fig.3E).

Abundant TUNEL staining was observed in cone photoreceptor nuclei after exposure to metronidazole (Fig.4), coincident with the decrease of transgene-expressing UV cones, confirming that the loss of mCherry fluorescence is in fact due to cytotoxin-induced ablation via apoptosis. The cell-specific expression of nitroreductase was at a sufficiently high level to form a viable toxin

upon the addition of metronidazole, confirming that this germline transmissible technique is valid for temporally inducible targeted-ablation of photoreceptors.

To test the hypothesis that metronidazole application also killed or damaged cells not expressing our transgene, we compared gross morphology of the remaining cones following prodrug application. Zebrafish *Tg(-5.5opn1sw1:EGFP)kj9* that produce GFP expression in the UV cones (Takechi et al., 2003) were crossed into *Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264* such that nitroreductase-mCherry expression was present in a subset of the UV cones and GFP was present in all UV cones. Zpr1, an antibody against an epitope of the red-green double cone photoreceptors, was applied to retinal cryosections to determine if neighbouring cells were affected by ablation. 24 hours following metronidazole treatment, the subset of UV cones expressing nitroreductase were ablated, while neighbouring double-cone pairs and UV cones lacking nitroreductase-expression persisted and appeared morphologically normal (Fig.5). Thus we were not able to support the hypothesis that adjacent cells were damaged. This is consistent with past work showing that metronidazole does not have a toxic bystander effect on neighbouring cells, including neighbouring retinal neurons (Curado et al., 2008; Pisharath et al., 2007; Edwards, 1993).

2.3.2. Effects of cone photoreceptor ablation on retinal stem cell proliferation.

I tested the hypothesis that our combination of transgenes and metronidazole that resulted in UV cone death subsequently led to proliferation in the retina. Abundance of proliferating cells was assessed in stem cell populations

known to occur in the retina (see background) via detection of BrdU incorporation.

Observations of retinal sections showed the expected incorporation of BrdU into the CMZ of all transgenic fish, both with and without metronidazole treatment, indicating that BrdU successfully integrated into the newly synthesized DNA of proliferating retinal neurons at the time of exposure (Kee et al., 2002). An increase in proliferating cells, excluding the CMZ, was quantified following metronidazole ablation. At the early time point following BrdU application (24 hours, that which most represents proliferation), a greater number of BrdU-positive cells were present in the INL and ONL in fish receiving metronidazole compared to those that received vehicle control (Fig.5A-D). 24 hours after ablation, fish treated with the prodrug showed an approximate 3-fold increase in BrdU labels in the INL ($p<0.001$, control $n=9$, experimental $n=10$) and an 8-fold increase in the ONL ($p=0.0004$) of the retina (Fig.6E, F). At 1 week after ablation, approximately 5-fold more cells in the INL ($p=0.0051$, control $n=2$, experimental $n=4$) and 15-fold more in the ONL ($p=0.068$) co-localized with BrdU, though small sample sizes preclude any interpretation of statistical significance. At 3 weeks post metronidazole ablation (1 month post-fertilization), 4-fold more BrdU-positive cells in the INL ($p=0.0123$, control $n= 2$, experimental $n=2$) and 13-fold more in the ONL ($p=0.07$) were present compared to non-metronidazole treated fish, though small sample sizes preclude any interpretation of statistical significance. Overall, the increase in proliferation following conditional ablation of the transgenic UV cone photoreceptors provides evidence

that the limited neuronal death of a subset of a single photoreceptor type was sufficient to trigger an injury response.

2.3.3. Regeneration following conditional targeted ablation of UV cone photoreceptors

I investigated the hypothesis that the ablation of a single cone photoreceptor subtype was insufficient to lead to regeneration of new cone photoreceptors. Larvae were first examined for evidence of regeneration at 5 days post metronidazole treatment. BrdU-positive detection of cells in the ONL of the retina indicated that the regenerating retinal neurons were recently proliferating (Fig.7B). Although the regenerating cells were not yet morphologically mature, in this transitional state, the co-localization of BrdU incorporation with the expression of nitroreductase-mCherry indicated that neuronal precursors had begun to differentiate, and had an established cone photoreceptor fate due to the expression of UV opsin (Fig.7). Other BrdU-positive cells were observed that did not co-localize with mCherry (Fig.7C). These retinal neurons potentially represent regenerating UV cones not possessing the nitroreductase transgene. It is also possible that these neurons had entirely different cell fates and differentiated into another type of cone or rod photoreceptor, or were actively proliferating rod precursor cells.

I observed the regeneration of morphologically mature cones 1 week after metronidazole ablation (Fig.8). BrdU-positive cells that co-localized with UV cone nitroreductase-mCherry transgene expression were detected (Fig.8A-D).

Regenerated UV cone photoreceptors were observed to be morphologically similar to newly generated UV cones in the peripheral expanding retina, preliminarily suggesting that the regenerated neurons are qualitatively normal (Fig.8A-D,E). Overall, assuming our treatments did not induce damage to other cells in the retina (consistent with data in 2.2.1.), these data refute the hypothesis above by demonstrating that ablation of a small set of cone photoreceptors is sufficient to induce regeneration of cone photoreceptors.

2.3.4. Identity of regenerating cone photoreceptors after the targeted ablation of a subset of UV cone photoreceptors

I tested the hypothesis that ablation of a particular cone type would lead to the regeneration of random rod and cone photoreceptor types. Nitroreductase-transgenic larval zebrafish were submitted to a series of treatments between 7 and 14 dpf with metronidazole to induce UV cone-specific ablation, and to incorporate BrdU into proliferating cells. The larvae were reared for 3 months, allowing the eyes to grow large enough to enable retinal dissections, freeing the neural retina from the RPE. An examination of the whole-mount neural retinas for BrdU-containing photoreceptors within the larval remnant was then performed.

The 3-month period for growth was presumed to be more than sufficient time for the regenerative response to be completed following cone ablation, based on recent similar work (Thummel et al., 2008). Among the eight fish retinas examined (control n=4, experimental n=4), the larval remnant in the adult retina

was observed to be extremely variable, consistent with past data (Allison et al., 2010). Similar to the adult zebrafish retina, the larval retina displays a mosaic pattern; however it is not as strictly defined as the intricate adult photoreceptor mosaic (Allison et al., 2010). Specifically, the precise rows of photoreceptors do not form until adulthood. Once maturity is attained, photoreceptors generated during larval development retain their larval pattern, making the larval remnant proximal to the optic nerve very distinct in that area (Allison et al., 2010). An obvious inconsistency in density and distribution of photoreceptor types in this region was observed (Fig.9A), limiting our ability to average the quantifications. Overall, however, it was observed that metronidazole treated nitroreductase-transgenic retinas contained more BrdU-positive cells in the ONL (average number of BrdU+ cells experimental=67.3±52, control=58±37, p=0.81), consistent with data in Figure 6, further supporting our hypothesis that UV cone ablation induces an increased level of proliferation. The data in this trial was not significantly different, as opposed to the data 24 hours post-ablation in Figure 6, which may be a result of methodological differences including that in the current trial, there was 5 days between BrdU treatments compared to 2 days in Figure 6.

I classified the BrdU-containing cells in the larval remnant into six categories based on opsin in situ hybridization, confocal microscopy and nuclear position. I focus here on those cells I could unambiguously categorize (see Methods) and thus consider three categories of regenerated (BrdU-containing) cells: i) UV cones; ii) BGR cones (other cone photoreceptors); iii) rod

photoreceptors. The abundance of each of these was tallied, and the percent contribution of each category to the total regenerated cells was determined.

In retinas that received vehicle only, the majority of cells containing BrdU were rods (mean of three retinas, 68.2±20%), as expected. UV cones (7.5±6%) and BGR cones (24.3±16%) were also detected, which was not expected. The appearance of cone photoreceptors in this area can be taken as evidence of remodelling of the larval remnant during maturation, via addition or movement of cones, consistent with past speculations (Allison et al., 2010). Alternatively, this may indicate that some cones are being killed in our transgenic fish regardless of metronidazole treatment; this is not consistent with data in Figure 5, however it cannot yet be excluded.

My data allowed me to test the hypothesis that ablation of UV cones would lead to an increase in the number of rod photoreceptors, as observed in the trout retina (Allison et al., 2006a; Allison, 2004). Unexpectedly, a smaller proportion of recently proliferating cells became rods (42.9%±20%) in larvae with UV cones ablated compared to controls without ablation (68.2%±20%). This observation demonstrates that when UV cones are selectively destroyed, the resulting gaps in the photoreceptor mosaic are not preferentially replaced by rods (Fig.9D). This data rejects the hypothesis that ablation of UV cones would lead to an increase in the number of rod photoreceptors.

My data also allowed me to determine the typical identity of regenerating cones after ablation of UV cones. Amongst BrdU-positive photoreceptors, retinas

receiving metronidazole had a greater proportion of UV cones (225%) and BGR cones (58%) compared to vehicle-treated controls.

Due to variability between the larval remnants of different fish, experimental retinas were examined on an individual basis as well (Fig.9A). To accomplish this, the values of each metronidazole-treated retina were normalized to the mean values of untreated retinas. Retinas #1 and #2 showed an overall increase in BrdU-positive UV cones when normalized against the controls (a 4-fold increase and a 2-fold increase, respectively) (Fig.9E, F). The number of BrdU-positive blue, green and red cones was similar regardless of metronidazole treatment. Retina #3 differed in that it showed fewer BrdU-positive UV cones when normalized against the control, while BrdU-positive blue, green or red cones increased 3-fold after normalization following metronidazole ablation (Fig.9G). Upon the selective ablation of a subset of UV cones, on average a new UV cones regenerates in their place 30% of the time, while other photoreceptor subtypes (blue, green or red cones) regenerate 70% of the time. However when compared to the abundance of BrdU+ cells in control retinas, it is apparent that this represents more than a doubling in abundance of BrdU+ UV cones.

In an adult zebrafish retina, the predicted ratio of cone subtypes in the mosaic is 1 UV: 1 blue: 2 green: 2 red (Allison et al., 2010). The expected larval remnant differs because it has fewer UV cones compared to the adult and the exact value is difficult to predict due to variability in the larval portion of the retina, therefore the predicted ratio is <1 UV: 1 blue: 2 green: 2 red (Fig.9H) (Allison et al., 2010). From the expected ratio, we predicted that in a normal

larval remnant, <17% of the BrdU-positive cells would be UV cones. The actual ratio of the mean BrdU-positive UV cones to the mean BrdU-positive BGR cones of control (DMSO treated) retinas was calculated (see Methods) and 24% of BrdU containing cones were UV cones. This ratio of 24% UV cones in the untreated retina is comparable to the <17% predicted value. The individual retinas #1 and #2 were similar, with ratios of 58% and 46% of BrdU-positive cones identifying as UV cones after metronidazole treatment, respectively (Fig.9J,K), which is much higher than the predicted normal ratio. Retina #3 differed greatly, with a ratio of only 6% of BrdU-positive cones being UV cones (Fig.9L). Overall for all three metronidazole-ablated retinas, the ratio of BrdU-positive UV cones differed greatly from the expected ratio.

2.4. Discussion

The transgenic zebrafish line *Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264* was engineered, and characterized in regards to its validity as a paradigm for precise cone photoreceptor ablation and regeneration. The exposure of live transgenic fish to metronidazole was first optimized in order to induce ablation of the targeted neuronal cell population expressing nitroreductase. Evidence of apoptosis in the ONL and the significant reduction in UV cones expressing nitroreductase-mCherry confirmed that the nitroreductase restricted to the UV cones was able to bind and reduce the introduced metronidazole. The resulting cytotoxin successfully induced apoptosis of the

cells selectively expressing nitroreductase. Additionally, reports that metronidazole does not produce a bystander effect at appropriate concentrations were supported in that the effects of metronidazole were apparently confined to the targeted cells (Curado et al., 2008; Pisharath et al., 2007; Edwards, 1993). This conditional targeted ablation technique employing the metronidazole-nitroreductase system is an efficient and effective method for removal of a specific cone photoreceptor population, providing both cell-type and temporal specificity.

This pharmaco-genetic method was also determined to be a valuable tool for regenerative studies. Following metronidazole-induced ablation of the transgene-expressing UV cones, a significant increase in proliferation was observed in both the INL and ONL of the retina. The proliferative response was detected 24 hours after metronidazole treatment and BrdU-positive cells were still detectable at 3 weeks post ablation, providing evidence that the ablation of a subset of photoreceptors is sufficient to stimulate proliferation and retinal regeneration. It is speculated that Müller glial cells in the INL are the source of the majority of neuronal proliferation, responding to retinal injury manifested as UV cone ablation (Bernardos et al., 2007; Thummel et al., 2008). The appearance of immature retinal neurons was observed at 5 days post metronidazole treatment as evidenced by the incorporation of BrdU in the nuclei of cells located in the ONL. Various BrdU-positive cells co-localized with nitroreductase-mCherry transgene expression at this time point, indicating that at least some of these precursors were expressing the UV specific opsin gene and therefore had already

determined a specific cone photoreceptor fate. The regeneration of morphologically typical mature cone photoreceptors expressing UV opsin were observed at 1 week following ablation, confirming the effectiveness of this targeted cone ablation method for regenerative investigations.

Previous literature has reported that when broad cell death or injury occurs in the retina, the resulting cone mosaic of the regenerated retina is significantly disrupted, with evidence of identical subtypes of cones regenerating close to or directly adjacent to each other (Cameron and Carney, 2000). As well, cell densities are greater in the regenerated retina, possibly contributing to the irregularity of the mosaic pattern (Cameron and Easter, 1995). These regenerative characteristics, combined with variability of the larval retina, indicate that the identification of a regenerated photoreceptor following UV cone ablation is difficult to predict. One possibility is that the ablated cones are replaced by only multipotent progenitors that regenerate into cones. It is also plausible that rod progenitors already present in the ONL replace the ablated cones with rod photoreceptors. The specificity of ablation does not necessarily infer that regeneration will be equally specific. We therefore set out to determine the proportion of regenerated cells containing BrdU that were UV cones.

We have provided evidence that following the exclusive ablation of a subset of UV cones and sufficient time for complete regeneration, rods, UV cones, and other cones were observed to be added to the larval remnant. The majority of these cells were blue, green and red cones and rods. Ratios of BrdU-containing UV cones to all other BrdU-positive cones demonstrated that the

DMSO treated fish had cone ratios similar to the predicted values. Overall for the three experimental retinas, the BrdU-positive cone ratios showed that metronidazole ablation results in a very different outcome in BrdU-positive cone identity compared to the expected and the calculated control ratios. This variation in the identity of regeneration may be an artefact of the larval retina. Due to the inconsistency in photoreceptor distribution and density of the larval retina and the lack of precision in the larval mosaic, potentially more freedom in cell fate determination is tolerated during regeneration in the juvenile form (Allison et al., 2010). Considering UV cones comprise about 17% of zebrafish cone photoreceptors, and much fewer than this in the larval remnant (Allison et al., 2010), we can conclude that a bias exists for UV cones to be generated in the place of ablated UV cones.

In cyprinid retinal development, cone photoreceptors differentiate in an organized manner, as defined by opsin expression, with red cones developing first, followed by green cones, then blue and UV cones being the last to differentiate (Stenkamp et al., 1997). As well, UV cones and blue cones are comparable in that they express visual pigments that are the only single cones and both are sensitive to short wavelengths of light (Raymond and Barthel, 2004). It is possible that the differentiation pathways between these two cone types are very similar, and therefore they may be able to respond to the same regenerative cues.

Previous studies in teleost fish have shown that opsin expression can be influenced by thyroid hormone (Allison et al., 2006a; Allison et al., 2004;

Veldhoen et al., 2006). Altering thyroid hormone levels in salmon induces mature UV cones to die (Allison et al., 2006a). This phenomenon is presumably regulated by the thyroid hormone receptors *TRα* and *TRβ2* due to their increased expression at the time of the photoreceptor death. Therefore based on evidence that SWS1 and SWS2 opsins are closely related and that UV cones have the potential to transform into blue cones, I speculate that the other non-UV cones that regenerated are blue cones.

The results strongly suggest that rod photoreceptors are not preferentially generated in the place of ablated UV cones as demonstrated by a decrease in the proportion of BrdU+ rods after ablation compared to non-treated retinas. Some factors that have previously been investigated to play important roles in rod and cone photoreceptor differentiation offer a potential interpretation. A zebrafish mutant dubbed *lots-of-rods* (*lor*) was identified to have an increased number of rod photoreceptors based on detection of rhodopsin and it was hypothesized that the extra rods were the result of a fate change in a single cone subtype (Alvarez-Delfin et al., 2009). The mutation was identified in the gene *Tbx2b*, a T-box transcription factor known to have roles in development including cell cycle control, limb, heart, and endoderm development (Alvarez-Delfin et al., 2009; Christoffels et al., 2004; Snelson et al., 2008). Further investigation showed that *lor* mutants had an increase in rods and a decrease in UV cones (Alvarez-Delfin et al., 2009). Rod precursors require the expression of another transcription factor, *Nrl*, as well as its target gene, *Nr2e3*, in order to differentiate into rod photoreceptors. If *Nrl* is underexpressed, photoreceptor precursors take on the

short-wavelength opsin and are fated to become UV cones (Alvarez-Delfin et al., 2009; Mears et al., 2001). Conversely, if *Nrl* is over-expressed, cone precursors differentiate into rods, expressing rhodopsin (Oh et al., 2007). From this investigation, the role of *Tbx2b* in regulating photoreceptor cell fate was characterized. *Tbx2b* represses rod cell fate by inhibiting the expression of *Nr2e3* and therefore promoting UV cone specification (Alvarez-Delfin et al., 2009).

With this knowledge of the pathway involved in regulating photoreceptor precursors to differentiate into either rods or UV cones, we can propose that the injury response in our experiment may have triggered rod precursors to switch cell fate, regenerating into UV cone photoreceptors instead. Since Müller glia receive a signal in response to ablation of UV cones to dedifferentiate into multipotent stem cells, it is possible that this injury response is also able to communicate with early stage rod progenitors already present in the INL (Raymond et al., 2006; Bernardos et al., 2007; Thummel et al., 2008). Distinct from the injury response, rod progenitors are always proliferating and migrating radially from the INL to the ONL of the retina (Raymond et al., 2006). Therefore some of these precursors would presumably be further along in the regenerative process upon the initial onset of damage compared to Müller glia which must first re-enter the cell cycle before migrating to the ONL and differentiating into a new cell fate (Raymond et al., 2006; Bernardos et al., 2007; Thummel et al., 2008). It is possible that the regenerative signalling pathway caused an upregulation of *Tbx2b* in these precursors, inhibiting the *Nrl/Nr2e3* interaction, and promoting a cell fate change from rod to UV cone. Therefore, we would see fewer rods with

BrdU incorporation because fewer progenitors fated to become rods were present at the time of BrdU exposure.

BrdU-positive cells in transgenic retinas treated with a vehicle-only control were found to consist mostly of rods as was expected due to retinal growth in the adult zebrafish (Perron and Harris, 2000; Kubota et al., 2002). As the existing retina expands, new rod photoreceptors are added to the central retina to fill in the gaps and maintain visual sensitivity (Otteson and Hitchcock, 2003; Morris et al., 2008). Unexpectedly, some proliferating cells became cone photoreceptors following control treatment, which we interpret as representing a remodelling of the larval remnant during maturation (Allison et al., 2010). A recent investigation in zebrafish of the larval and adult photoreceptor mosaic determined that the larval remnant of the mature retina does not reorganize into the precise row mosaic pattern (Allison et al., 2010). They do however suggest that the larval remnant may undergo remodelling by the loss or addition of cone photoreceptors or by cell movements which contribute to the larval mosaic (Allison et al., 2010; Allison et al., 2006a; Biehlmaier et al., 2001; Eglen, 2006).

Table 1. Transgenic lines used to express nitroreductase (*nfsB*) in various cells.

Transgenic line	Description	Background	Source
Tg(-5.5opn1sw1:Gal4VP16)ua3016	Gal4-VP16 (activates UAS when present) in UV-sensitive cone photoreceptors	*Tg(-5.5opn1sw1:EGFP)kj9*	This work
Tg(-5.5opn1sw1:nfsb-mCherry)ua3003	Nitroreductase expressed in UV-sensitive cone photoreceptors, fused to mCherry	*Tg(-5.5opn1sw1:EGFP)kj9*	This work
Tg(-3.5opn1sw2:nfsb-mCherry)ua3001	Nitroreductase expressed in blue-sensitive cone photoreceptors, fused to mCherry	*Tg(-5.5opn1sw1:EGFP)kj9*	This work
Tg(UAS-E1b:NfsB-mCherry)c264	nitroreductase in cells that express Gal4VP16, fused to mCherry	AB	Davison et al 2007
Tg(-5.5opn1sw1:EGFP)kj11	GFP in UV-sensitive cones, used here to clarify anatomy and expression	WIK	Takechi et al 2003
Tg(hsp70l:nfsb-mCherry)[*]	Heat-shock promoter driving nitroreductase	*Tg(-5.5opn1sw1:EGFP)kj9*	This work

[*]used in transient expression experiments only, i.e. no stable transgenic created

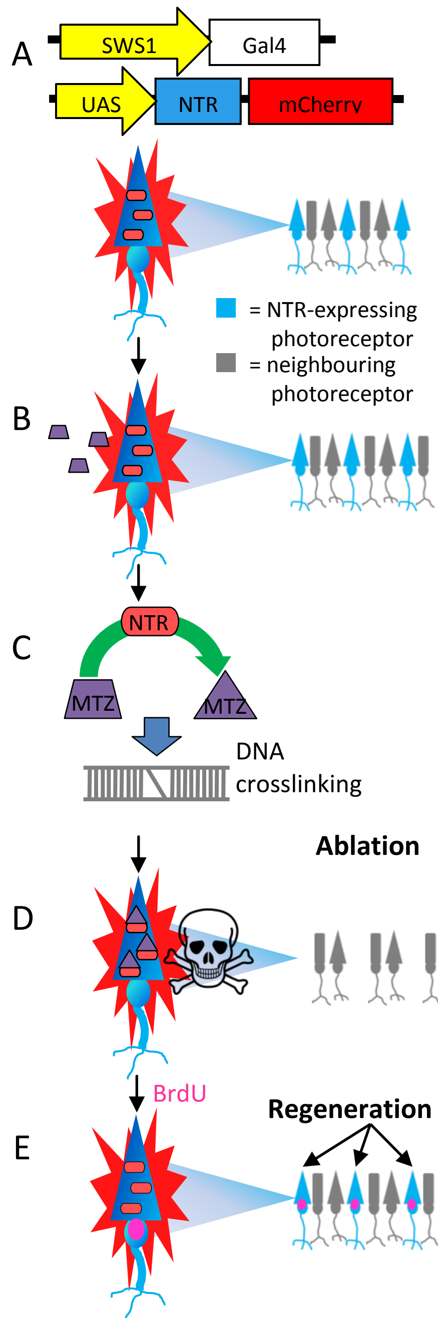


Figure 2. Regeneration investigated via a cell-specific ablation method. UV cone photoreceptors exclusively express the NTR-mCherry transgene (A). Upon treatment with a MTZ solution, NTR converts MTZ into a cytotoxin (B, C), resulting in ablation of only the cones expressing NTR (D), without disruption to neighbouring cells. Following cell death, BrdU incorporates into proliferating cells. Regeneration of the targeted photoreceptors occurs once the MTZ treatment is removed (E).

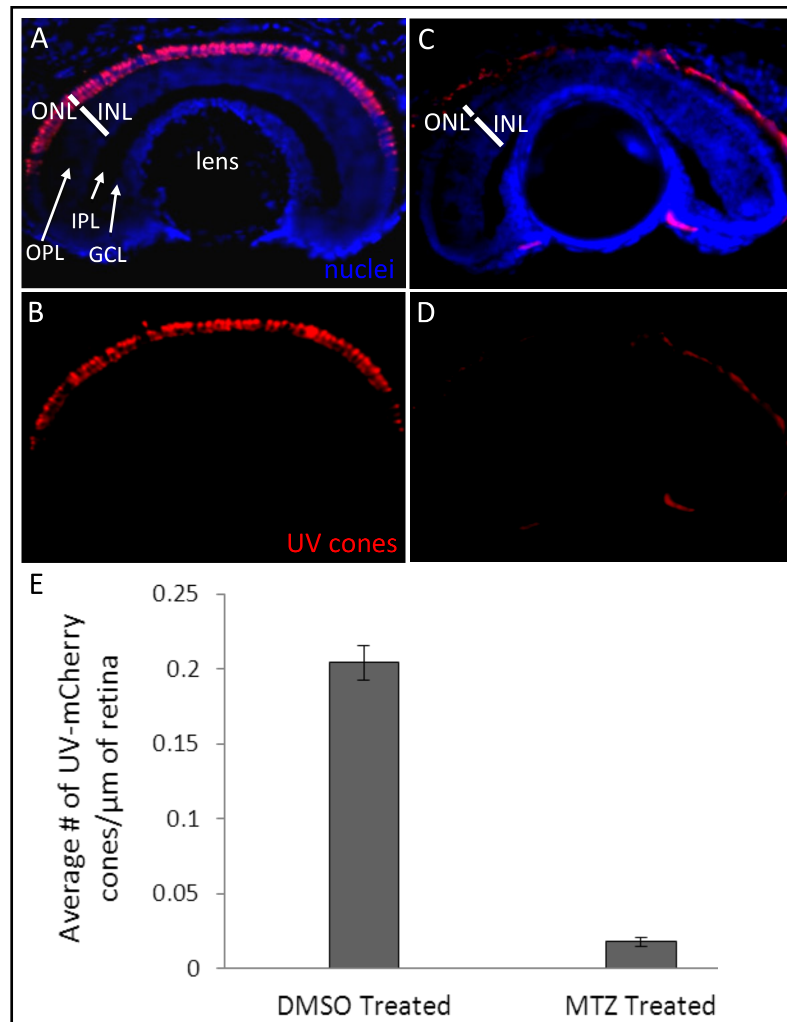


Figure 3. UV cone photoreceptor cell death by MTZ-NTR mediated cell specific ablation. *Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264* fish treated with a control 0.2% DMSO solution maintained NTR-mCherry expression and cell death was not induced (A, B). *Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264* fish treated with 10mM MTZ for 48hrs, lost the mCherry fluorescence due to ablation of the targeted UV cones (C, D). Note: The detected red fluorescence in C and D is auto-fluorescence due to a longer exposure compared the A and B. Quantification of UV cones expressing NTR-mCherry after treatment with the prodrug MTZ (E) revealed a significant decrease in the number of cones expressing mCherry fluorescence in the ONL compared to vehicle-treated controls ($p<0.0001$; DMSO treated n=10, MTZ treated n=8).

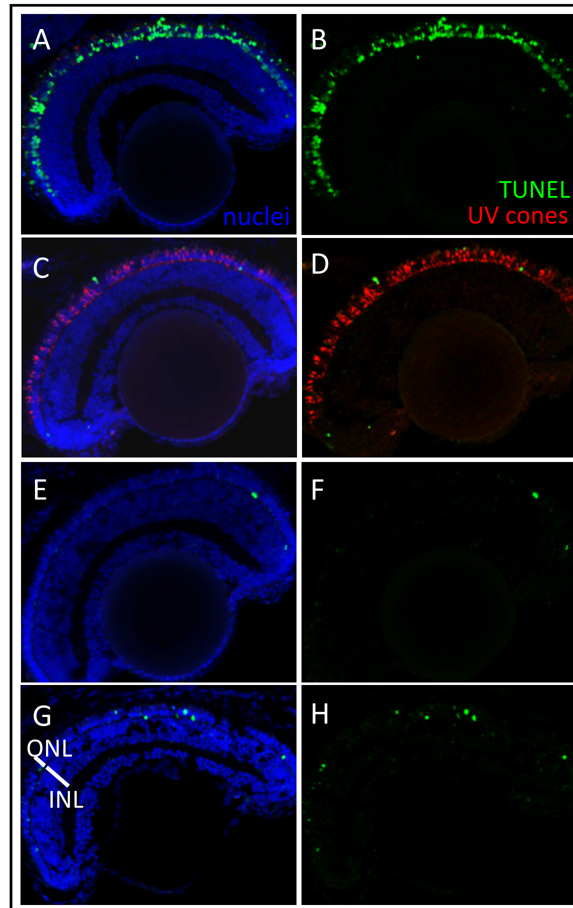


Figure 4. TUNEL detection of apoptotic cells following MTZ-NTR ablation. An abundance of apoptotic cells were detected in the ONL of the retina of *Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264* fish treated with 10mM MTZ for 48hrs (A, B) compared to the controls. Very little apoptosis was found in the ONL of *Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264* fish treated with a control 0.2% DMSO solution (C, D). Non-transgenic siblings treated with either 10mM MTZ (E, F) or 0.2% DMSO (negative control) (G, H) for 48hrs possessed few apoptotic cells as well.

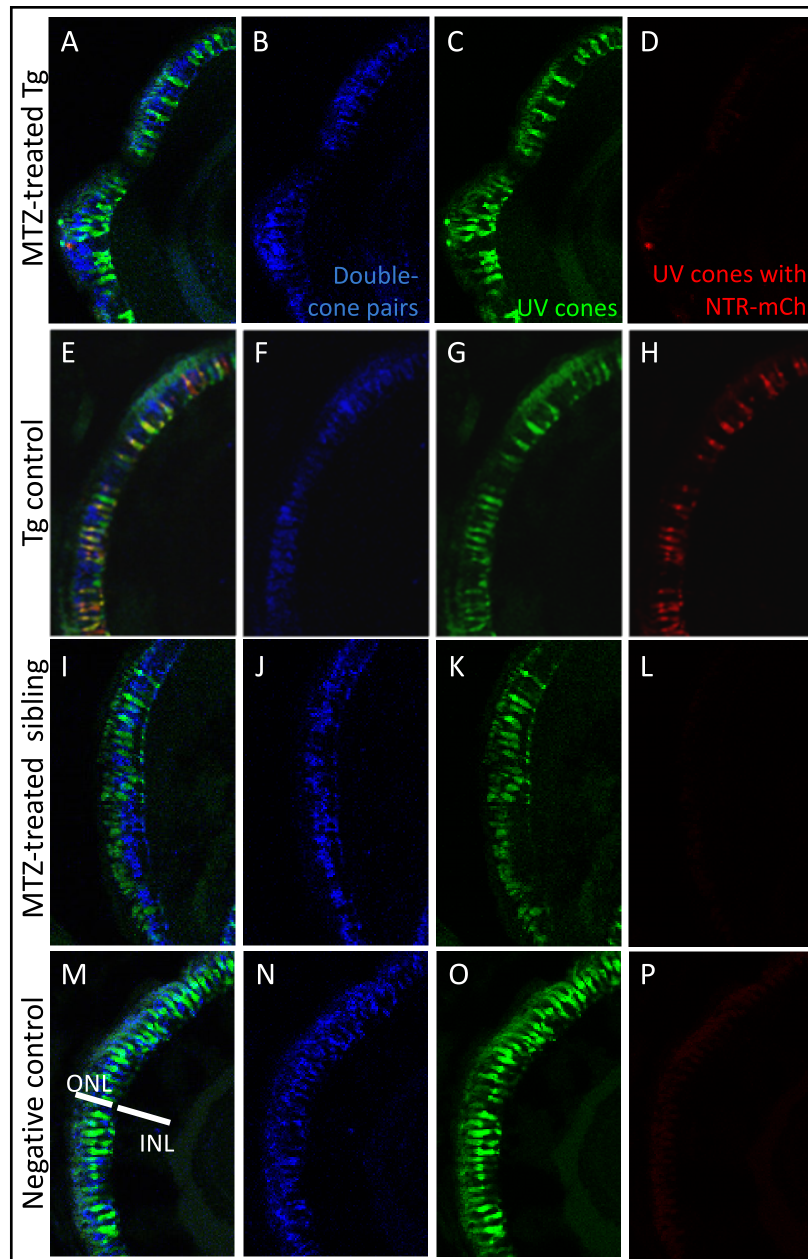


Figure 5. Morphological analysis of photoreceptors indicates that metronidazole does not create a bystander effect. UV cones expressing NTR were ablated after the addition of MTZ (A, D), while *nfsB*-mCherry persisted in transgenic fish treated with DMSO (E, H). 24 hours after ablation, UV cones lacking NTR-expression (GFP) appeared morphologically normal (C) compared to all controls (G, K, O). Red-green double cone pairs were detected with the antibody zpr1. Zpr1 labelling was consistent following MTZ ablation (B) compared to the controls (F, J, N). A-D: nitroreductase-transgenic fish treated with 10mM MTZ for 48hrs, resulting in ablation of targeted UV cones; E-H: nitroreductase-transgenic fish treated with a control 0.2% DMSO solution; I-L: non-transgenic siblings treated with 10mM MTZ for 48hrs; M-P: negative control of non-transgenic sibling treated with a control 0.2% DMSO solution.

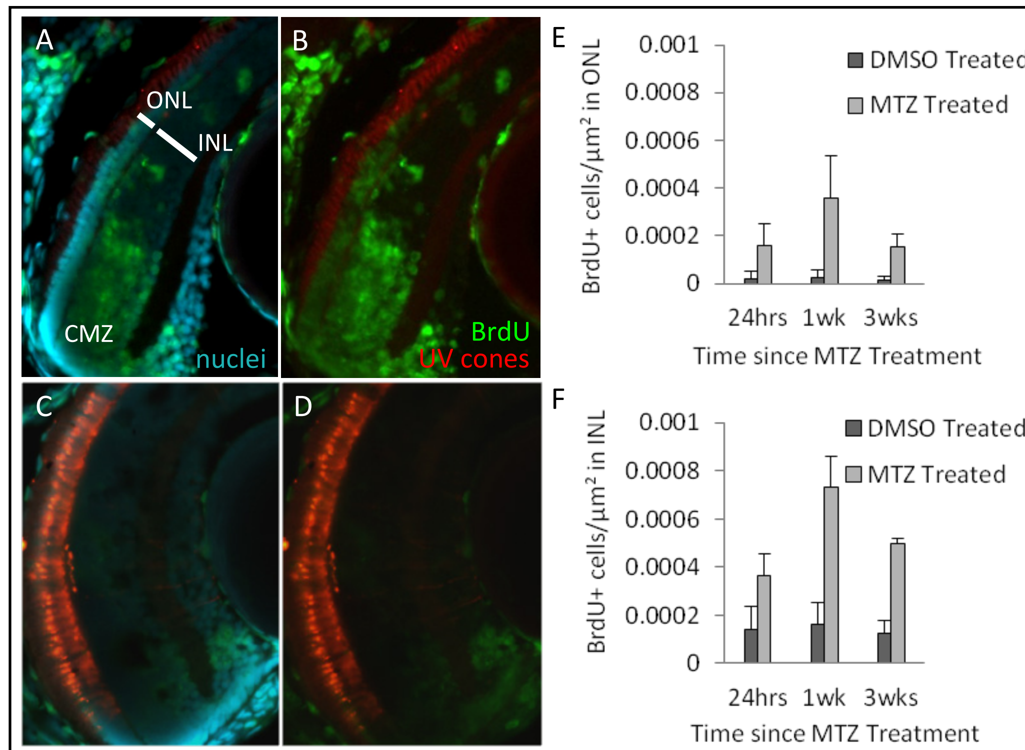


Figure 6. Following MTZ-NTR ablation of UV cones expressing the transgene, there was an increase in proliferating cells as evidenced by the increased number of BrdU-positive cells in the inner and outer nuclear layers of the retina. *Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264* fish treated with MTZ showed a significant increase in proliferating cells in the retina at 24 hours after ablation (A, B) compared the transgenic fish treated with a DMSO control solution (C, D). BrdU incorporated into the CMZ of all fish with and without cone ablation, as expected (A-D). Proliferating cells were quantified in the ONL and INL at 24 hours, 1 week and 3 weeks after ablation (E, F) (24 hours after ablation: ONL $p=0.0004$, INL $p<0.001$, DMSO treated n=9, MTZ treated n=10; 1 week after ablation: ONL $p=0.068$, INL $p=0.0051$, DMSO treated n=2, MTZ treated n=4; 3 weeks after ablation: ONL $p=0.07$, INL $p=0.0123$, DMSO treated n=2, MTZ treated n=2).

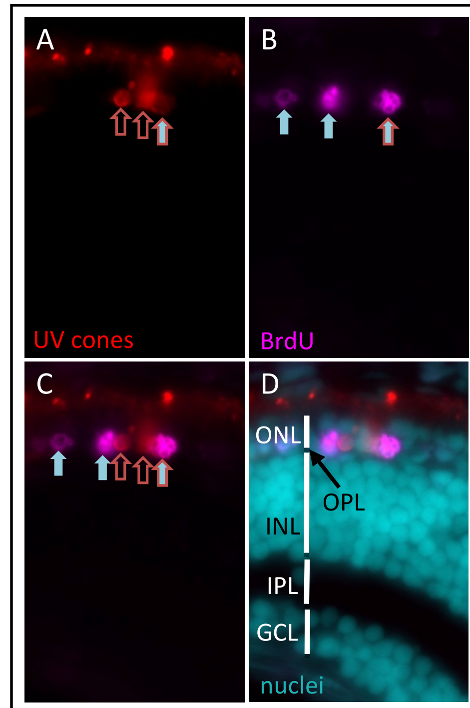


Figure 7. Early regeneration following prodrug cell ablation. Regenerating UV cones were observed in larvae at 5 days after MTZ treatment. Co-localization of these cells with BrdU-positive detection indicated that the regenerating cones were recently proliferating. The right-most cell (A-C) is double labelled for BrdU and UV opsin (mCherry) (filled arrow with red outline; has not yet differentiated to a cone morphology). Other UV cones have begun to reappear (empty red arrows), presumably outside of the window of BrdU application. Other BrdU-positive photoreceptors are detectable (filled arrows), likely representing rods or nascent UV cones.

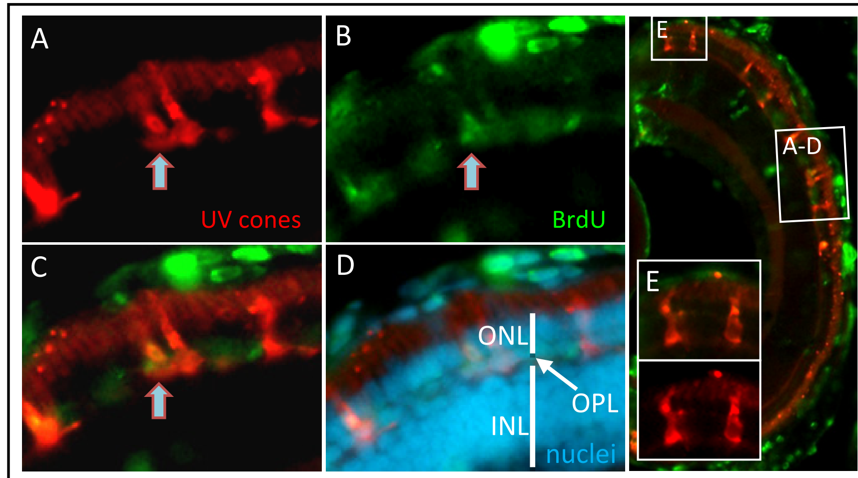
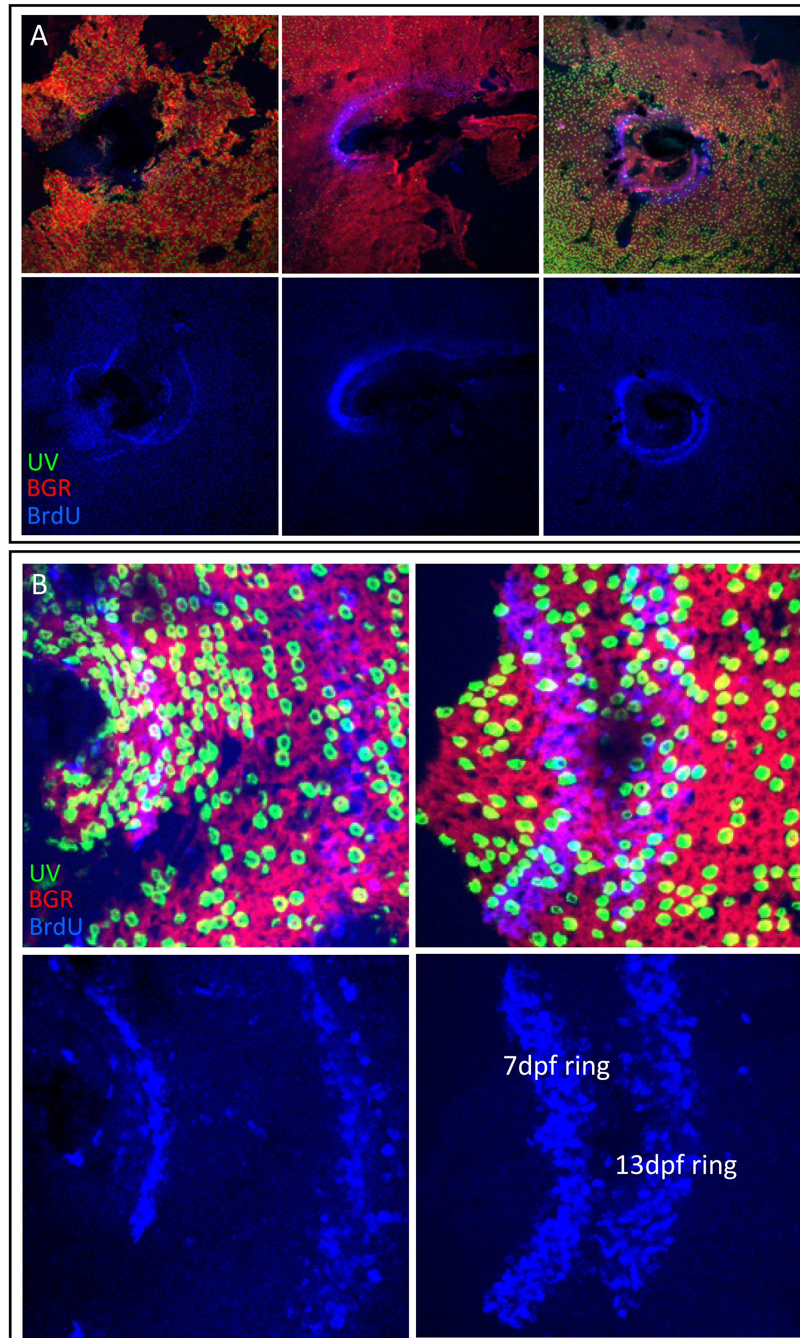
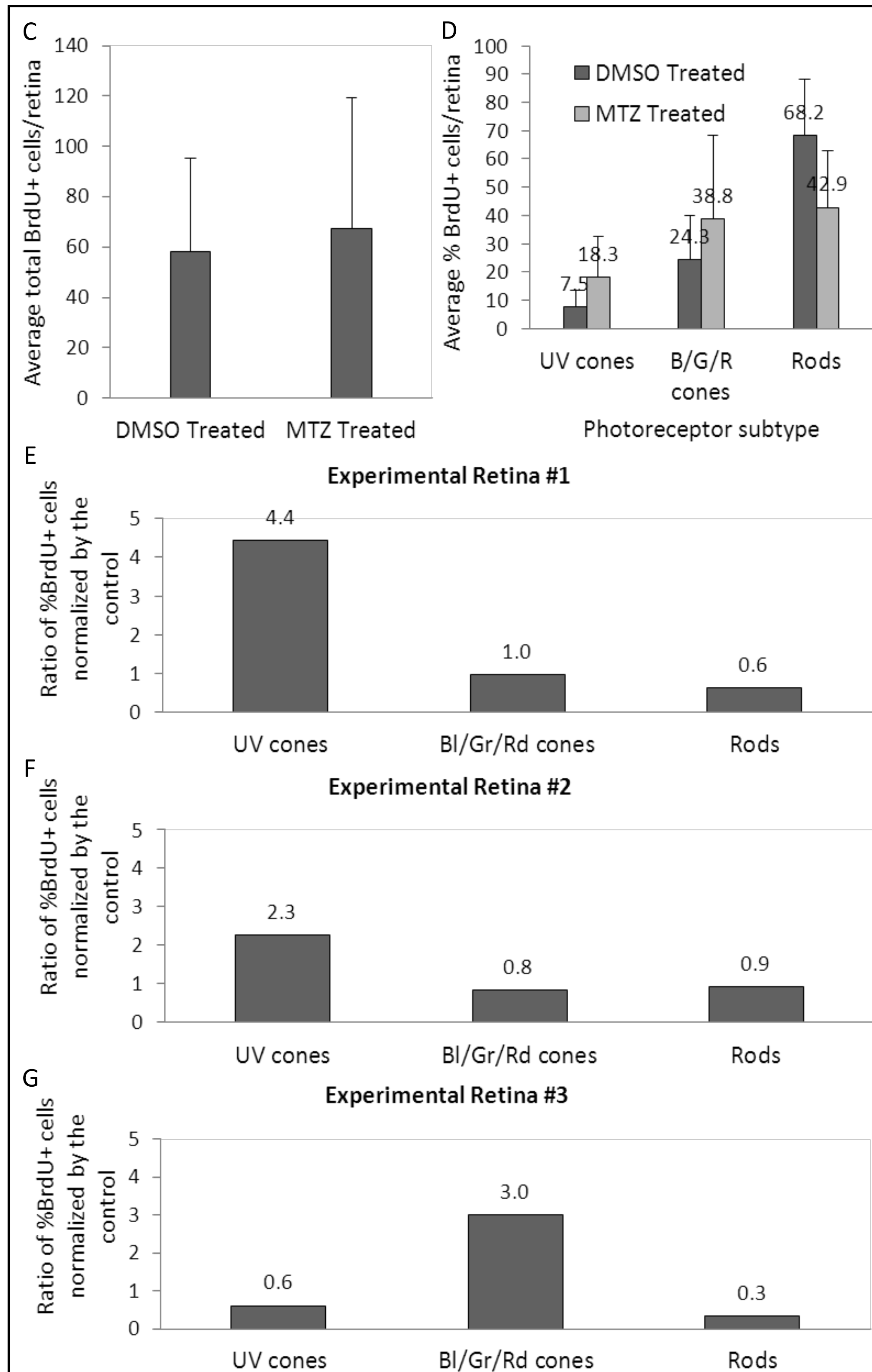


Figure 8. Regeneration of morphologically mature UV cones following prodrug ablation. 1 week after MTZ ablation, the regeneration of morphologically mature cones was observed. BrdU-positive cells co-labelled with UV cone mCherry expression (A-C; arrow). The regenerated UV cones are morphologically similar to newly generated UV cones in the expanding retina (A-D, E), preliminarily suggesting that the regenerated cones are qualitatively normal.


A
UV
BGR
BrdU
B
UV
BGR
BrdU
7dpf ring
13dpf ring



C
Average total BrdU+ cells/retina
140
120
100
80
60
40
20
0
DMSO Treated
MTZ Treated
D
Average % BrdU+ cells/retina
DMSO Treated
MTZ Treated
7.5
18.3
24.3
38.8
68.2
42.9
UV cones
B/G/R cones
Rods
Photoreceptor subtype
E
Experimental Retina #1
Ratio of %BrdU+ cells normalized by the control
4.4
1.0
0.6
UV cones
Bl/Gr/Rd cones
Rods
F
Experimental Retina #2
Ratio of %BrdU+ cells normalized by the control
2.3
0.8
0.9
UV cones
Bl/Gr/Rd cones
Rods
G
Experimental Retina #3
Ratio of %BrdU+ cells normalized by the control
0.6
3.0
0.3
UV cones
Bl/Gr/Rd cones
Rods

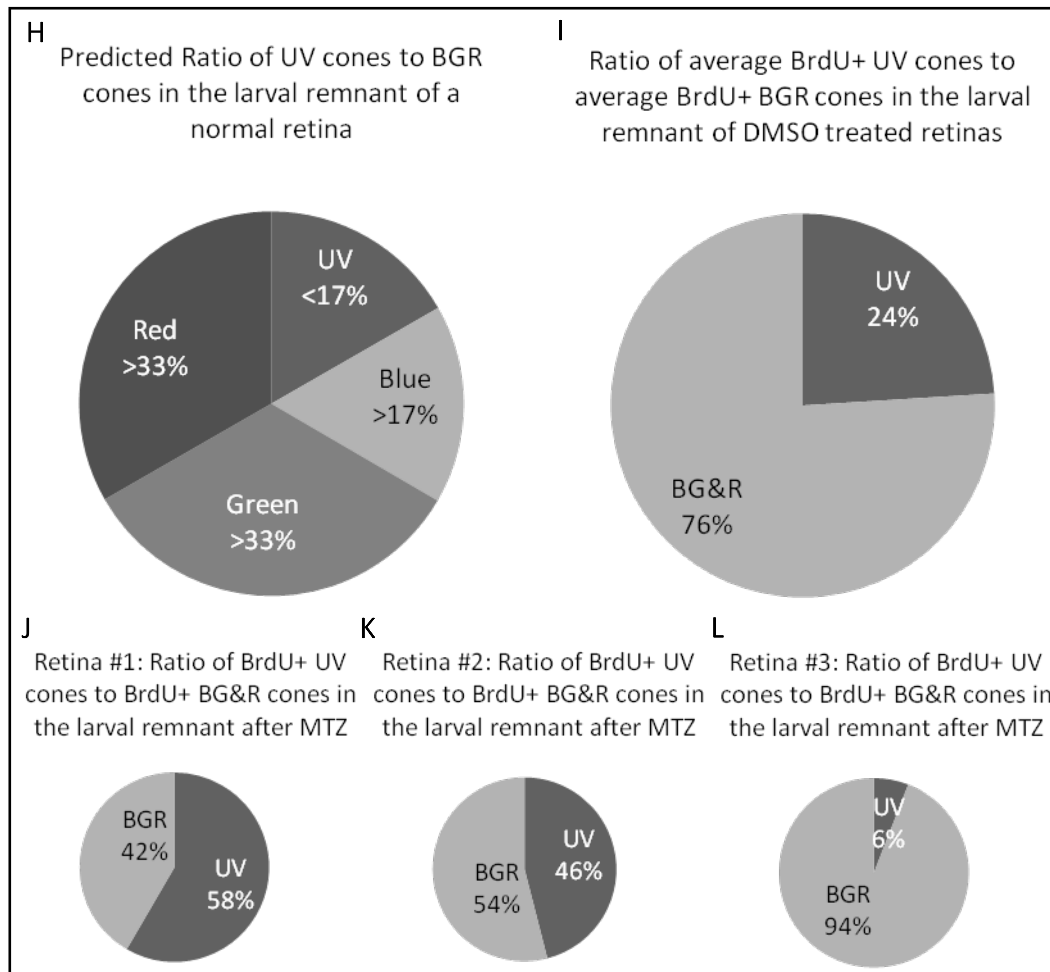


Figure 9. BrdU-positive cells were identified as UV cones, BGR cones and rods in *Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264* fish following UV cone ablation and regeneration, with a bias towards a UV cone identity. Photoreceptor density and distribution are extremely variable in the larval remnant of the adult retina (A). The two rings of BrdU-positive cells represent cells that were proliferating in the CMZ at the time of BrdU exposure, which were not included in the analysis (A, B). The inner ring (7 dpf ring) are cells from the CMZ at 7 dpf and the outer ring (13 dpf ring) represents CMZ cells from 13-14 dpf (B). Only BrdU-positive cells located between the two rings or inner to the 7 dpf ring were analyzed for co-localization with a photoreceptor subtype (B). The average number of BrdU-positive cells was similar for MTZ treated and vehicle control retinas (C; $p=0.81$, DMSO treated $n=3$, MTZ treated $n=3$), which was different to the data in Figure 5, but may be a result of variations in BrdU treatment. BrdU-positive cells were classified into subtypes (UV cones, BGR cones and rods). The percentages of BrdU-positive UV cones and BrdU-positive BGR cones, respectively, increased following ablation compared to the control, while the percentage of BrdU-positive rods decreased (D). The ratios of BrdU-positive photoreceptor subtypes for individual MTZ treated retinas were normalized against the

mean for the controls. Retinas #1 and #2 showed an increase in the proportion of BrdU-positive UV cones (E, F) while Retina #3 showed a higher proportion of BrdU-positive BGR cones (G). The actual control ratio (I) of BrdU-positive UV cones to BGR cones was found to be similar compared to the predicted cone ratio (H). BrdU-positive cone ratios for each of the experimental retinas showed very different UV cone ratios overall compared to the control and predicted (J-L).

Chapter 3 Understanding the ineffectiveness of the metronidazole-nitroreductase ablation system on *Tg(SWS1:nfsB-mCherry)* zebrafish

3.1. Metronidazole experimental trials with Tg(SWS1:nfsB-mCherry)and Tg(SWS2:nfsB-mCherry)

In our first generation of this technology, transgenic fish were engineered (as described in 2.2.3 above) to express nitroreductase fused to the fluorescent reporter gene mCherry under the direct control of the SWS1 opsin promoter [*Tg(SWS1:nfsB-mCherry)ua3003*] or SWS2 opsin promoter [*Tg(SWS2:nfsB-mCherry)ua3001*], resulting in construct expression specifically in the UV cones or blue cones, respectively. Fish ranging in age from 4 dpf to 2 weeks post-fertilization that were expressing cone-specific nitroreductase were submitted to numerous variations of metronidazole treatment (5-10mM for 24-60 hours) in order to optimize the photoreceptor ablation-inducing dosage.

3.1.1. Metronidazole treatment failed to produce photoreceptor death in this version of the technology

Ablation experiments were unsuccessful when employing the *Tg(SWS1:nfsB-mCherry)*. Treatment with 5-10mM metronidazole, with and without DMSO, for 24-48 hours did not induce detectable photoreceptor cell ablation, as determined by the lack of obvious reduction in the number of cells with mCherry fluorescence (Fig.10). BrdU, employed to detect proliferating cells, incorporated into the CMZ as expected. There was no increase in BrdU-

positive cells in the INL or ONL of nitroreductase transgenic fish treated with metronidazole compared to those treated with vehicle only control (Fig.11), indicating that proliferative response was not initiated and therefore the photoreceptor ablation did not occur.

A small increase in overall toxicity to the animal was observed in fish age 5 dpf or older treated with a metronidazole concentration of 7.5mM or more. Metronidazole is known to be toxic at high concentrations (Curado et al., 2008). Previous reports recommend that when applying this conditional ablation method to juvenile or adult zebrafish (older than 5dpf), 5mM should be the maximum concentration of a metronidazole solution (Curado et al., 2008). Therefore this increase in toxicity was reasonable and was not detrimental to the overall effectiveness of the experiments.

3.2. Potential problems of the metronidazole-nitroreductase ablation method applied to Tg(SWS1:nfsB-mCherry)

3.2.1. Addressing the construct as a potential problem

A potential explanation for the lack of ablation upon metronidazole exposure was that there may have been a problem with the construct expressing the nitroreductase-mCherry fusion protein. Due to the nature of the fusion construct, the two genes are fused in frame and translation results in a single polypeptide demonstrating the functions of both original proteins. A problem in

either gene sequence could result in the degradation of mRNA of the entire fusion product and affect the function of both components. Therefore, the presence of mCherry fluorescence in the UV or blue photoreceptors confirmed fusion protein and nitroreductase expression. The construct used to make these transgenic fish was checked by sequencing genomic DNA from these transgenic lines to confirm no deleterious mutations had occurred.

To determine if there was another problem with *nfsB*-mCherry, the functionality of the construct was examined by demonstrating nitroreductase-mediated ablation in a separate tissue type. The heat shock protein Hsp70 promoter was employed to drive the expression of the nitroreductase-mCherry fusion construct throughout the zebrafish (Blechinger et al., 2002). Juveniles (5 dpf) were observed to transiently express nitroreductase-mCherry most distinctively in the muscle, heart and lens following heat shock (HS) (1 hour at 38°C) (Fig.12). Treatment with both 10mM metronidazole solution and heat shock resulted in cell ablation throughout the fish, whereas treatment with metronidazole or heat shock alone did not. High overall toxicity (death) only in the fish receiving both metronidazole and heat shock (percent of surviving fish: i) *nfsB*+HS+MTZ= 14.3%; ii) *nfsB*+HS= 94.4%; iii) *nfsB*+MTZ= 100%; iv) wildtype+HS+MTZ= 71.4%; v) wildtype+HS= 87.5%; vi) wildtype+MTZ= 76.2%) provided evidence that the *nfsB*-mCherry fusion construct we created expressed functional nitroreductase. Thus the *nfsb*-mCherry fusion protein we created, which was different from the one driven by the UAS promoter in Chapter

2, was functional and not the deficiency that led to the failure of this first generation of our technology.

3.2.2. Nitroreductase expression as a potential problem

Another possible reason for the inadequacy of the metronidazole-nitroreductase system using *Tg(SWS1:nfsB-mCherry)* and *Tg(SWS2:nfsB-mCherry)* fish is that nitroreductase in the cone photoreceptors may have been unavailable or expressed at inadequate levels. The concentrations of nitroreductase and metronidazole may not have formed a sufficient amount of cytotoxin to induce ablation. Prodrug-metabolizing activity is dependent upon the amount of functional nitroreductase protein in a cell (Grohmann et al., 2009). A cancer therapy study considered nitroreductase protein availability and examined the effectiveness of the nitroreductase-prodrug ablation system in mammalian cells (Grohmann et al., 2009). The *E. coli* nitroreductase-GFP fusion protein was found to be expressed at low levels in mammalian cells and had a tendency to form aggregates in the cytoplasm. Nitroreductase protein aggregation is most likely due to the conflicting codon usage among prokaryotes and eukaryotes (Grohmann et al., 2009; Sharp et al., 1988). The prokaryotic codon usage pattern is not conserved in taxonomically distant species, therefore expressing prokaryotic genes in a eukaryote is disadvantageous and translation often suffers (Sharp et al., 1988). To investigate nitroreductase protein expression and conditional ablation by the prodrug CB1954 in mammalian cells, Grohmann et al. (2009) optimized nitroreductase activity by designing a synthetic nitroreductase

gene that was more suited for eukaryotic expression. Protein expression significantly increased, cytoplasmic nitroreductase aggregates did not occur and sensitivity to the prodrug was improved following optimization (Grohmann et al., 2009).

Another factor that may have contributed to the lack of ablation in our transgenic lines is the targeted tissue, photoreceptors. The nitroreductase-prodrug method has been reported to be less efficient and effective in neurons compared to other cell types. SH-SY5Y cells, human derived neuroblastoma cells, expressing nitroreductase were not ablated upon treatment with the prodrug CB1954 (Grohmann et al., 2009). Therefore we can hypothesize that metronidazole ablation problems with the *Tg(SWS1:nfsB-mCherry)* and *Tg(SWS2:nfsB-mCherry)* lines are a result of delayed or hindered translational processing of prokaryotic nitroreductase in a eukaryotic organism, combined with expression in a tissue partially resistant to prodrug ablation. Indeed the *nfsb*-mCherry in these lines appeared to be enriched at the scleral end of the nucleus, rather than distributed throughout the cytoplasm, consistent with it being unable to exit the endoplasmic reticulum. The amount of protein expressed per cone appeared to be comparable in the models discussed here compared to the successful models in Chapter 2. The mosaic nature of transgene expression in the latter precluded quantifying this qualitative assessment on Western blot. Future generations of this technology ought to optimize codon usage for zebrafish.

3.3. Comparing the Tg(SWS1:nfsB-mCherry) line to the Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264 line

3.3.1. Gal4-VP16 Toxicity

The potent transcriptional activator Gal4-VP16 is composed of a fusion of the acidic activation domain of the herpes simplex virus protein, VP16, to the DNA-binding domain of Gal4 (Berger et al., 1992; Sadowski et al., 1988). In the herpes simplex virus, VP16 is involved in infection initiation, as it promotes the transcription of early viral genes. VP16 exploits host genes with the ability to recognize DNA sequences in their promoters by attaching to them with its amino-terminal region (McKnight et al., 1987; Triezenberg et al., 1988). With these viral characteristics, Gal4-VP16 is an efficient and effective transcriptional activator when applied to the Gal4-UAS amplification system.

However, the extreme efficiency of the VP16 activator also has its drawbacks. Gal4-VP16 has been observed to bind non-specifically to other promoters not encoding the targeted UAS, resulting in the inhibition of transcription of those genes (Berger et al., 1992; Sadowski et al., 1988; Gill and Ptashne, 1988). In yeast, when the Gal4-VP16 protein non-specifically binds near transcription factor II D (*TFIID*) binding sites, the formation of transcription complexes is promoted (Berger et al., 1992). Components of the complex are present in a limited manner within the cell and their availability is reduced by the

excessive assembly of these complexes, resulting in toxicity due to growth inhibition (Berger et al., 1992; Sadowski et al., 1988; Gill and Ptashne, 1988).

3.3.2. Investigating VP16 toxicity for a role in metronidazole-nitroreductase ablation

The nitroreductase transgenic line employing the Gal4-UAS system [*Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264*] was investigated to determine if the potentially toxic Gal4-VP16 expression in the UV cones was contributing to the metronidazole-mediated cell death. The high levels of VP16 in the cones could conceivably disrupt transcriptional functions enough to weaken those photoreceptors, increasing their sensitivity to metronidazole-nitroreductase ablation.

To determine if Gal4-VP16 was playing a role in photoreceptor death other than amplifying nitroreductase expression and thus sensitizing photoreceptors to cytotoxic reduced metronidazole, *Tg(SWS1:Gal4)ua3016* zebrafish were crossed to the transgenic line *Tg(SWS1:nfsB-mCherry)ua3003*, and their progeny were submitted to metronidazole treatment as previously described in the ablation experiments. These fish are expected to express an abundance of Gal4-VP16 similar to the successful second generation of our technology described in Chapter 2, but with the first generation *nfsb*-mCherry construct that this chapter seeks to troubleshoot. Following metronidazole treatment and recovery, analysis was performed on retinal cryosections to detect apoptosis and to determine if nitroreductase transgenic UV cone ablation was induced. Levels

of apoptosis in the ONL of *Tg(SWS1:Gal4)ua3016* retinas were observed to be unchanged in the presence or absence of nitroreductase-expressing cones upon exposure to metronidazole, and no noticeable reduction in mCherry containing cells was apparent (Fig.13). These results contrast retinas in Chapter 2, where 91% of UV cones were ablated and TUNEL label was abundant. As well, these results are not observably different compared to retinas with UV cones expressing *nfsb* alone (i.e. not expressing Gal4-VP16). Therefore, the proposed toxicity of Gal4-VP16 does not appear to induce a weakened photoreceptor phenotype and does not contribute to the susceptibility of ablation by the metronidazole-nitroreductase cytotoxin.

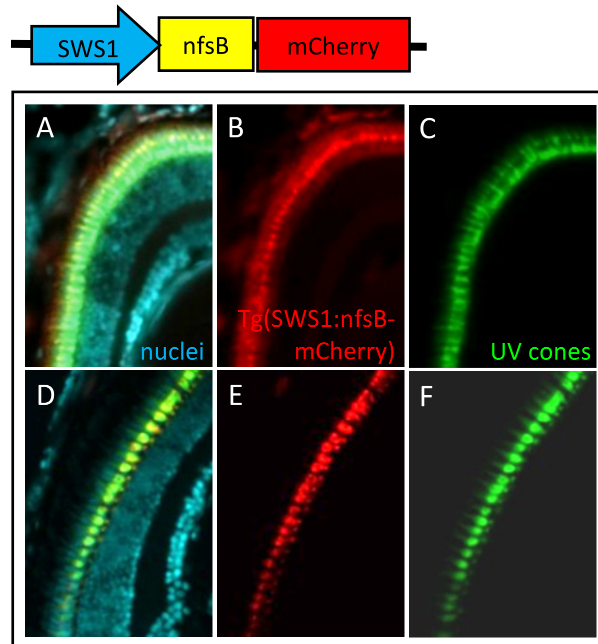


Figure 10. The metronidazole-nitroreductase method failed to induce photoreceptor ablation in *Tg(SWS1:nfsB-mCherry)* fish. 6 dpf fish were treated with 10mM MTZ (A-C) or 0.2% DMSO (D-F) for 48 hours. Following metronidazole exposure, no ablation was observed and mCherry persisted in the UV cones.

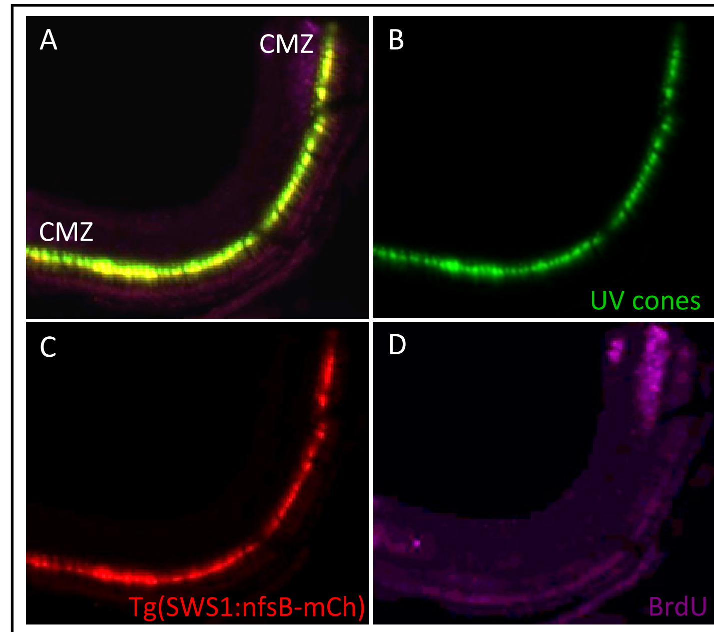


Figure 11. Retinal regeneration was not observed in *Tg(SWS1:nfsB-mCherry)* fish treated with BrdU following MTZ exposure. Fish were allowed to grow up for 2 weeks after the attempted prodrug ablation. BrdU was detected in the CMZ, but not in the INL or ONL.

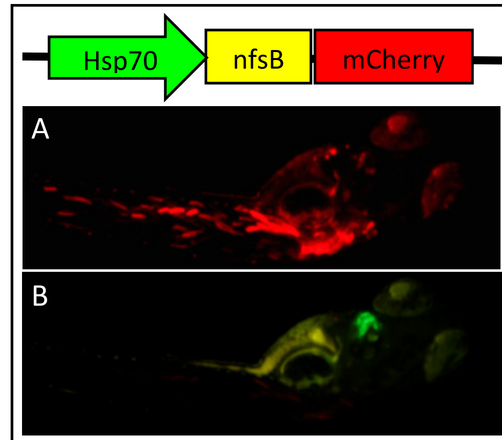


Figure 12. Following heat shock, *nfsB*-mCherry was mosaically expressed in the muscles, heart and lens of fish transiently expressing the transgene. Heat shock was applied for 1 hour at 38°C in combination with 10mM MTZ. Overall toxicity resulting in death to the fish was observed 24-48 hours following the heat shock.

SWS1 nfsB mCherry

X

SWS1 Gal4

MTZ-treated Tg(SWS1:nfsB-mCh/SWS1:Gal4)

A UV cones

B TUNEL

C Tg(SWS1: nfsB-mCh)

Tg(SWS1:nfsB-mCh/ SWS1:Gal4) control

D

E

F

MTZ-treated Tg(SWS1:Gal4)

G

H

I

Tg(SWS1:Gal4) negative control

J ONL INL

K

L

MTZ-treated Tg(SWS1:Gal4 /UAS:nfsB-mCh)

M Tg(SWS1: Gal4/UAS: nfsB-mCh)

N

Figure 13. Gal4-VP16 toxicity was not observed to have an influence on the metronidazole-nitroreductase ablation method. TUNEL detection was employed to label apoptotic cells. Fish expressing both *Tg(SWS1:Gal4)* and *Tg(SWS1:nfsB-mCherry)* demonstrated similar levels of apoptosis in the ONL following MTZ treatment (A-C) compared to DMSO treated controls (D-F). The same was observed for siblings expressing *Tg(SWS1:Gal4)* but not nitroreductase (G-L). The positive control, *Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264* treated with MTZ, displayed high levels of apoptosis in the ONL, as expected.

Chapter 4 Future Directions[1]

4.1.1. Optimizing a zebrafish model for absolute ablation of targeted cone photoreceptors

The current *Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264* zebrafish line exhibits mosaic expression of nitroreductase in the UV cone photoreceptors. Therefore metronidazole exposure only induces ablation in the subset of UV cones expressing the transgene while other UV cones continue to persist in the retina. The engineering of a transgenic line where 100% of a particular subtype of cone photoreceptors contained nitroreductase would allow for the complete ablation of targeted cells. This would simplify analysis of the identity of regenerated cell types, including via identifying regenerated photoreceptors based upon their position in the cone mosaic rather than by BrdU incorporation.

The effects of the total loss of a particular cone photoreceptor subtype to the function of the retina are unknown. Furthermore, whether the absolute loss of a photoreceptor subtype induces an altered regenerative response compared to partial ablation of the cell type needs to be addressed. The magnitude of cone photoreceptor death may influence the regenerative product. Therefore regenerative studies employing the two differing lines would be complimentary in that comparisons of the injury response and regeneration could be made (Montgomery et al., 2010).

4.1.2. Optimizing a zebrafish model for persistent expression of nitroreductase

Observations of our *Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264* zebrafish at 1-3 months post-fertilization have demonstrated variability and reduction of nitroreductase-mCherry expression in the photoreceptors of the mature fish. As the fish age, nitroreductase expression appears to progressively decrease in most of the cells.

In zebrafish, it has been previously reported that transgenes under the control of the Gal4-UAS system will initially display strong expression, but occasionally it is disrupted over time, resulting in mosaic or absent expression despite the persistent activity of Gal4-VP16 (Goll et al., 2009). The UAS sequence employed in the formation of our transgenic line is a multicopy sequence composed of 14 UAS repeats. DNA methylation is known to target tandem repeats. The 14X UAS is additionally vulnerable due to its CpG-rich regions, where a cytosine nucleotide occurs next to a guanine in the linear sequence, because methylation is particularly attracted to those sites (Garrick et al., 1998; Giniger et al., 1985). It was recently confirmed that methylation of 14X UAS was correlated to transcriptional silencing of transgene expression in zebrafish (Goll et al., 2009; Li et al., 2007).

DNA-binding proteins have been observed to trigger demethylation at recognition sites, reactivating silenced transgenes (Lin et al., 2000; Matsuo et al., 1998). Therefore the loss of transgene expression is partially reversible by increasing Gal4-VP16 activator levels (Goll et al., 2009). However, this solution is not favourable given that increasing Gal4-VP16 levels would likely increase

VP16-mediated toxicity in the cell (Berger et al., 1992; Sadowski et al., 1988; Gill and Ptashne, 1988). In order to improve on our ablation model, the engineering of a transgenic zebrafish line expressing *nfsB*-mCherry under the promotion of a UAS sequence composed of fewer repeats is necessary. Fewer tandem repeats of UAS would render the transgene less attractive to methylation, resulting in nitroreductase expression in the UV cones throughout the life cycle.

4.2. Directed cell-specific ablation of photoreceptors in the adult zebrafish retina

Applying the genetic-chemical ablation method to cone photoreceptors in the mature *Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264* zebrafish retina would be advantageous in that our spectrum for experimenting and analyzing regeneration would be broadened. Exposing adult zebrafish to metronidazole by immersing them in a solution has been shown to effectively induce nitroreductase-dependent rod cell death (Montgomery et al., 2010). Therefore the ease of the metronidazole ablation procedure that has been optimized in larvae is transferable to the adult.

An important characteristic of the adult zebrafish retina is that the cone photoreceptors are arranged in a precise mosaic with a predictable pattern (Raymond and Barthel, 2004; Allison et al., 2010). Ablating specific cones in the mature retina presents us with the opportunity to exploit the mosaic as a tool in regenerative studies (Alvarez-Delfin et al., 2009). Specifically, the extent of

influence that the established photoreceptor pattern exerts on regenerating cells could be addressed. Adult fish give us the opportunity to perform experiments that would otherwise not be reasonable in larvae.

An intriguing and novel technology anticipated to be applied to research of adult retinal regeneration is the *in vivo* lens developed through a collaboration of the Lehmann and Allison labs at the University of Alberta (unpublished). The lens is non-invasive and allows us to see through the zebrafish lens to the back of the retina and focus on specific regions of photoreceptors in longitudinal studies. With this new technology, we have the opportunity to observe the ablation of targeted photoreceptors *in vivo* and follow the progression of cone regeneration within the same live fish.

4.3. Reconstituted caspase: an alternative method for targeted cone photoreceptor ablation

Caspases are known for their integral roles in apoptosis (Thornberry and Lazebnik, 1998). They become active upon the formation heterotetramers consisting of two active sites, each formed by a pair of large and small subunits (Thornberry and Lazebnik, 1998; Chelur and Chalfie, 2007). Caspase-3 in particular, a downstream component in the caspase-apoptosis cascade, directly cleaves proteins that lead to cell death (Chelur and Chalfie, 2007). Recently, the existence of individual non-active large and small subunits was exploited for selectively targeting cells for apoptosis by expressing the two subunits so that

they only overlapped in specific cells (Chelur and Chalfie, 2007). The engineering of a heat-inducible caspase method for conditional cone ablation to study retinal regeneration in zebrafish has begun. The small caspase subunit p12 is continuously expressed in the targeted photoreceptor subtype, driven by the specific opsin promoter. A second transgene contains the heat shock protein *Hsp70* promoter driving the large p17 domain. Upon heat shock, the large subunit is expressed and caspase is activated only in cells containing both p12 and p17 caspase domains, brought together by leucine zippers (Chelur and Chalfie, 2007). It is expected that activated caspase will induce the robust ablation of the targeted photoreceptors, and retinal regeneration can subsequently be examined.

4.4. Assessing the functional integration of regenerated photoreceptors

The regeneration of a neuronal cell is an impressive process, but would be futile if the cell did not functionally integrate into the existing tissue system upon regeneration. Neuronal regeneration in the zebrafish is readily observable, but the importance of assessing the functionality of the regenerated cells is often overlooked. Therefore, a topic for investigation is whether the regenerated photoreceptors functionally integrate into the injured retina, forming synapses with the second order neurons.

In a degenerating retina, second order neurons undergo remodelling, therefore it is conceivable that similar modifications would occur following

photoreceptor ablation (Gaillard and Sauve, 2007). However, the effect of these second order neuron alterations on visual function and regeneration remain unknown. Many behavioural assays and electrophysiological tools have been specifically adapted to zebrafish for examination of the functionality of regeneration (Fleisch and Neuhauss, 2006; Makhankov et al., 2004).

4.4.1. Assessing functional integration using electrophysiology

The ERG (electroretinogram) is a routinely used tool for studying degeneration of retinal neurons in the zebrafish and has been adapted for assessment of regenerative function in the teleost fish retina (Mensinger and Powers, 2007; Allison et al., 2006b). This non-invasive technique measures light-induced changes of electrical activity in the eye, which are recorded by the instrumentation and interpreted into an ERG trace (Makhankov et al., 2004; Saszik et al., 1999; Seeliger et al., 2002). The vertebrate ERG output is expressed as three distinct waves (Fleisch et al., 2011). An initial low-amplitude negative a-wave reflecting photoreceptor activity is followed by the large positive b-wave, mainly representing second- order (ON bipolar) neuron activity. The d-wave appears at light offset and is indicative of postsynaptic activity involved in the OFF response (Makhankov et al., 2004; Dowling, 1987).

4.4.2. Assessing functional integration using visually-evoked behavioural assays

Behavioural responses are another mode to assess functional integration of regenerated neurons into existing circuits. The loss of certain behaviours upon the ablation of neurons, as well as the recovery of those behaviours once the cells have regenerated, can be assessed by a number of well-established paradigms in the zebrafish.

Visually-evoked behaviours of zebrafish can be easily stimulated and are readily accessible for measurement and genetic analysis of functional vision (Fleisch and Neuhauss, 2006; Neuhauss, 2003). The optokinetic response (OKR) is a robust assay for visual function measuring zebrafish eye movements in response to a moving stimulus (grating) (Brockerhoff, 2006; Easter and Nicola, 1997; Huang and Neuhauss, 2008; Rinner et al., 2005). Ablation of cone photoreceptors for example, would be expected to impair colour detection in zebrafish larvae. Consequently they would not be able to track the movements of coloured objects. Cone photoreceptor regeneration would lead to recovery of functional colour vision and improved performance in the OKR assay could be observed.

In the optomotor response (OMR), motion in a particular direction is simulated and zebrafish will swim in the direction of the perceived motion (Neuhauss et al., 1999; Orger and Baier, 2005). Failure of zebrafish to respond to the OMR stimulus can be attributed to diverse underlying causes, and thus the OMR can be used for studying regeneration of various CNS tissues. Swimming arrest will occur following retinal ablation, and functional regeneration should be able to (at least partially) restore the optomotor response. As the OMR is not only

dependent on a functional retina, but also on the correct propagation of visual stimuli to the fish optic tectum, regeneration of the optic nerve can also be tested (Fleisch et al., 2011).

4.4.3. Tracing retinal neural circuits using barley lectin

The application of neuronal tracers to our ablation and regeneration model offers us the opportunity to visualize the neuronal circuitry involved in a functional photoreceptor. A tracing method that has proven effective in mapping connectivity is the use of plant lectin expressed in targeted neurons (Horowitz et al., 1999). Lectins bind carbohydrate residues of *N*-acetylglucosamine and sialic acid and are capable of undergoing transneuronal transport (Fabian and Coulter, 1985). Barley lectin expressed in olfactory sensory neurons of transgenic mice allowed for visualization of the connectivity in the olfactory system. The lectin transport was traced through the axons of sensory neurons all the way to the olfactory cortex in the brain (Horowitz et al., 1999).

Currently available in the Allison lab is a transgenic construct composed of barley lectin (BL) fused to the mCherry fluorescence reporter gene for expression in a targeted photoreceptor subtype. Preliminary observations of transient larvae injected with *Tg(SWS1:BL-mCherry)* show mosaic expression of barley lectin in the photoreceptor axons (data not shown). This technology would allow us to observe remodeling of second order neurons following ablation as well as the integration of regenerated photoreceptors back into the functional retina.

4.5. Characterizing retinal regeneration by manipulating stem cell fate of regenerating photoreceptors

Studies of teleost retinas have confirmed that thyroid hormone levels have an evident effect on photoreceptor cell fate during development and maturation (Veldhoen et al., 2006). Retinal stem cells also express thyroid hormone receptors and thyroid hormone metabolizing enzymes such as deiodinase (Allison et al., 2006a). Thyroid hormone T4 gets converted by deiodinases to the active T3 form, where it can then bind to thyroid hormone receptors and control the transcription of targeted genes (Thompson and Potter, 2000). Thyroid hormone has been observed to both activate and repress transcription, and therefore can influence multiple neuronal mechanisms such as proliferation, differentiation and migration (Thompson and Potter, 2000; Harpavat and Cepko, 2003). We are proposing to further characterize retinal regeneration by altering thyroid hormone T4 levels in order to alter retinal stem cell fate.

For this investigation, the *Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264* zebrafish line would be employed to induce the exclusive ablation of UV cone photoreceptors. The regenerating retinal progenitors could then be manipulated by the application of thyroid hormone T4 or methimazole (MMI), a common drug which inhibits T3 and T4 synthesis (Allison et al., 2006a; Shiroozu et al., 1986).

The preceding research employing *Tg(SWS1:Gal4-VP16)ua3016;Tg(UAS-E1b:NfsB-mCherry)c264* zebrafish represents a first step towards selectively and progressively characterizing retinal regeneration for all cone photoreceptor subtypes. The metronidazole-nitroreductase method to genetically and conditionally direct cone ablation allows us to minimize the factors influencing regeneration and pinpoint genetic pathways regulating cell fate determination. With the successful implementation of this ablation and regeneration paradigm, the evolutionary mysteries of neuronal regeneration are waiting to be unlocked.

Bibliography

Abler A, Chang C, Ful J, Tso M,Lam T (1996) Photic injury triggers apoptosis of photoreceptor cells. Res Commun Mol Pathol Pharmacol 92:177-189.

Ali MA, Anctil M, (1976) Retinas of fishes: An atlas. The University of Michigan: Springer-Verlag.

Allison WT (2004) Rainbow trout as a model of retinal photoreceptor death and regeneration.

Allison WT, Barthel LK, Skebo KM, Takechi M, Kawamura S,Raymond PA (2010) Ontogeny of cone photoreceptor mosaics in zebrafish. J Comp Neurol 518:4182-4195.

Allison WT, Dann SG, Veldhoen KM,Hawryshyn CW (2006a) Degeneration and regeneration of ultraviolet cone photoreceptors during development in rainbow trout. J Comp Neurol 499:702-715.

Allison WT, Veldhoen KM,Hawryshyn CW (2006b) Proteomic analysis of opsins and thyroid hormone-induced retinal development using isotope-coded affinity tags (ICAT) and mass spectrometry. Molecular Vision 12:655-672.

Allison W, Barthel L, Yan B, Chi C, Ross J, Yang X, Kuang C, Goldman D, Kawamura S,Raymond P (2005) Genetic analysis of the cone photoreceptor mosaic in zebrafish. Invest Ophthalmol Vis Sci 46:

Allison W, Haimberger T, Hawryshyn C,Temple S (2004) Visual pigment composition in zebrafish: Evidence for a rhodopsin-porphyropsin interchange system. Vis Neurosci 21:945-952.

Alvarez-Delfin K, Morris AC, Snelson CD, Gamse JT, Gupta T, Marlow FL, Mullins MC, Burgess HA, Granato M,Fadool JM (2009) Tbx2b is required for ultraviolet photoreceptor cell specification during zebrafish retinal development. Proc Natl Acad Sci U S A 106:2023-2028.

Anlezark G, Melton R, Sherwood R, Coles B, Friedlos F,Knox R (1992) The bioactivation of 5-(aziridin-1-Yl)-2,4-dinitrobenzamide (Cb1954) .1. Purification and properties of a nitroreductase enzyme from Escherichia-coli - a potential enzyme for antibody-directed enzyme prodrug therapy (adept). Biochem Pharmacol 44:2289-2295.

Balciunas D, Davidson A, Sivasubbu S, Hermanson S, Welle Z,Ekker S (2004) Enhancer trapping in zebrafish using the Sleeping Beauty transposon. BMC Genomics 5:62.

Barthel L,Raymond P (2000) In situ hybridization studies of retinal neurons. Vertebrate Phototransduction and the Visual Cycle, Pt B 316:579-+.

Berger S, Pina B, Silverman N, Marcus G, Agapite J, Regier J, Triezenberg S,Guarente L (1992) Genetic isolation of Ada2 - a potential transcriptional adapter required for function of certain acidic activation domains. Cell 70:251-265.

Bernardos RL, Barthel LK, Meyers JR,Raymond PA (2007) Late-stage neuronal progenitors in the retina are radial Muller glia that function as retinal stem cells. Journal of Neuroscience 27:7028-7040.

Biehlmaier O, Neuhauss S,Kohler K (2001) Onset and time course of apoptosis in the developing zebrafish retina. Cell Tissue Res 306:199-207.

Bilotta J, Saszik S,Sutherland S (2001) Rod contributions to the electroretinogram of the dark-adapted developing zebrafish. Developmental Dynamics 222:564-570.

Blader P, Lam C, Rastegar S, Scardigli R, Nicod J, Simplicio N, Plessy C, Fischer N, Schuurmans C, Guillemot F,Strahle U (2004) Conserved and acquired features of neurogenin1 regulation. Development 131:5627-5637.

Blechinger S, Evans T, Tang P, Kuwada J, Warren J,Krone P (2002) The heat-inducible zebrafish hsp70 gene is expressed during normal lens development under non-stress conditions. Mech Dev 112:213-215.

Braisted J, Essman T,Raymond P (1994) Selective regeneration of photoreceptors in goldfish retina. Development 120:2409-2419.

Brand A,Perrimon N (1993) Targeted gene-expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401-415.

Bridgewater J, Springer C, Knox R, Minton N, Michael N,Collins M (1995) Expression of the bacterial nitroreductase enzyme in mammalian cells

renders them selectively sensitive to killing by the prodrug CB1954. Eur J Cancer 31A:2362-2370.

Bringmann A, Pannicke T, Grosche J, Francke M, Wiedemann P, Skatchkov SN, Osborne NN,Reichenbach A (2006) Muller cells in the healthy and diseased retina. Prog Retin Eye Res 25:397-424.

Brockerhoff SE (2006) Measuring the optokinetic response of zebrafish larvae. Nature Protocols 1:2448-2451.

Cameron D,Carney L (2000) Cell mosaic patterns in the native and regenerated inner retina of zebrafish: Implications for retinal assembly. J Comp Neurol 416:356-367.

Cameron D,Easter S (1995) Cone photoreceptor regeneration in adult fish retina - phenotypic determination and mosaic pattern-formation. Journal of Neuroscience 15:2255-2271.

Cameron D, Gentile K, Middleton F,Yurco P (2005) Gene expression profiles of intact and regenerating zebrafish retina. Molecular Vision 11:775-791.

Chelur DS,Chalfie M (2007) Targeted cell killing by reconstituted caspases. Proc Natl Acad Sci U S A 104:2283-2288.

Chinen A, Hamaoka T, Yamada Y,Kawamura S (2003) Gene duplication and spectral diversification of cone visual pigments of zebrafish. Genetics 163:663-675.

Christoffels V, Hoogaars W, Tessari A, Clout D, Moorman A,Campione M (2004) T-box transcription factor Tbx2 represses differentiation and formation of the cardiac chambers. Developmental Dynamics 229:763-770.

Collin SP (2008) A web-based archive for topographic maps of retinal cell distribution in vertebrates. Clinical and Experimental Optometry 91:85-95.

Curado S, Stainier DYR,Anderson RM (2008) Nitroreductase-mediated cell/tissue ablation in zebrafish: a spatially and temporally controlled ablation method with applications in developmental and regeneration studies. Nature Protocols 3:948-954.

Curcio C, Sloan K, Kalina R,Hendrickson A (1990) Human photoreceptor topography. J Comp Neurol 292:497-523.

Davison JM, Akitake CM, Goll MG, Rhee JM, Gosse N, Baier H, Halpern ME, Leach SD,Parsons MJ (2007) Transactivation from Gal4-VP16 transgenic insertions for tissue-specific cell labeling and ablation in zebrafish. Dev Biol 304:811-824.

Dowling J (1987) Functional and pharmacological organization of the retina. Arch Neurol 44:1208-1208.

Easter S,Malicki J (2002) The zebrafish eye: developmental and genetic analysis. Results Probl Cell Differ 40:346-370.

Easter S,Hitchcock P (2000) Stem cells and regeneration in the retina: What fish have taught us about neurogenesis. Neuroscientist 6:454-464.

Easter S,Nicola G (1997) The development of eye movements in the zebrafish (Danio rerio). Dev Psychobiol 31:267-276.

Edwards D (1993) Nitroimidazole drugs - action and resistance mechanisms .1. Mechanisms of action. J Antimicrob Chemother 31:9-20.

Eglen SJ (2006) Development of regular cellular spacing in the retina: theoretical models. Mathematical Medicine and Biology-a Journal of the Ima 23:79-99.

Fabian RH,Coulter JD (1985) Transneuronal transport of lectins. Brain Res 344:41-48.

Felmer R, Cui W,Clark A (2002) Inducible ablation of adipocytes in adult transgenic mice expressing the E-coli nitroreductase gene. J Endocrinol 175:487-498.

Fimbel SM, Montgomery JE, Burket CT,Hyde DR (2007) Regeneration of inner retinal neurons after intravitreal injection of ouabain in zebrafish. Journal of Neuroscience 27:1712-1724.

Fischer J, Giniger E, Maniatis T,Ptashne M (1988) Gal4 activates transcription in Drosophila. Nature 332:853-856.

Fleisch VC, Fraser B,Allison WT (2011) Investigating regeneration and functional integration of CNS neurons: Lessons from zebrafish genetics and other fish species. Biochimica Et Biophysica Acta-Molecular Basis of Disease 1812:364-380.

Fleisch VC,Neuhauss SCF (2006) Visual Behavior in Zebrafish. Zebrafish 3:191-201.

Furukawa T, Morrow E,Cepko C (1997) Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. Cell 91:531-541.

Gaillard F,Sauve Y (2007) Cell-based therapy for retina degeneration: The promise of a cure. Vision Res 47:2815-2824.

Garrick D, Fiering S, Martin D,Whitelaw E (1998) Repeat-induced gene silencing in mammals. Nat Genet 18:56-59.

Gill G,Ptashne M (1988) Negative effect of the transcriptional activator Gal4. Nature 334:721-724.

Giniger E, Varnum S,Ptashne M (1985) Specific DNA-binding of Gal4, a positive regulatory protein of yeast. Cell 40:767-774.

Goll MG, Anderson R, Stainier DYR, Spradling AC,Halpern ME (2009) Transcriptional silencing and reactivation in transgenic zebrafish. Genetics 182:747-755.

Grohmann M, Paulmann N, Fleischhauer S, Vowinckel J, Priller J,Walther DJ (2009) A mammalianized synthetic nitroreductase gene for high-level expression. BMC Cancer 9:301.

Grunberg E,Tisworth E (1973) Chemotherapeutic properties of heterocyclic-compounds - monocyclic compounds with 5-membered rings. Annu Rev Microbiol 27:317-346.

Harada T, Harada C,Parada LF (2007) Molecular regulation of visual system development: more than meets the eye. Genes Dev 21:367-378.

Harpavat S,Cepko C (2003) Thyroid hormone and retinal development: An emerging field. Thyroid 13:1013-1019.

He S,Masland R (1997) Retinal direction selectivity after targeted laser ablation of starburst amacrine cells. Nature 389:378-382.

Helsby N, Ferry D, Patterson A, Pullen S,Wilson W (2004) 2-Amino metabolites are key mediators of CB 1954 and SN 23862 bystander effects in nitroreductase GDEPT. Br J Cancer 90:1084-1092.

Hitchcock PF,Raymond PA (2004) The teleost retina as a model for developmental and regeneration biology. Zebrafish 1:257-271.

Hitchcock P, Myhr K, Easter S, Mangionesmith R,Jones D (1992) Local regeneration in the retina of the goldfish. J Neurobiol 23:187-203.

Horowitz L, Montmayeur J, Echelard Y,Buck L (1999) A genetic approach to trace neural circuits. Proc Natl Acad Sci U S A 96:3194-3199.

Huang Y,Neuhauss SCF (2008) The optokinetic response in zebrafish and its applications. Frontiers in Bioscience 13:1899-1916.

Hughes D, Schade R,Ontell M (1992) Ablation of the fetal mouse spinal-cord - the effect on soleus muscle cytoarchitecture. Developmental Dynamics 193:164-174.

Jager RD, Mieler WF,Miller JW (2008) Medical progress: Age-related macular degeneration. N Engl J Med 358:2606-2617.

Julian D, Ennis K,Korenbrot J (1998) Birth and fate of proliferative cells in the inner nuclear layer of the mature fish retina. J Comp Neurol 394:271-282.

Kee N, Sivalingam S, Boonstra R,Wojtowicz J (2002) The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. J Neurosci Methods 115:97-105.

Kubota R, Hokoc J, Moshiri A, McGuire C,Reh T (2002) A comparative study of neurogenesis in the retinal ciliary marginal zone of homeothermic vertebrates. Dev Brain Res 134:31-41.

Kunz Y (2006) Review of development and aging in the eye of teleost fish. Neuroembryol Aging 4:31-60.

Kwan KM, Fujimoto E, Grabher C, Mangum BD, Hardy ME, Campbell DS, Parant JM, Yost HJ, Kanki JP,Chien C (2007) The Tol2kit: A multisite Gateway-based construction kit for Tol2 transposon transgenesis constructs. Developmental Dynamics 236:3088-3099.

Lee S, Lee B, Ruiz E,Pfaff S (2005) Olig2 and Ngn2 function in opposition to modulate gene expression in motor neuron progenitor cells. Genes Dev 19:282-294.

Li F, Papworth M, Minczuk M, Rohde C, Zhang Y, Ragozin S,Jeltsch A (2007) Chimeric DNA methyltransferases target DNA methylation to specific DNA sequences and repress expression of target genes. Nucleic Acids Res 35:100-112.

Lin C, Hsieh M, Li Y, Li S, Pearson D, Pollard K,Chuan L (2000) Protein N-arginine methylation in subcellular fractions of lymphoblastoid cells. J Biochem 128:493-498.

Lindmark D,Muller M (1976) Anti-trichomonad action, mutagenicity, and reduction of metronidazole and other nitroimidazoles. Antimicrobial Agents Chemother 10:476-482.

Liu K,Fetcho J (1999) Laser ablations reveal functional relationships of segmental hindbrain neurons in zebrafish. Neuron 23:325-335.

Ma W, Yan R, Xie W,Wang S (2004) A role of ath5 in inducing neuroD and the photoreceptor pathway. Journal of Neuroscience 24:7150-7158.

Maier W,Wolburg H (1979) Regeneration of the goldfish retina after exposure to different doses of ouabain. Cell Tissue Res 202:99-118.

Makhankov Y, Rinner O,Neuhauss S (2004) An inexpensive device for non-invasive electroretinography in small aquatic vertebrates. J Neurosci Methods 135:205-210.

Marotte L, Wyedvorak J,Mark R (1979) Retinotectal reorganization in goldfish .2. Effects of partial tectal ablation and constant light on the retina. Neuroscience 4:803-&.

Masai I, Stemple D, Okamoto H,Wilson S (2000) Midline signals regulate retinal neurogenesis in zebrafish RID B-9404-2008. Neuron 27:251-263.

Matsuo K, Silke J, Georgiev O, Marti P, Giovannini N,Rungger D (1998) An embryonic demethylation mechanism involving binding of transcription factors to replicating DNA RID B-7622-2008. EMBO J 17:1446-1453.

McCalla D, Kaiser C,Green M (1978) Genetics of nitrofurazone resistance in Escherichia-coli. J Bacteriol 133:10-16.

McKnight J, Kristie T,Roizman B (1987) Binding of the virion protein mediating alpha-gene induction in Herpes-Simplex virus-1-infected cells to its cis site requires cellular proteins. Proc Natl Acad Sci U S A 84:7061-7065.

Mears A, Kondo M, Swain P, Takada Y, Bush R, Saunders T, Sieving P,Swaroop A (2001) Nrl is required for rod photoreceptor development. Nat Genet 29:447-452.

Mensinger AF,Powers MK (2007) Visual function in regenerating teleost retina following surgical lesioning. Vis Neurosci 24:299-307.

Meyers RA, (2007) Genomics and genetics: from molecular details to analysis and techniques. pp 1228. Weinheim, Germany: Wiley-VCH.

Montgomery JE, Parsons MJ,Hyde DR (2010) A novel model of retinal ablation demonstrates that the extent of rod cell death regulates the origin of the regenerated zebrafish rod photoreceptors. J Comp Neurol 518:800-814.

Morris AC, Scholz T,Fadool JM (2008) Rod progenitor cells in the mature zebratish retina. Recent Advances in Retinal Degeneration 613:361-368.

Neuhauss S (2003) Behavioral genetic approaches to visual system development and function in zebrafish. J Neurobiol 54:148-160.

Neuhauss S, Biehlmaier O, Seeliger M, Das T, Kohler K, Harris W,Baier H (1999) Genetic disorders of vision revealed by a behavioral screen of 400 essential loci in zebrafish. Journal of Neuroscience 19:8603-8615.

Newman E (1996) Regulation of extracellular K+ and pH by polarized ion fluxes in glial cells: The retinal Muller cell. Neuroscientist 2:109-117.

Nishida A, Furukawa A, Koike C, Tano Y, Aizawa S, Matsuo I,Furukawa T (2003) Otx2 homeobox gene controls retinal photoreceptor cell fate and pineal gland development. Nat Neurosci 6:1255-1263.

Oh ECT, Khan N, Novelli E, Khanna H, Strettoi E,Swaroop A (2007) Transformation of cone precursors to functional rod photoreceptors by bZIP transcription factor NRL. Proc Natl Acad Sci U S A 104:1679-1684.

Organisciak D,Winkler B (1994) Retinal light damage - Practical and theoretical considerations. Prog Retin Eye Res 13:1-29.

Orger M,Baier H (2005) Channeling of red and green cone inputs to the zebrafish optomotor response. Vis Neurosci 22:275-281.

Otteson D, D'Costa A,Hitchcock P (2001) Putative stem cells and the lineage of rod photoreceptors in the mature retina of the goldfish. Dev Biol 232:62-76.

Otteson D,Hitchcock P (2003) Stem cells in the teleost retina: persistent neurogenesis and injury-induced regeneration. Vision Res 43:927-936.

Penn J (1985) Effects of continuous light on the retina of a fish, Notemigonus-crysoleucas. J Comp Neurol 238:121-127.

Perron M,Harris W (2000) Retinal stem cells in vertebrates. Bioessays 22:685-688.

Perron M, Kanekar S, Vetter M,Harris W (1998) The genetic sequence of retinal development in the ciliary margin of the Xenopus eye. Dev Biol 199:185-200.

Pisharath H, Rhee JM, Swanson MA, Leach SD,Parsons MJ (2007) Targeted ablation of beta cells in the embryonic zebrafish pancreas using E-coli nitroreductase. Mech Dev 124:218-229.

Raymond P (1985) The unique origin of rod photoreceptors in the teleost retina. Trends Neurosci 8:12-17.

Raymond P,Barthel L (2004) A moving wave patterns the cone photoreceptor mosaic array in the zebrafish retina. Int J Dev Biol 48:935-945.

Raymond P, Hitchcock P, (2000) How the neural retina regenerates. In: Results and Problems in Cell Differentiation, Vol. 31 (Fini M ed),Springer-Verlag.

Raymond P, Reifler M,Rivlin P (1988) Regeneration of goldfish retina - Rod precursors are a likely source of regenerated cells. J Neurobiol 19:431-463.

Raymond P,Rivlin P (1987) Germinal cells in the goldfish retina that produce rod photoreceptors. Dev Biol 122:120-138.

Raymond PA, Barthel LK, Bernardos RL,Perkowski JJ (2006) Molecular characterization of retinal stem cells and their niches in adult zebrafish. Bmc Developmental Biology 6:36.

Rinner O, Rick J,Neuhauss S (2005) Contrast sensitivity, spatial and temporal tuning of the larval zebrafish optokinetic response. Invest Ophthalmol Vis Sci 46:137-142.

Rodieck RW, (1998) The first steps in seeing pp 546. Sunderland, Massachusetts, USA: Sinauer Associates.

Sadowski I, MA J, Triezenberg S,Ptashne M (1988) Gal4-Vp16 is an unusually potent transcriptional activator. Nature 335:563-564.

Sastry S,Jayaraman R (1984) Nitrofurantoin-resistant mutants of Escherichia-coli - Isolation and mapping. Molecular & General Genetics 196:379-380.

Saszik S, Bilotta J,Givin C (1999) ERG assessment of zebrafish retinal development. Vis Neurosci 16:881-888.

Scheer N,Campos-Ortega J (1999) Use of the Gal4-UAS technique for targeted gene expression in the zebrafish. Mech Dev 80:153-158.

Schmitt E,Dowling J (1999) Early retinal development in the zebrafish, Danio rerio: Light and electron microscopic analyses. J Comp Neurol 404:515-536.

Searle P, Chen M, Hu L, Race P, Lovering A, Grove J, Guise C, Jaberipour M, James N, Mautner V, Young L, Kerr D, Mountain A, White S,Hyde E (2004) Nitroreductase: A prodrug-activating enzyme for cancer gene therapy. Clinical and Experimental Pharmacology and Physiology 31:811-816.

Seeliger M, Grimm C, Jaissle G, Zrenner E, Reme C, Humphries P, Biel M, Fariss R, Redmond T,Wenzef A (2002) Genetic separation of rod and cone-driven activity in RPE65 knockout mice identifies the rods as the source of vision in a form of lebers congenital amaurosis. Invest Ophthalmol Vis Sci 43:U1020-U1020.

Shahinfar S, Edward D,Tso M (1991) A pathological-study of photoreceptor cell-death in retinal photic injury. Curr Eye Res 10:47-59.

Sharp P, Cowe E, Higgins D, Shields D, Wolfe K,Wright F (1988) Codon usage patterns in Escherichia-coli, Bacillus-subtilis, Saccharomyces-cerevisiae, Schizosaccharomyces-pombe, Drosophila-melanogaster and Homo-sapiens - a review of the considerable within-species diversity
. Nucleic Acids Res 16:8207-8211.

Shen Y,Raymond P (2004) Zebrafish cone-rod (crx) homeobox gene promotes retinogenesis. Dev Biol 269:237-251.

Sherpa T, Fimbel SM, Mallory DE, Maaswinkel H, Spritzer SD, Sand JA, Li L, Hyde DR,Stenkamp DL (2008) Ganglion cell regeneration following whole-retina destruction in zebrafish. Developmental Neurobiology 68:166-181.

Shiroozu A, Okamura K, Ikenoue H, Sato K, Nakashima T, Yoshinari M, Fujishima M,Yoshizumi T (1986) Treatment of hyperthyroidism with a small single daily dose of methimazole. Journal of Clinical Endocrinology & Metabolism 63:125-128.

Snelson CD, Santhakumar K, Halpern ME,Gamse JT (2008) Tbx2b is required for the development of the parapineal organ. Development 135:1693-1702.

Stenkamp D, Barthel L,Raymond P (1997) Spatiotemporal coordination of rod and cone photoreceptor differentiation in goldfish retina. J Comp Neurol 382:272-284.

Szel A, Rohlich P, Caffe A, Juliusson B, Aguirre G,Vanveen T (1992) Unique topographic separation of 2 spectral classes of cones in the mouse retina. J Comp Neurol 325:327-342.

Takechi M, Hamaoka T,Kawamura S (2003) Fluorescence visualization of ultraviolet-sensitive cone photoreceptor development in living zebrafish. FEBS Lett 553:90-94.

Takechi M, Seno S,Kawamura S (2008) Identification of cis-acting elements repressing blue opsin expression in zebrafish UV cones and pineal cells. J Biol Chem 283:31625-31632.

Teakle E, Wildsoet C,Vaney D (1993) The spatial-organization of tyrosine hydroxylase-immunoreactive amacrine cells in the chicken retina and the consequences of myopia. Vision Res 33:2383-2396.

Thompson C,Potter G (2000) Thyroid hormone action in neural development. Cerebral Cortex 10:939-945.

Thornberry N,Lazebnik Y (1998) Caspases: Enemies within. Science 281:1312-1316.

Thummel R, Kassen SC, Enright JM, Nelson CM, Montgomery JE,Hyde DR (2008) Characterization of Muller glia and neuronal progenitors during adult zebrafish retinal regeneration. Exp Eye Res 87:433-444.

Triezenberg S, Kingsbury R,McKnight S (1988) Functional dissection of Vp16, the trans-activator of Herpes-Simplex virus immediate early gene-expression. Genes Dev 2:718-729.

Valente R, Capella L, Monteiro R, Rumjanek V, Lopes A,Capella M (2003) Mechanisms of ouabain toxicity. Faseb Journal 17:1700-+.

Veldhoen K, Allison WT, Veldhoen N, Anholt BR, Helbing CC,Hawryshyn CW (2006) Spatio-temporal characterization of retinal opsin gene expression during thyroid hormone-induced and natural development of rainbow trout. Vis Neurosci 23:169-179.

Venitt S,Croftonsleigh C (1987) The toxicity and mutagenicity of the antitumor drug 5-aziridino-2,4-dinitrobenzamide (Cb1954) is greatly reduced in a nitroreductase-deficient strain of Escherichia-coli. Mutagenesis 2:375-381.

Vihtelic T, Doro C,Hyde D (1999) Cloning and characterization of six zebrafish photoreceptor opsin cDNAs and immunolocalization of their corresponding proteins. Vis Neurosci 16:571-585.

Vihtelic T,Hyde D (2000) Light-induced rod and cone cell death and regeneration the adult albino zebrafish (Danio rerio) retina. J Neurobiol 44:289-307.

Vugler A, Lawrence J, Walsh J, Carr A, Gias C, Semo M, Ahmado A, da Cruz L, Andrews P,Coffey P (2007) Embryonic stem cells and retinal repair. Mech Dev 124:807-829.

Watanabe M, Ishidate M,Nohmi T (1990) Nucleotide-sequence of Salmonella-typhimurium nitroreductase gene. Nucleic Acids Res 18:1059-1059.

Wenzel A, Grimm C, Samardzija M,Reme C (2005) Molecular mechanisms of light-induced photoreceptor apoptosis and neuroprotection for retinal degeneration RID B-9245-2008. Prog Retin Eye Res 24:275-306.

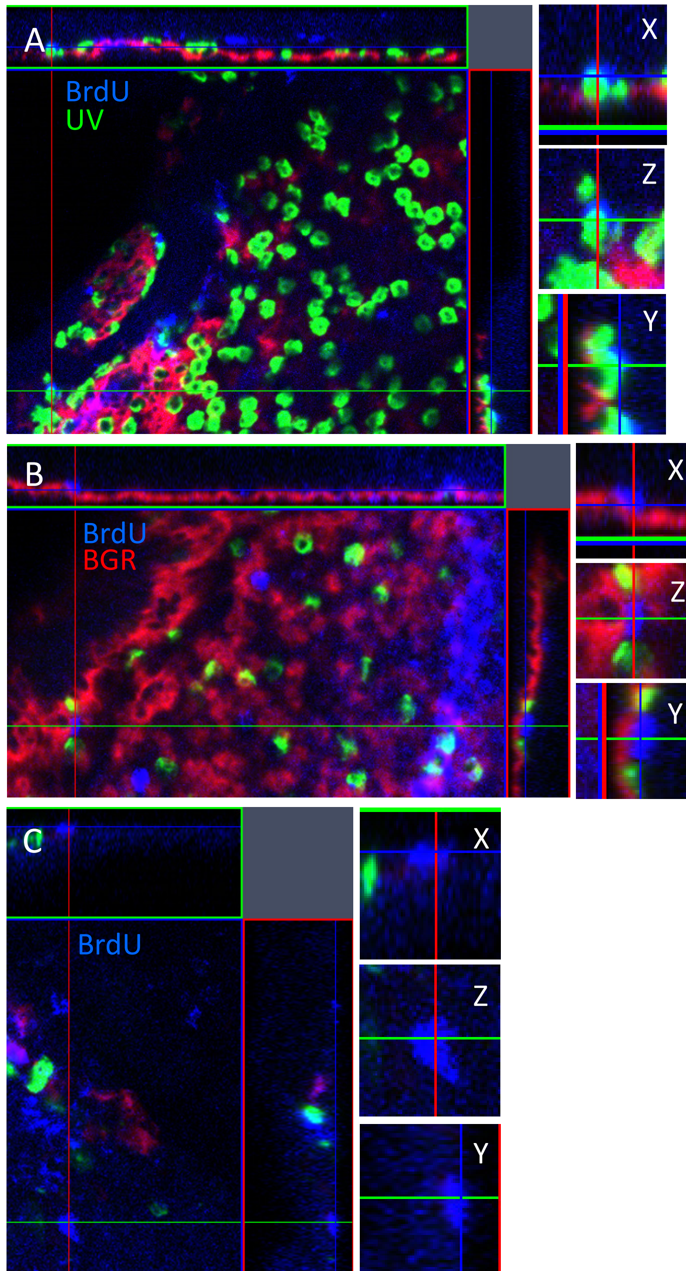
Westerfield M, (2000) The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio). Eugene, Oregon, USA: University of Oregon Press.

Westerfield M, (1995) The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio). Eugene, Oregon, USA: University of Oregon Press.

Wetts R, Serbedzija G,Fraser S (1989) Cell lineage analysis reveals multipotent precursors in the ciliary margin of the frog retina. Dev Biol 136:254-263.

Wu D, Schneiderman T, Burgett J, Gokhale P, Barthel L,Raymond P (2001) Cones regenerate from retinal stem cells sequestered in the inner nuclear layer of adult goldfish retina. Invest Ophthalmol Vis Sci 42:2115-2124.

Appendix



Supplementary Figure 1. Confocal z-stack analysis to determine the identity of BrdU-positive photoreceptors. A 3-dimensional analysis was performed using the ZEN microimaging software to allow for the visualization of BrdU in the nucleus co-localizing with opsin expression. Photoreceptors were divided into 3 unambiguous categories: BrdU-positive co-localizing with UV opsin (A), BrdU-positive co-localizing with BGR opsin (B), and non-colocalizing BrdU-positive rods (C).