Development of Transgenic Techniques for the Study of Adult Newt Retinal Regeneration

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Contents

Abstract

The order Urodela (newts and salamanders) has the strongest ability to regenerate body parts following tissue injury. Previous research have shown that adult newts are capable of regenerating their limbs, heart, tail, spinal cord, jaw, brain, lens, and retinal tissue. The underlying molecular mechanisms of newt regeneration remain an unsolved mystery, despite being first reported by Spallanzani in 1760`s. This study focuses on newt retinal regeneration. The retina is a multilayer neural tissue involved in vision. Following the removal of the retina (retinectomy), the newt can regenerate a fully functional retina. Previous studies have identified the retinal pigment epithelium (RPE) layer of the newt as a primary cell source for retinal regeneration. Use of the newt RPE layer as a model to study retinal regeneration is useful because this layer is composed of a single cell type, specific markers are available (RPE65), and techniques to remove the neural retina without the damaging the RPE layer have been established. During early retinal regeneration the RPE layer dedifferentiates into a "stem cell like state", referred as RPE derived cells. These RPE derived cells undergo cellular reprogramming, lose their RPE65 expression, and regenerate the neural retina by a precess called transdifferentiation. Currently, there is no system to examine functional gene analysis in the newt *in vivo* (such as gene knockdown or gain of function).

Technical limitations such as: the inability to sequence the newt genome (due to its large size) and lack of molecular techniques to manipulate gene function in the newt, have decelerated the progress in this field. Therefore to overcome these obstacles, this study has established an effective method to generate transgenic newts, using a coinjection of *I-SceI* endonuclease with a target transgene into the one cell stage of the newt. This transgenic protocol can be used to study newt regeneration in general. To take full advantage of this transgenic protocol, this study also applied it to newt retinal regeneration. In order to drive transgene expression and later manipulate gene function specifically in the RPE layer, this study identified the newt RPE65 promoter capable of

driving exogenous genes in the RPE layer of transgenic newts. In addition, this study generated for the first time dual promoter and reporter transgenic newts for studies in retinal regeneration. Lastly this research applied the invaluable CreER^{T2} loxP recombinase system, a power tool for gene recombination, in the newt for the first time by injecting two transgenes simultaneously into the one cell stage. Transgenesis by *I-SceI* provides one way to examine retinal regeneration *in vivo*. This study provides essential and fundamental techniques to generate transgenic newts, and applications for newt retinal regeneration studies.

Abbreviations

4-OHT	4-Hydroxytamoxifen
AmCyan	*Anemonia majano* cyan fluorescent protein or cDNA
AP1	Adaptor Primer 1
AP2	Adaptor Primer 2
CCAAT	CCAAT-enhancer binding proteins (C/EBP)
$CreER^{T2}$	Modified cre recombinase containing the estrogen receptor T2
DMSO	Dimethly sulfoxide
EGFP	Enhanced green fluorescent protein, cDNA size 720bp
GSRP1	Gene specific reverse primer 1, derived from the the newt RPE65 mRNA
GSRP2	Gene specific reverse primer 2, derived from the the newt RPE65 mRNA
Holt medium	Holtfreter`s embryo medium or solution for newts.
HS4	The chicken β-globin core insulator, size 250bp
HS42X	Two copies of the chicken β-globin insulator core, size 500bp
MOK-2	Mouse Krüppel/TFIIIA-related zinc finger proteins
IRBP	Interphotoreceptor retinoid-binding protein, as known as RBP3 (retinal-binding protein)
I-SceI	*I-SceI* is a commercial intron-encoded endonuclease, derived from the mitochondria of *Saccharomyces cerevisiae*.
LB	Luria Broth
mCherry	Red fluorescent protein, modified from DsRed
pA	mRNA polyadenylation signal
pCAGG`s	The chicken beta actin promoter, size 1.7kb
npRPE65	The newt (*Cynops pyrrhogaster*) RPE65 promoter (-560 to 0bp)

nRPE65	The newt retinal pigment epithelium protein measuring 65kD
OCT-1	Octamer-1 transcription factor
PCE1	Photoreceptor Conserved Element 1
NF-Y/CCAAT	Nuclear transcription factor Y/CCAAT-enhancer binding proteins
RET/PCE1	Ret proto-oncogene/Photoreceptor Conserved Element 1, a TFBS
RPE	Retinal Pigment Epithelium
SL	Swimming larva capable of swimming having developed gills
TB	Tail bud stages

Chapter One: General Introduction

1.1 Urodela Regeneration

The order Urodela (newts and salamanders) possesses an elegant and remarkable ability to regenerate damaged tissue. Newts and salamanders are diverse amphibians distributed in most parts of the world, except Antarctica. Their habitat consists of cool environments with access to land and water. In the case of the newt, their life cycle consist of an egg, a larva, a juvenile (by metamorphosis) and an adult stage (sexually mature). The most common species of Urodela currently used in regenerative biology and medical research are the following: *Ambystoma mexicanum* (Mexican Axolotl), *Cynops pyrrhogaster* (Japanese firebelly newt), *Pleurodeles Walt* (Iberian newt), and *Notophthalmus viredenscens* (The North American newt).

In 1768 Lazzaro Spallanzani first reported the salamanders` ability to regenerate its tail, by repeating the experiment several hundred times (Spallanzani, 1768; Tsonis and Fox, 2009). Decade's later researchers observed that other tissues in the newt also had the ability to regenerate such as the: heart, brain, retina, lens, jaw, limbs, and spinal cord (Brockes and Kumar, 2002; Ferretti et al., 2003; Brockes and Kumar, 2005; Chiba et al., 2006; Carlson, 2007; Singh et al., 2010; Tanaka et al., 2011). Researchers continue to be baffled and amazed at this phenomenon. Without a doubt knowledge gained in newt regenerative research will provide powerful insights in regenerative medicine and biology. Some major examples include spinal cord injury, retinal degeneration, degenerative arthritis, and myocardial scaring.

It is important to note that newts are the only adult vertebrates capable of strong body-parts regeneration. Other lower vertebrates such as the fish (Poss et al ., 2002; Raya et al., 2003), frog

(Gross, 1969) and axolotl (Sobkow, 2006) have certain abilities to regenerate tissue, but are not equivalent to the newt (Sandoval-Guzman et al., 2014). In addition, the axolotl (an aquatic salamander) remains at larval stage, never metamorphosing to adulthood, a process called neoteny, therefore its difficult to study mature tissue in this model. In contrasts newts fully metamorphose into the adult stage. Thus the newts` ability to regenerate tissue at an adult stage makes them an excellent model organism for Regenerative Biology and Medicine.

We currently have a better understanding of molecular and cellular events during newt regeneration since the time of Spallanzani. It is important to define what regeneration in the newt implies. Humans can "heal" damaged tissue; such as injury to the skin or liver damage to a certain extent. In contrast general tissue-regeneration of the adult newt includes the following events: dedifferentiation, reprogramming and transdifferentiation (cell-type switching) of terminally differentiated tissue from an adult stage (Sandoval-Guzman et al., 2014; Eguchi et al, 2011; Jopling et al., 2011;). In addition salamander regeneration maintains a “positional memory” theses are molecular programs that keep cell-type specific information (or regional information) in order to properly regenerate that lost tissue (Poss, 2010; Kragl et al., 2009).

Newt regeneration has also been observed to have a unique relationship with cancer. For example attempts to induce cancer in the newt limb using carcinogens resulted in mostly abnormal limbs without tumor formation (Tsonis and Eguchi, 1982). Humans on the other hand are more susceptible to cancers, when exposed to carcinogens. In a more recent study, it was shown that newt limb regeneration requires regulation of p53, for proper formation of the blastema (Yun et al, 2013). p53 is a tumor suppressor, involved in genome stability (Ryan et al., 2001), and promotion of differentiation pathways in mammals (Molchadsky et al.,, 2010)

To elaborate more on these terms, I will use retinal regeneration as an example and the research interest of this study for applications.

1.2 Newt Retinal Regeneration

The vertebrate eye is a hallmark of evolution and a critical sense organ for the organisms survival. The vertebrate eye contains a light sensing multilayer tissue called the retina. The retina in higher and lower vertebrates is found in the back of the eye. Light enters the cornea and penetrates the lens, strikes the retinal layers, which transmits a signal through the optic nerve to occipital part of the brain for processing. The retina of the newt is composed of the following layers: ganglion cell, inner plexiform, inner nuclear, outer plexiform, outer nuclear, and the retinal pigment layer. The retinal pigment epithelium (RPE) is a monolayer of cells located between the choriod and the photoreceptors (Kennedy et al., 1998). The retinal pigment has several key functions in vision: secretion, phagocytosis, involvement in the visual cycle, glia, epithel transport, light absorption, forms part of the blood retinal barrier, and maintains the photoreceptors nourished (Strauss, 2005; Kennedy et al., 1998). The newt's retinal pigment epithelium (RPE) has an additional function, retinal regeneration (Chiba, 2013; Mitashov, 1996).

The Swiss naturalist Charles Bonnet is first credited for performing and publishing studies on newt lens and retinal regeneration in 1781(Mitashov, 1996). A hundred years later studies on newt retinal regeneration began to increase and several researchers reached the same conclusion; that the newt has a flawless skill to regenerate the lens and retina (Philipeaux, 1880; Griffini and Marcchio, 1889; Colucci, 1891; Wolff, 1895). The primary cell source for retinal regeneration is the RPE layer, the secondary cell source are stem/progenitors cells of the retinal marginal area, and the third cell source are bipolar like cells of the retina itself (Chiba and Mitashov, 2007; Chiba et al., 2006; Mitashov, 1996.) This study will focus on the primary cell source, the RPE layer, shown in (**Figure1)**.

How does the newt regenerate the retina from the retinal pigment epithelium (RPE layer)? The current working hypothesis of the newt early retinal regeneration has been proposed by Chiba and Mitashov (2007), it is illustrated in (**Figure 2)**. During retinal regeneration several genes in the RPE cells are up-regulated and down-regulated. RPE cells dedifferentiate into a stem cell like state, two particular gene that are up-regulated the stem cell maker Pax-6 "the master control of the eye" and Msi-1, a critical a gene involved in post-transcriptional regulation, proliferative activity of gliomas and melanomas (Chiba et al., 2006; Kaneko et al., 1999). Interestingly, RPE cells lose expression of a specific marker RPE65 (Chiba et al, 2006) and slowly begin to lose their pigmentation (Chiba and Mitashov, 2007; Chiba et al., 2006; Moshiri et al., 2004; Mitashov, 1996). RPE cells lose their identity, and reprogram into retinal progenitors cells, early retinal progenitor cells (expressing Pax6, Msi-1, Chx-1, and Notch) continue to poses some pigmentation a characteristic from the RPE cells. Melanin from RPE cells takes a long time to breakdown, in classical experiments it was used to track RPE derived cells during retinal regeneration (Mitashov, 1996). These retinal progenitor cells derived from RPE cells, transdifferentiate to regenerate the neural retina. How exactly newt RPE cells dedifferentiate is unknown.

1.3 Direction of the Field

(**Figure 3)** summarizes the some major highlights in the field of newt retinal regeneration. The field of retinal regeneration is shifting from histological observation of regeneration towards gene identification and gene manipulation of regeneration. To understand the molecular mechanisms in the newt regeneration (in general) it is crucial to obtain genomic information (mRNA transcripts, contigs, and proteomic data) during the regenerative process. Unfortunately one limitation when working with newts is their huge genome, in general all newts and salamanders have large genomes. For example, the Japanese newt genome (for *Cynops pyrrhogaster*) has a C-

value of 37.8 picograms (approximately 18 Giga basepairs) (Licht and Lowcock, 1991). This size has really made it difficult for researchers to seek unique genes involved during regeneration. A huge insight in this field regarding the salamander genome size was that it is composed of long intron regions (Smith et al., 2009). Nonetheless researchers from several labs around the world are eager and currently focusing on genomic information. In 2013 Abdullayev et al., generated a *de novo* reference transcriptome for the newt *Notophthalmus viridescens*, they found 118,893 transcripts with a N50 of 2016 nucleotides, and 19,903 newt proteins in during regeneration (Abdullayev et al., 2013), these still need to be further examined. In a different study by other researchers examined microarray analysis of gene expression during newt regeneration, they found several factors related in cell cycle and DNA repair during regeneration (Sousounis et al 2013). Unpublished data from Dr. Chiba`s Lab of Regenerative Physiology are also examining genomic data during retinal regeneration of the newt (Personal Communication).

Newt regeneration is approaching a genetic direction, there is another limitation to consider. How can researchers test genes involved during regeneration? It is essential that these genes are tested in order to provide evidence it is necessary to examine their functional role during regeneration. Manipulating gene expression in the newt is a critical step in understanding their ability to regenerate. Currently only one study has examined gene function in the newt, this study used morpholinos to knockdown Pax6 expression in vitro and *in vivo* during lens regeneration from dorsal pigmented iris, results showed that Pax6 expression is necessary for early events in lens regeneration (Madhavan et al., 1996). Morpholinos are a useful gene tools (for modifying RNA splicing or inhibiting miRNA activity and maturation) and gene knock down. Morpholinos are very expensive (this limits the amount of experiments) and difficult to make. The delivery of morpholinos to mature regenerating tissue (without tampering with it) or those that are in the process of later regeneration is challenging *in vivo.* Thus researchers working with morpholinos can

only examine early events. This study proposes an alternative solution to study functional gene analysis in the regenerating newt, *in vivo* manipulation by transgenesis.

1.4 Transgenesis in the Newt

Transgenesis is the process of introducing foreign DNA into a host genome, with the goal of altering and manipulating the hosts` gene expression. Transgenesis is a very powerful tool for *in vivo* experimentation, such as gain of function or lose of function of a particular gene of interest. Before the findings of this study, transgenesis in the newt was limited to two papers, one published by Mikita et al., 1995 and the second by Ueda et al., 2005. Thus transgenesis in the newt has very few techniques and references available. This is largely due to the difficultly that comes with working with the newt in the lab settings, I would like to mention three challenges with transgenesis in the newt.

(1) One of the major problems with generating transgenic newts is the difficultly of obtaining fertilized eggs to generate F0 transgenic lines (Ueda et al., 2005). Breeding newts in the lab is a difficult task, researchers generally purchase or collect them from rice fields, of which many females are used but contain very few fertilized eggs (Ueda et al., 2005). Furthermore, in some cases females are sacrificed to collect eggs. Amphibians have been declining at a global scale (Stuart et al., 2004; Pounds et al., 2006), and the newt is not an exception. The Ministry of Environment of Japan has announced that *Cynops pyrrhogaster* is also declining (Ministry of Environment of Japan; http://www.env.go.jp/press/press.php?serial=7849). Furthermore rearing fertilized eggs to adult stages requires laborious work, especially if large sample sizes are used. Thus, at the current moment there are no reports or published papers using F1 transgenic newts or established transgenic lines.

(2) The second problem is mosaic transgene expression. The 1980's marked the expansion

of vertebrate transgenic studies. Studies were done by carefully microinjecting embryos, Biologist realized that specific genes could be introduced, examine gene expression and compare their transgenic organism with a wild type (Stuart et al., 1988). Transgenic studies were done on: fruit flies (Rubin and Spradling, 1982), sea urchins (McMahon et al., 1985), frogs (Etkins, 1982; Etkins et al., 1984), and mice (Gordon and Ruddle, 1981; Palmiter and Brinster, 1986). Earlier experiments microinjected linearized or circular DNA with reporters into one-cell stage of embryos. In general the results were mosaic with decreasing expression as the organism developed (Rusconi and Schaffner, 1981; Etkins et al. 1984; Andres et al., 1984; Etkins and Pearman, 1987). It is important to note that mosaic refers to uneven or non-uniform transgene expression in the target cells or tissue. Mosaic expression generally occurs because the transgene fails to insert at the one cell stage of embryos or suffer from positional effect (Tabin, et al., 2012; Perrimon, 2008; Vleminckx, 2008; Nakatsuki, 2002). Makita et al., 1995 succeeded in introducing transgenes into the newt, but ultimately led to the mosaic expresion.

(3)The third problem with generation of transgenic newt are technical, involving survivorship. Before this study there was no conventional protocol to generate a transgenic newt at a practical level. Some labs used different size injection needles, injection volume to the one cell stage, others dejellied eggs with forceps or chemicals. Furthermore these methods have not been examined in details to test there survivorship. This was confusing because one can not determine if the injected embryos die due to the transgene itself, or the injection, pre/post culture conditions, or abnormalities caused by poor egg quality. To give a solid example of this, Ueda et al., 2005 successfully generated full expression transgenic newt, but survival was less than 2%, we can only speculate why survival was so low.

Lastly, their are few labs working with newt regeneration, compared to those working with model organism such as the frog, mouse, and the fly. Many scientist lost interest in regeneration

(Okada, 1996; Brockes and Kumar, 2002.) The following is a relevant excerpt from Professor TS Okada from the University of Osaka who followed the history of regenerative research and gives us insight why the decline occurred:

> "*Later on, regeneration research seemed to retire from the leading part in the recent history of developmental biology, replacing its role to studies of early embryonic development….. There are several reasons for the recent decline in popularity of regeneration research. Probably, the fact that none of such really major scientific issues in developmental biology like the discovery of the organizer, or of the multipotentiality of somatic nuclei in development, has not come out of the regeneration studies, may have resulted in the failure to attract the interest of many scientists to this particular subject. The phenomenon of regeneration itself is in many respects too complex to be accommodated well in the modern trends in developmental biology. Regeneration was, and still is, a tremendously difficult subject to grasp in terms of entity or of element. An introduction of some recent techniques at that time like biochemistry, electron microscopy and others, did not help much.* ***Furthermore, there was no possibility of a genetic approach, since no mutation was known (in principle, even now), which affects regeneration***. "
>
> - TS Okada, 1996

With this in mind it is essential to generate an easy way to produce transgenic newts with the goal to altering gene expression during regeneration. Transgenesis will serve as one way for studying newt regeneration.

1.5 Research Purpose

The purpose of this study are the following:

1. To establish a simple and efficient method for generating transgenic newts with full expression.
   a. Development of mating tank conditions to obtain large amounts of fertilized eggs in the lab settings.
   b. Collection and preparation of fertilized eggs, microinjection conditions, post injection culture conditions until metamorphosis.
   c. Application of *I-SceI* co-injection with transgene construct to improve transgenic efficiency in the newt.
2. Applications of this transgenic protocol (1) for regenerative studies in the newt.
   a. Identification of the newt RPE65 promoter region capable of driving transgene expression at the RPE layer, a source for retinal regeneration.

b. Transgenic assay of the newt RPE65 promoter during development and metamorphosis, examining tissue specificity.

c. Comparison of the newt RPE65 promoter region with known vertebrates.

d. Generation of Dual promoter/reporter transgenic newts for tracking RPE cells during retinal regeneration. In addition to cell tracking during regeneration, this dual promoter/reporter system can help visual RPE cells during regeneration for single cell transcriptome analysis.

e. Generation of $CreER^{T2}$ loxP transgenic newts by two construct injection. Cre loxP system allow threes basic recombination events: inversion, translocation (homologous recombination), and deletion. This study will examine only the deletion recombination event with a transgene as a reporter excision.

Chapter Two: Generation of Transgenic Newts by *I-SceI*

2.1 Methods: Two Tank Mating System

To overcome the difficultly of collecting fertilized eggs in the lab, this study developed two mating tank system depicted in **Figure 4A**. The conditions are the following: tank size aquarium tank (60-cm width; 30-cm depth; 45-cm), natural light was used by keeping tanks near windows, room temperature was kept at 18°C, water inside the tanks was kept between 14-18°C (fluctuated during winter) regulated by a heater/thermostat system, external filter/circulation system was used, water remained still the majority of the day, a filter system was used for 4 hr/day for cleaning, water was changed when needed, rocks were added to allow newts to hide and rest (critical to minimize stress), and water level was set to ~15 cm (height).

Adult newts (tail to snout length: ~9 cm male and 11-12 females) were purchased from Mr. Kazuo Ohuchi in Chiba Prefecture, Japan. A ratio of 1–2:3 male to female were used per tank. Four to six days before the day of egg collection, females are injected with 30U of Gonadotropin (Gonatropin 3000; Asuka Seiyaku, Japan. stored at 4°C) in the subcutaneous layer of the abdomen every 2 days. Plastic vinyl ribbons were added into the tanks to allow females to lay their eggs and

to prevent them from eating their own eggs. In the morning (dawn) of the microinjection, eggs are collected early between 6-8 am because eggs must be injected during one cell stage. Eggs collected after 8 am begin to develop into second cell stage. All developmental stages of *C. pyrrhogaster* were followed according to the standard table of Okada and Ichikawa, 1947. Animals were cared according to University of Tsukuba Animal Use and Care Committee.

Two tanks were designed to allow females to lay eggs in one tank, while females in the second tank were rest or recovering from previous oviposition (egg laying). In other words when Tank 1 females layed eggs, (collection time), Tank 2 females rested (recovered), this process was alternated every two weeks for continuous eggs collection during the mating season of the Japanese newt (*Cynops pyrrhogaster*) shown in **Figure 4B**. Using these two tank system, newt were monitored daily to examine fertilization percentage and daily eggs collected per day/ per month shown in **Figure 4C**.

2.2 Methods: Fertilized Egg Preparation

Newt and salamander eggs contain several layers of jelly coating (Hiyoshi et al., 2007; Okamoto, 1972), which protect the eggs from drying out, and play an important role in fertilization (Onitake and Matsuda, 1984). In order to microinject material into the one cell stage it is necessary to remove the jelly, leaving only the vitelline membrane. The vitelline membrane is essential for proper development of newt embryos because it provides an early physical environment without it the embryos will collapse and lose their shape. In order to remove the jelly layers, a classical technique used in *Xenopus* (Okamoto, 1972) was modified for the newt. Alternatively egg jelly can be removed by using fine forceps (Kragl et al., 2009) but this quickly becomes tedious and damages several eggs. Therefore newts eggs were treated with 2% sodium thioglycolate in 0.5X Holtfreters solution, pH 10 (from herein called dejelly solution). After dejellying eggs, they were carefully rinsed with chilled 0.5X Holtfreters solution ten times to

remove jelly debris and residual dejelly solution. Dejellied eggs were transferred to terasaki dishes containing 6% Ficoll, 0.5X Holt medium, penicillin-streptomycin, pH 7.6 (from herein called microinjection egg medium). Dejellied eggs were kept on ice or inside the refrigerator until microinjection. A summary of egg preparation is shown in **Figure 5A**.

From the time of collection to rearing mature adults, modified Holtfreters solution was used for treating, rinsing, and manipulating. Preparation of 1x Modified Holtfreters (Holt) solution, contained the following (g/l) 3.5 NaCl, 0.05 KCl, 0.1 $CaCl_2$, and 0.2 $MgCl2{\cdot}6H_2O$. For diluted versions of H solution (i.e. 0.5xH, 0.2xH), pH was adjusted to 7.5 with $NaHCO_3$, unless otherwise as mentioned. All solutions for dejellied eggs/embryos were sterilized by a syringe filter of 0.2-mm pore size (DISMIC- 25cs, Cellulose Acetate; Advantec, Japan) and stored at 4°C.

2.3 Methods: Co-Microinjection of a Transgene with *I-SceI*

In the 2000`s vertebrate transgenesis shifted to a different approach, enzyme-mediated transgenesis was used to help transgene insertion into the genome. The fish, *Xenopus*, and Axolotl transgenesis have examined a different approach, the co-injection of DNA with *I-SceI* meganuclease demonstrated to highly increase transgene expression and decrease mosaic expression (Thermes et al., 2002; Ogino et al., 2006; Pan et al., 2006; Kragl et al., 2009). *I-SceI* is an endonuclease isolated from *Saccharomyces cerevisiae*, this enzyme has a 18 bp recognition site (TAGGGATAACAGGGTAAT), this sequence is found once in 7 X 10^{10} bp, approximately once in the human genome (Jacquier and Dujon, 1985). It is important to note, that unlike the transgenic REMI (restriction enzyme mediated integration) method, *I-SceI* will rarely cut the genomic DNA. Rather it only digest the injected transgene (Thermes et al, 2002). The exact mechanisms of *I-SceI* transgenesis insertion is unknown, but current speculations will be discussed later in Chapter 6. *I-SceI* has not been applied to newt transgenesis; therefore it is unknown if the transgene (efficiency)

will work with I-SceI in the newts large genome.

Microinjection mix is define as the solution that will be injected into the one cell stage of the embryos. It consists of 4 key components, *I-SceI* enzyme, plasmid DNA with reporter gene, *I-SceI* buffer, and phenol red. In this study a plasmid construct containing pCAGGs-EGFP (Sce) (kindly provided by Dr. Elly M. Tanaka, Max Planck Institute of Molecular Cell Biology and Genetics, Leipzig, Germany; see Sobkow et al., 2006) was used to test transgene efficiency with *I-SceI*. CAGG`s is the CAGGs—chicken b-actin promoter combined with IE CMV enhancer, as described by Sobkow et al., 2006. The plasmid DNA containing pCAGGs-EGFP was amplified in JM109 cells (Takara) and cultured with standard techniques. pCAGGs-EGFP plasmid was purified using Endofree Plasmid Maxi Kit (Qiagen, Valencia, CA), recovered in nuclease-free water, and stored at −80°C. Immediately after arrival *I-SceI* enzyme was aliquoted and stored at −80°C.

Microinjection mix (solution) contained 1× *I-SceI* buffer (New England Biolabs, Ipswich, MA), 0.25–1 U/µl of *I-SceI* meganuclease enzyme (New England Biolabs), 0.01% phenol red (stock: 0.1% phenol red dissolved with 0.3M NaOH), and 0.01–0.2 µg/µl of a plasmid DNA construct, (**Figure 5B)**. Red phenol was used to help visual microinjection into the egg. Fresh injection solution mix was used in each experiment; solutions were incubated at 37°C for 0–60 minutes and placed on ice until use.

Grinded glass capillary were prepared as shown in (**Figure 5B),** they were mounted on a holder that was fixed to a motorized micromanipulator (MP-330; Narishige) next to a fluorescence stereomicroscope (Leica M165 FC); the holder was connected to an injector PV820 Pneumatic Picopump (World Precision Instruments, Sarasota, FL). Immediately before injection, a 2-µl drop of the injection solution was placed on a parafilm sheet (Pechiney Plastic Packaging, Chicago, IL) mounted on ice and front filled into the

micropipette using an aspirator connected to the injector. Terasaki dish containing dejellied embryos (at one-cell stage, prepared as mentioned above) were placed under the stereomicroscope, the tip of the micropipette was inserted into the one-cell embryo up to ~50 μm in depth **Figure 5C**. The site of injection was on the superior animal pole section. Approximately 1-4 nanoliters were injected into the one cell stage.

2.4 Methods: Post Microinjection Culture and Rearing Conditions

I tested 1–4-nl (range) of microinjection mix in order to examine the optimal volume per embryo; the microinjection mix was injected at a pressure of 4-6 PSI (pounds per square inch) for 80–300 msec. The injection volume was regulated by changing the duration of injection time. All calibrations were estimated according to the World Precision Instruments operation manual. After all embryos (approximately 96) on Terasaki plates were injected using the same micropipette in ~30 min, they were transferred to an incubator (CN-25C; Mitsubishi Engineering, Japan/M-200; TAITEC, Japan) and kept at 14°C. The following day embryos were reared in 0.5X, pen-strep, pH 7.4, rearing solution was lowered to 0.2X Holt until tailbud (TB) stage. After tailbud stage embryos were transferred to 22°C and continue to be reared until swimming larvae stage (SL). Developmental stages were determined according to the criteria of Okada and Ichikawa (1947). At blastula stage 9-10 , transgenic newts containing CAGG`s promoter driving EGFP can be screened for the first time (**Figure 5C)** and (**Figure6 A** and **B)**. Here I defined embryos at stage 9–10, 17–21, 22–27, and 40–42 as blastula, neurula, tail-bud, and swimming larvae, respectively.

2.5 Conclusion

The methods described here for generation of transgenic newts were described in detail

during work done during my 5 year doctoral program, and published by Casco-Robles et al 2010 and Casco-Robles et al 2011. Here I provide a concise methodology to focus on the applications of this transgenic system in the following chapters. From herein all transgenic newts are generated using this protocol. The results of these methods demonstrated that *I-SceI* can be applied to the newt, (**Figure6)**. In summary, optimization of *I-SceI* improved transgene expression and reduced mosaic expression in the F0 generation (**Table 1**). Most importantly, this transgenic method was examined during regeneration, a critical propose for regenerative studies. So far it has shown promise, (**Figure 7)**. This transgenic system needs to be exploited, next I focus my attention to retinal pigment epithelium, a cellular source for retinal regeneration.

Chapter Three: The Newt RPE65 Promoter Can Drive Expression in the RPE Layer of Transgenic Newts

3.1 Methods: Identification of the Newt RPE65 Promoter Partial Region

In order to drive exogenous gene expression specifically to the RPE layer of the newt, it is necessary to identify a specific marker or protein expressed in this tissue, and identify its promoter region. There are currently only two newt promoters identified a lens promoter (Ueda et al., 2005) and a limb promoter, Prod1, which contains unique regulatory elements during regeneration (Shaikh et al, 2011). Prod1 and lens promoter are not specific to the RPE layer. There are currently two known specific proteins expressed in the RPE layer. (1) RPE65, is a visual cycle protein weighing 65 kiloDalton, it has been examined carefully in the newt as being specific to the RPE layer (Chiba et al., 2006). It is important to mention that RPE65 promoter region has been identified in the mouse (Boulanger et al., 2000) and human (Nicoletti et al.,1998). (2) Another RPE layer specific marker protein is BEST1, which the promoter sequence has been identified in the

mouse and used to generate transgenic mice (Iacvelli et al., 2011). Since BEST1 protein expression has not been detected in the newt, I reasoned that identifying the newt RPE65 promoter was the current option for this study.

3.1.1 Methods: Genome Walking and PCR conditions

3.1.2 Methods: Sequencing Conditions

Sequencing was carried out using Big Dye Terminator DTv3.1 (Applied Biosystems), using M13 Forward and Reverse Primers (mentioned above). Following Big Dye reactions were done according to the manufactures recommendations, samples were cleaned with Centri-Sep Spin Columns (Applied Biosystems) and dissolved in with 15 micro-liters of Formamide (Applied Biosystems) at 95°C for 2 minutes. Samples were kept on ice, and later transferred to 64 well sequencing plates (Applied Biosystems). Samples were sequenced on a ABI3130 using the instrument protocol Rapid seq36_pop7_v3 and analysis protocol KB_3130_pop_v3.

3.1.3 Results: The Newt RPE65 partial upstream promoter sequence

A partial upstream region was sequenced containing a matching sequence of the 5`UTR ( 5`untranslated region) from the known newt RPE65 mRNA(mention above) and shown in (**Figure 10B)**. This identified sequence extended up to -560 bp 5` from the mRNA +1 position, shown in (**Figure 10B)**. This positive bacterial clone was stored in 15% glycerol in LB mix solution and stored at -80°C for future use.

3.2 Comparison of Newt RPE65 Promoter Sequence Higher and Lower Vertebrates

Because there are only 2 known reported promoter regions in the newt (mentioned above), and the RPE is a cellular source for retinal regeneration, it was curious to compare this newt upstream region with other vertebrates. There are currently no studies focusing on regulatory elements for the regenerating newt. Thus, understanding what is unique and different in the newt is insightful to this field.

3.2.1 Methods used for Alignment and Software

The newt RPE65 promoter region was first compared to the known higher and lower vertebrate RPE65 regions in the databases. The species and accession numbers used were the following: human (*H. sapien,* NG_008472), Mouse (*M. musculus*) Accession A F 2 7 2 7 1 2 9 7 Cow (*B. taurus,* NW_003103871), Dog (*C. familiaris*, NW_876321), Chicken (*G. gallus,* NC_006095), Frog (*X. tropicalis,* NW_003163757), Fish (*Danio rerio,* NW_001879345). In all cases only the -560bp to 0bp promoter region was used for alignment. Clustal W Omega (EMBL http://www.ebi.ac.uk/Tools/msa/clustalo/) was used to align the multiple high and lower vertebrates promoter regions, (**Figure 11)**. Using the Clustal W alignment the percent identity and phylogram were generated to compare the newt promoter among other vertebrates, (**Figure 12)**. An additional alignment was done using Promoter T-Coffee or Pro-coffee (http://tcoffee.crg.cat/apps/tcoffee/

do:procoffee), this aligns homologous promoter regions and searches for tentative transcription factor binding sites, (**Figure 13)**.

3.2.2 Methods: Identification of TFBS

To find reliable transcription factor binding site sequences, I compared the the human and the mouse upstream promoter regions to the newt. The mouse (Boulanger et al., 2000) and human (Nicoletti et al.,1998) promoter regions have been examined by DNA foot-printing, which demonstrates sequence specificity of DNA binding factors. The other lower and higher vertebrates promoter region mentioned early have not been examined. Therefore, a separate Clustal W alignment was done using only the newt, mouse, and human RPE65 promoter region (-560 to 0 bp). Because promoter regions have inversions and translocations, I used Promoter Wise (http://www.ebi.ac.uk/Tools/psa/promoterwise/). This software identifies regions that have been changed in direction or position, shown in **Figure 14A**.

Because the newt is currently the only known vertebrate to regenerate the retina at the adult stage, unique tentative transcription factor sites were examined for all 8 species using MatInspector (Carthariius et al 2005) online version (http://www.genomatix.de/cgi-bin/UMapps/register.pl). This programs allows multiple promoter regions to be examined at once, producing individual tables and group tables to identify similarities and differences. BioBase Match 1.0 and Patch 1.0 (http://www.gene-regulation.com/pub/programs.html#patch) running Transfac 6.0 were used to confirm results from MatInspector. Tentative transcription factors were scored by Matrix similarity ≥0.85 and Core similarity = 1.0, results were filtered and unique tentative transcription factors elements only found in the newt were selected, **Table 2**.

3.2.3 Results: The Newt RPE65 Promoter Contains Conserved TFBS

Alignment using clustal W of the newt RPE65 promoter did not reveal any immediate conserved regions among all lower and higher vertebrates RPE65 promoter regions, as shown in (**Figure 11)**. Furthermore percent identity of clustal w alignment showed very weak similarity among others species, the frog having the closest similarity with the newt by approximately 40%, (**Figure 12A)**. Phylogram also revealed that the newt promoter has higher base pair substitutions in this region compared to other vertebrates, (**Figure 12B)**. Because these results did not provide much insight into the newt RPE65 promoter, I turned my attention to comparing TFBS (transcription factor binding sites) that might be common among lower and higher vertebrates. Tentative TFBS can be identified using algorithms from conserved sequence motifs of other vertebrates. Promoter T-coffee alignment provides tentative TFBS distribution among multiple alignments. Several "hot spots" were detected among higher vertebrates and the newt promoter, shown in red in (**Figure 13)**.

To examine TFBS in more detail an additional alignment with the mouse, human, and newt was done and known human and mouse TFBS and Promoter Wise results were added to this alignment, (**Figure 14)**. Promoter wise revealed the newt RPE65 promoter region (-560 to 0bp) contains the following human or mouse TFBS: RET/PCE1 (ggatttagaga), CCAAT (tttttgcaat), IRBP (ttctgtt), MOK-2 (tgccttttttttat), OCT1 (ttatgtaaa), PCE1 (taactaaattgaattaacggt), and NF-Y/CCAAT Box (agggggattggcccg). When compared to the positions among the mouse the human RPE65 promoter region (-560 bp to 0 bp), these TFBA sites in the newt were shifted up stream to the 5` end (translocated), or were in antisense (inversions).

Examination of TFBS of all 8 species, and filtering tentative unique TFBS revealed only in the newt are summarized in **Table 2**. Interestingly, the Yamanaka factor c-Myc/Max (in heterodimer form) was found in the newts RPE65 promoter region, in plus and minus strand. Another unique tentative TFBS were: Odd skipped related factor 1 (involved in embryonic structures), ZIC factor 2 (involved in embryonic structures of the nervous systems), and the "SIX1" Sine Oculis homeobox 1

(involved in embryonic structures).

3.3 Transgenic Assay of pRPE65 Promoter Activity

To examine if the newt RPE65 promoter can drive exogenous gene expression *in vivo*, the promoter and 5`UTR were added into an mCherry reporter cassette containing *I-SceI* for transgenesis. Transgenic animals containing npRPE65-mCherry were monitored for promoter activity during development and at the RPE layer of mature larvae. RPE65 protein expression has been weakly detected at stage 42 in the center of the RPE layer (Chiba et al., 2006), therefore I decided to examine promoter activity at stages 55-59 to allow sufficient time for RPE65 expression.

3.3.1 Methods: npRPE65-mCherry Transgene Construct

3.3.2 ImmunoHistochemistry

The retinal layer produces minor red auto fluorescence depending on fixation procedures, and the RPE layer is heavily pigmented making it difficult to see the cell body clearly. In order to visualise mcherry fluorescence directly (without the need of antibodies), I examined fixation conditions. After several trials the following resulted in a good signal. Newt larvae were fixed at stage 55-59. All stages were based on the newts standard developmental table by Okada and Ichikawa (1947).

Larvae were fixed in 4%PFA, 1xPBS, 0.25% Gluataraldehyde, at pH 7.4-7.6 for 6 hours at room temperature. To remove excess fixatives samples were later rinsed with 1xPBS in the following rinse cycle: 15 min, 15 min, 30 min, 30 min, 1 hour, 1 hour, and 2 hours. Samples were later transferred to 1XPBS with 30% sucrose and kept at 4°C over night. The next day samples were cryosectioned using O.C.T medium (Tissue-Tek), at 2µm per tissue section. Sections were rinsed with 1XPBS , treated with DAPI (dilution 1:25,000) and mounted on 90% glycerol and observed under a confocal microscope using an mCherry filter.

To confirm mCherry expression in the RPE layer of transgenic animals, additional sections were used for standard immunohistochemistry. Primary antibody: Rabbit dsRed polyclonal antibody, 1:500 (Clontech), Secondary antibody: Goat anti-rabbit IgG conjugated. Sections were treated with ABC blocking kit (Vector labs), and later with immunoreactivity DAB substrate (Vector labs). In negative controls, sections were not treated with the primary antibody, dsRed polyclonal antibody. Pigmentation was removed from the RPE layer by bleaching the slides with 1.5% sodium azide and 15% hydrogen peroxide.

3.3.3 Results: npRPE65 Promoter can Drive Expression in the RPE layer

The newt RPE65 promoter activity, *in vivo* assay, was first detected in the blastula stage 10, shown in (**Figure 16)**. Approximately 25% ±5 blastula embryos showed RPE65 promoter activity. To examine at the RPE layer, transgenic animals that expressed the mCherry fluorescent reporter were monitored until larval stages 55-59. At tail bud stage 27, (organogenesis) promoter activity was confined to neural tissue such as the eye cup, forebrain and developing nervous system, (**Figure 17A)**. At stage 31, RPE65 promoter activity gradually shifted to the forebrain, anterior developing neural tissue, and the eye, (**Figure 17B)**. By stage 39, the RPE65 promoter activity in the forebrain decreased, and the mcherry expression was detected in the eye, as shown in (**Figure 17C)**.

At the target stage 59, the eye of the newt is highly pigmented, expression of RPE65 promoter had decreased in the surrounding eye, mcherry was no longer visible macroscopically in the body, (**Figure 18A and B).** Therefore, sections of eye were prepared. (**Figure 18D)**, shows that newt eye contains the major retinal layers by stage 59 and RPE65 promoter activity was detected in the RPE layer. To examine this promoter activity in detailed, immunohistochemitry staining (primary antibody dsRed) was used. The RPE layer was bleached to removed pigmentation. For visualisation DAB immunoreactivty was used. (**Figure18E1 and 2)** shows sections expressing positive DAB in the RPE layer only, notice expression is detected in the cytosol and not the nucleus. mCherry is a cytosolic protein. (**Figure 18F)**, shows a negative control section derived from the same transgenic without the primary antibody (dsRed). This promoter activity in the eye was observed at 40% from the positive derived blastula embryos monitored, (**Figure 18C)**. The remaining %60 positive npRPE65-mcherry had RPE65 promoter activity in the RPE layer but also had off target mCherry expression in he ONL (photoreceptor layer) and INL (horizontal, amacrine, bipolar cells), shown in (**Figure 19A2 and A3)**. These transgenic larvae were also examined at the same stage 59, when the RPE layer was pigmented , and the rental layers were development,

(**Figure 19A1).**

3.4 Conclusion

The newt RPE65 promoter region (-560 to +148bp) can successfully drive exogenous transgene expression in the RPE layer of F0 generation transgenic larvae. Promoter activity was detected in early developmental stages before maturation of the RPE layer. In some cases, variations of mcherry expression outside of the RPE layer were detected in INL and ONL. This off target expression "leaky expression" should be examined in more detail to improve specificity for future RPE transgenic gene manipulation.

Chapter Four: Generation of a Dual Promoter/Reporter Transgenic Newts

One of the biggest challenges examining a single promoter transgene, is the uncertainty, if the tissue specific transgene was inserted correctly at the one cell stage. For example, if RPE65 promoter transgene inserts into the 1, 3 or 4 cell stage, there is no way of knowing. Therefore an internal control in necessary and critical to assure that all cell types carrying the transgene. Thus, I developed another set of transgene constructs, this time using the general CAGG`s promoter derived from the chicken beta actin gene, together with the newly identified RPE65 promoter. CAGG`s was examined previously during the development of the transgenic protocol, this promoter expression is known to be active at blastula stage (Thermes et al., 2002; Ogino et al., 2006; Pan et al., 2006; Kragl et al., 2009) and in the newt, (**Figure 6** and **7).**

4.1 Methods: Dual promoter/Dual Reporter Transgene System

The following primers containing the BstxI restriction enzyme sites were used to amplify npRPE65-mCherry-pA Forward primer 5`—3`: ccaccgcggtggCGACGGCCCGGGCTGGTAAAAAGC) and Reverse primer 5`—3` (ccaccgcggtggACATTGATGAGTTTGGACAAACC), producing BstxI-npRPE65-mCherry-pA-BstxI. This PCR products was cleaned and ligated (as described above) into the previously used pCAGG`s-EGFP-pA (Clontech EGFP N1, modified version and kind gift from Elly Tanaka, Max Planck Insitute) cassette, into the BstxI restriction site. Using the same restriction enzyme two possible insertion patterns of npRPE65-mCherry-pA were possible sense or antisense, as shown in (**Figure 21A)**. These two trangenes were transformed into HST02 *E.coli (*Takara*)* because standard JM109 (Takara) competent cells yielded coiled plasmids of these transgenes. Plasmid DNA was isolated and purified (as describe above using transgenic protocol). These trangenes were used to generate transgenic newts containing two promoter/two reporter transgenes. Transgenic newts were monitor during early development, later metamorphosis (to assure maturity), and eye sections of the retina were taken examine the RPE layer (methods same as described above).

4.2 Methods: Application of the Chicken Beta Insulator HS4 to the Dual promoter/ Dual Reporter Transgene System

Transgenesis by *I-SceI* is a random insertion of the transgene. When a tissue specific promoter is used with *I-SceI* transgenesis it brings a new challenge. Leaky expression is one of the major problems in all transgenic model organism, frog (Vleminckx et al., 2008), mouse (Nakatsuji et al., 2002), Axolotl (Tabin et al., 2012), and drosophila (Perrimon et al., 2008). Leaky expression occurs due to positional effect, in other words, where the transgene is inserted, will have a consequence on its expression, see (**Figure 20)**. If a transgene is inserted near a regulatory element such as an enhancer region, this can cause off target activity of RPE65 promoter. Therefore, to

protect RPE65 promoter in a two promoter/ two reporter system it is important to examine ways to protect the transgene from positional effect. One way to protect transgenes from positional effect is to use insulators.

Insulators occur naturally in the vertebrate genome, they help protect against enhancers and promoters interaction from neighbouring genes. One of the most studied insulators is the chicken beta-globin insulator (1.2kb), containing functional core (250bp) called the HS4 site (Bell et al., 1999; Chung et al., 1993; Recillas-Targa et al., 1999; Recillas-Targa et al., 2002). The chicken beta insulator 1.2kb can been successfully used in transgenic mouse containing dual promoters (Hasegawa and Nakatsuji, 2002) and the core HS4 insulator has also been applied to transgenic F0 *Xenopus* (Sekkali et al.,2008). It is also important to mention that two promoter in a single transgene can interefere with their activity (Hasegawa and Nakatsuji, 2002), therefore it is also important to use insulators between to promoters in a transgenic cassette. In order to protect RPE65 promoter, the chicken HS4 insulator was applied.

The chicken HS4 core (250bp) was a kind gift from Felsenfeld Group from the National Institute of Health, USA. Three dual core HS4 copies were inserted into the CAGG`s-EGFP vector two cores one at a time, at restriction sites: *XhoI/BstxI, Afl2/Dra3* and at the *BstxI/SpeI*, shown in (**Figure 21B)**. The (BstxtI) npRPE65-mcherry-pA (BstxI) was added last to generate two promoter/ reporter trangenes containing the 3 (2X HS4), shown in (**Figure 21C**). These trangenes containing multiple repeats of HS4 were transformed into the Stbl3 competent *E. coli* cells (Introgen) to avoid recombination inside the bacterias. *E.coli* are know to be sensitive to DNA repeats. Trangenes from (**Figure 21 A and C)**, were microinjected into the one cell stage of the newt to generate four transgenic lines. They were examined at early blastula stage, metamorphosis, and retinal sections were prepared as mention above to examine RPE65 promoter activity at the RPE layer.

4.3 Results: Insulator protects the newt RPE65 Promoter from Positional Effect

pCAGG`s-EGFP was used here as an internal control to assure that npRPE65-mCherry was also inserted evenly in all cells. At early blastula stage 10 when embryos expressed EGFP, the newt RPE65 promoter had severe leaky expression, when the promoters were placed in opposite direction, (**Figure 22D)**. It was interesting that under the same opposite directions, but using the HS4 insulator, RPE65 promoter continued to express mcherry at blastula, (**Figure 22C)**. When the promoters were oriented in the same direction, without HS4, RPE65 promoter activity was also detected but to minor extent compared to transgenes containing promoters in opposite direction, (**Table 3).** Promoters in the same direction containing HS4, showed the minimum RPE65 activity at blastula stage. Its important to note that all four transgene containing different orientations with or without HS4 produced RPE65 activity at blastula, but the frequency varied. (**Table 3)**, summarizes the total number of eggs microinjected, expression of EGFP+ at blastula, expression of mCherry/EGFP+ at blastula, and blastula survival of these four transgenic lines containing two promoter/ two reporter construct. (**Figure 22)** depicts the promoter orientation from best (top) to worst (bottom). The internal control pCAGG`s-EGFP was always expressed and never silenced at blastula stage, (**Table3).** Two promoter interference was observed in transgenes without the HS4 insulators, pCAGG`s strongly influenced npRPE65 promoter activity (**Figure 23)** shows a representative larva expressing mCherry in a manner similar to pCAGG`s-EGFP. Survivorship to larval stage was improved with the HS4 insulator, but only when the promoters were placed in the same direction (**Table 3)**.

Representatives of each transgene were reared to metamorphosis, (**Figure 24A1,B1,C1,D1)**. Off target npRPE65 activity could be been in the lens of metamorphosing newts without insulation, and minimized (**Table 4**) when HS4 was used, (**Figure 24 A3, B3, C3, D3)**. Transgenic newts

without HS4 insulator in opposite direction at metamorphosis had leaky expression of npRPE65-mCherry, which appeared to be similar to that of pCAGG`s, (**Figure 24D)**. Transgenic newts with HS4 and promoters in the same direction, showed strong protection against positional effect, (**Figure 24A)**.

Examination of these transgenic lines in the retina and RPE layer showed similar results. Transgenes containing no HS4 and promoters in opposite direction showed off target expression in ONL and INL, (**Figure 25P-T)**. Transgenic animals with HS4 and promoters in the same direction had noticeable protection of in the neural retina, (**Table 4** and **Figure 25A-E).** RPE65 promoter activity was never silenced in the RPE layer, it was always detected with DAB immunoreactivity, (**Figure 25D,I,N,S and Table4)**.

4.4 Conclusion

Two promoter/ Reporter transgenic construct can be inserted into the newt genome by *I-SceI,* this is the first report in the newt. The chicken insulator HS4 core sequence can protect against leaky tissue-specific promoter actiivty. This study tested the chicken HS4 core for the protection of RPE65 promoter. This HS4 insulator can improve (protect) tissue-specific promoter in the newt. In all four transgenes, RPE65 promoter activity was observed in the RPE layer, it was never silenced by positional effect or pCAGG`s. The orientation of the insulator with respect to the promoter must be in the same direction. This improves protection from positional effect. It is possible to improve this insulation by adding additional copies of the HS4 insulator or using a stronger insulator.

Chapter Five: Generation of CreER^{T2} loxP Transgenic Newts

5.1 The CreER^{T2} loxP System

The Cre loxP system is a powerful genetic tool. It was originally derived from a P1 bacteriophage. Cre recombinase recognizes the 34bp loxP sites, which occurs once in every 10^{18} bp of DNA length. Two loxP sites are used to that allow 3 basic recombination events to occur depending on the loxP site orientation: deletion, inversion, and translocation (or homologous recombination) (Nagy, 2000; .) See (**Figure 27)** for depiction of these events. Unpublished data from (Mat and Nagy, 2000) note that cre recombinase can excise the loxP site up to 400kb distance in vitro, and that longer distances have shown reduced efficiency (Ramirez-Soliz et al., 1995) In lower vertebrates the Cre loxP system has just recently been used in the axolotl (Whited et al., 2012), frog (Roose et al., 2009), and fish (Hans et al., 2009). It has never been applied to the newt.

5.2 Obstacles and Consideration for Generating CreERT2 loxP Newts

The use of Cre loxP system in most model organism involves making two transgenic lines (1) one F0 generation line containing Cre, and (2) and a second transgenic line containing the loxP sites. These two F0 transgenic lines are later crossed to generate a F1 generation Cre loxP organism. This method is practical for model organism with short generation times. In the newt this poses a time limitation because the average newt generation time is approximately 1.5 to 2 years it would take 3 years to generate Cre loxP newt at the F1 generation, (**Figure 26)**.

Another point to consider when using the Cre loxP system is the control of recombination. Standard Cre is an exogenous gene that is translated into protein (enzyme) and cut the loxP sites in early development. This is a technical limitation if the target recombination needs to be cut at a more developed or adult stages. In order to apply Cre loxP to the newt at the adult stage for retinal regeneration, an alternative Cre is needed. Cre-ER^{T2} is a modified version of Cre containing the

mouse estrogen receptor. Cre-ER^{T2} after translation is found in the cytoplasm of the cell, but adding Tamoxifen (a chemical that binds to ER^{T2}) allows Cre-ER^{T2} to enter the nucleus and excise the loxP site. Therefore, Cre-ER^{T2} can be temporally activated, and using the RPE65 promoter will allow me to have a temporal-tissue specific recombination of target genes during newt retinal regeneration . Ultimately gene manipulation can be achieved in the regenerating newt.

5.3 Methods

In order to examine the Cre-ER^{T2} loxP system the two separate trangenes were designed. CreER^{T2} was obtained from Addgene. Using the two promoter reporter transgenes in the same direction with HS4 (mentioned above), the mCherry reporter was removed and replaced with CreERT2. EGFP was removed and replaced with YFP. An additional transgene was designed containing the two copies of loxP sites in the same direction (ATAACTTCGTATAGCATACATTATACGAAGTTAT), underline sequence contains the loxP core. Two tandem repeats of the loxP sites were inserted into the *AccI-KpnI* of pCAGG`s -AccI-KpnI-mCherry-pA vector, shown in (**Figure 28)**. AmCyan reporter gene (Clontech) was introduced between these two loxP sites by adding *AscI* and *AsiSI* restriction sites to Amcyan-pA using PCR (Forward primer: taggcgcgccATGGCTCTTTCAAACAAGTTTA and reverse primer: tagcgatcgcTGCAGTGAAAAAAATGCTTTAT), product AscI-AmCyan-pA-AsiSI was inserted in between the loxP sites, (**Figure 28)**. The final loxP reporter and npRPE65-CreERT2 transgene are shown in (**Figure 29A)**.

To overcome the the newts long generation time, I microinjected two transgene simultaneously into the one cell stage of the newt, to generation CreERT2 loxP newts at the F0 generation, as shown in (**Figure 26)**. The transgenic protocol was modified for optimization of two transgenes. Two nanoliters of the microinjection mix was injected into the one cell stage of the

newt. The optimal DNA concentration per egg (pg/egg) was examined in a series of microinjects, testing for survival and expression of YFP and Amcyan at blastula stage, shown in (**Table 5)**. DNA ratio was kept 1:1. At blastula stage transgenic newts were screened, those expressing Amcyan and YFP meant the two transgenes simultaneously were inserted. These transgenic embryos were monitored to any signs of mcherry expression. Good candidates should not expression mcherry. (**Table 5)** summarises expression pattern of two construct microinjection.

Good candidates were reared until larva stage 55-59. OH-Tamoxifen (hydroxyl) is a metabolite of Tamoxifen, it was used to activate $CreER^{T2}$, which meant it transported from cytoplasm into the nucleus. Because $CreER^{T2}$ has not been examined in the newt, I first examined toxicity concentrations on wild type larvae using DMSO or Ethanol, shown in (**Table 6)**. DMSO or Ethanol was mixed into the animals rearing solution, 0.1XHolfreters, pH 7.6. OH-Tamoxifen was added to both solutions at different concentrations (ranging from 0.5 to 100 μM). Larvae were fully submerged in these solutions. To determine treatment time, animals were placed inside DMSO or Ethanol solutions with or without Tamoxifen and monitor at a fixed time intervals, (**Table 6)**. Additionally I examined OH-Tamoxifen injection into the abdominal cavity of larva, but experiments failed because the solution leaked out from the abdominal cavity.

After and before Tamoxifen treatment transgenic larvae expressing YFP/AmCyan were examined for presence of mcherry reporter. After tamoxifen treatment expression of Amcyan should slowly decrease. By 36-48 hours expression should not be detected. This is due to cytosolic fluorescent protein still present, and require time for degradation. Cryosections of the retina and RPE layer were prepared to examine site specific deletion recombination of this system (as describe above).

5.4 Results: Two Transgene can be Simultaneously Introduced into the Newt Genome to Generate Cre-ER^{T2} loxP Newts

Insertion of two separate transgenes (CreER^{T2} and loxP) can be applied with *I-SceI*. A total working DNA concentration of 80 pg/egg (40 pg of each transgene, shown in **Figure 29A**) can produced an effective balance between survival and no mcherry expression, (**Table 5)**. DNA concentration higher than 80 pg/egg led to higher embryos expressing both transgenes, but survivorship was compromised at later stages, and mcherry+ blastula embryo cases increased. In contrast reducing DNA concentration led to fewer YFP+/AmCyan+ blastula embryos. Transgenic blastula embryos containing YFP+/AmCyan+/mCherry- (were grouped as good candidates) were reared and monitored, (**Figure 30A)**. At other developmental stages these good candidates did not express mCherry+ , (**Figure 30 B** and **C)**. At swimming larva stages transgenic animals expressing transgenes npRPE65-CreER^{T2} and loxP-Amcyan-loxP-mcherry, (**Figure 29A)**, were treated with OH-Tamoxifen. (**Table 6)** summarizes pharmacological treatment using OH-Tamoxifen. Ten µM was a practical concentration to activated CreER^{T2} into the nucleus, mcherry could be detected within 6hrs. Concentrations greater than 10µM were toxic to larvae. Concentration less than 10µM required longer time intervals to detect mcherry. DSMO 1% had minimum side-effects compared to ethanol, **(Table 6)**. Retinal sections showed site-specific activation of CreERT2 recombination in the RPE layer, of F0 transgenic animals treated with 10µM of OH-Tamoxifen (**Figure 31A and B)**. Negative control groups, without OH-Tamoxifen did not express mcherry (**Figure31 C and D**). Leaky mcherry was not detect in the retinal layers with or without OH-Tamoxifen, (**Figure31)**.

5.5 Conclusion

Insertion of two transgenes can be achieved using *I-SceI* transgenes. Using two separate transgenes containing HS4 one can generate F0 CreER^{T2} loxP newts. Here I generated site specific (RPE layer) CreER^{T2} loxP newts for the first time. This system can be temporally activated using 10µM of OH-Tamoxifen. This method saves experimentation time by directly generating CreER^{T2} loxP newts in the the F0 generation.

Chapter Six: General Discussion

6.1 New Findings

This study aimed at improving the genetic tools for studies in newt retinal regeneration. The newt RPE65 promoter region (-560 to 0 bp) was identified. Previously for the newt there were only two promoters identified a lens promoter (Ueda et al., 2005) and a limb promoter, Prod1, which contains unique regulatory elements and is regulated by MEIS1(a homebox thats activated by retinioc acid) during regeneration (Shaikh et al, 2011). Regulatory elements and their interaction on the promoter of genes involved in the newt regeneration are still new to research. This study found a third newt promoter, (npRPE65) capable of driving exogenous gene expression in the new RPE layer, a cellular source for retinal regeneration. Interestingly the human and mouse TFBS (transcription factor binding sites): RET/PCE1, CCAAT, IRBP, MOK-2, OCT1, PCE1, and NF-Y/ CCAAT Box were found inside the newt RPE65 promoter region -560 to 0 in different positions and orientations. I would like to discuss their relevant roles in the retina of vertebrates. RX homeodomain protein is necessary for the development of the eye, injected RX mRNA *Xenopus* embryos developed ectopic retinal tissue and hyperproliferation of the neural retina (Kimura et al 2000). RET/PCE1 (ret proto-oncogene/photoreceptor conserved element 1) sequence allows RX to bind to this site, interesting RET/PCE1 is found on the promoters of photoreceptor specific genes, for example the human RX can bind and activate the TATA-less arrestin promoter and IRBP promoter (Kimura et al., 2000). The IRBP (interphotoreceptor retinoid-binding protein , also known as retinol binding protein 3) was another TFBS found in the newt pRPE65 promoter, IRBP is located between the retinal pigment epithelium and the photoreceptor cells (http:// www.genecards.org/cgi-bin/carddisp.pl?gene=RBP3). IRBP is involved in the mature retina where it helps in the visual cycle. Interestingly MOK-2 (a kruppel/TFIIIA-related zinc finger protein)

DNA binding site is found within the newt RPE65 promoter region, MOK2 negatively modulates IRBP (Arranz, et al., 2001). Furthermore *in vivo* and *in vitro* studies have shown that MOK2 works as a repressor when in binds to the IRBP promoter site (Dreuillet et al., 2002), this may suggest that MOK2 may have repressor activity on RPE65 expression, during early retinal regeneration RPE65 is down-regulated (Chiba et al, 2006). OCT1 (also known as POU-domain transcription factor 1) DNA binding site is located in the newt RPE65 promoter region. Studies on the mouse RPE65 promoter activation showed that OCT-1 is a key player for promoter activity to occur (Boulanger et al., 2000). Lastly the NF-Y/CCAAT Box is commonly found in eukaryotic promoters, highly conserved, and NF-Y binding participates in histone post translational modifications by recruitment of relevant enzymes (Nardini, et al 2013).

Further research is needed to examine the above TFBS in the newt, such as DNA foot-printing. Nonetheless, the mouse (Boulanger et al., 2000) and human (Nicoletti et al.,1998) RPE65 promoter have been described as basal, containing all the necessary elements proximal to the 5`UTR region. For example, the human RPE65 promoter region -450, -262 and -83bp to +39bp are capable of driving luciferase reporter expression in ARPE19 cultured cells (Nicoletti et al., 1998). Here I used the -560 to +148 (5`UTR), to drive expression of mCherry in the RPE layer of F0 transgenic newts.

This is the first time that the newt RPE65 promoter activity was monitored from early development to a target stage expressing RPE65 protein. It is unknown if RPE65 is expressed during early development (blastula stage 10 and tailbud stage 27), the earliest protein detection in the newt has been reported at stage 42 (Chiba et al., 2006). In addition we don`t known if the newt RPE65 promoter is capable of driving other RPE65 alternative isoforms. Here I reporter RPE65 is primary expressed in the RPE layer at stage 59, was occasionally expressed in the ONL of the photoreceptors. It is important to note that this study examined RPE65 promoter activity before

adulthood. Studies in the aquatic tiger salamander have reported RPE65 mRNA expression in the cone cells of the photoreceptors (Ma et al., 1998a). In young mammals mouse, cow, and rabbit RPE65 protein expression has been in the photoreceptor layer by IHC (Znoiko et al., 2002). Human embryonic kidney cells have also been reported to express RPE65 mRNA (Ma et al., 2002b).

Shaikh et al.,2013 found that the MEIS (homeoprotein) and TF (transcription factors) has a regulatory function during limb regeneration. In the newt transcription factors or regulatory elements are just starting to be studied. Here I identified tentative candidates TFBS of the newt RPE65 promoter such as c-Myc/MAX, OSR1, SIX1, ZIC2. c-Myc is an oncogene which is a critical player for cellular reprogramming and pluripotency. In addition, OSR1, ZIC2, and SIX1 are all embryonic or neural embryonic TF`s. Because this field is just beginning with genomic data collection, these tentative TFBS provided a starting point for genes that could be present in early retinal regeneration from the RPE layer.

Two promoter two reporter transgenic animals are useful tools, especially if one promoter is tissue-specific and the other is a general promoter. This study developed a fundamental npRPE65/ pCAGG`s transgene, and assayed it transgenic newts. It was important to protect this two promoter system from each promoter and genomic positional effect by using an insulator sequence (the chicken HS4). The direction of the promoter and HS4 insulator was critical for proper protection, and this should be considered when designing future transgenes in the newt. Most recent studies inserting plasmid by transfection (Shaikh et al, 2011) or generation of transgenic salamanders by *I-SceI* have not applied insulation (Khattak et al., 2013). This should be carefully examined when inserting a gene at random into the genome, or less results will be inconsistent. For example experiments generating Cre Axolotls using $CreER^{T2}$ by Whited et al 2012 reported that the $CreER^{T2}$ loxP system does not work due to leaky expression of the loxP reporter. On the other hand

Sandoval-Guzman et al., 2014 applied Cre-loxP for genetic fate mapping using the same Axolotl species. Here I recommend to use insulation to protect transgenes form positional effects.

6.2 Applications

6.2.1 Dual promoter/reporter transgenic system for tracking

One application of this system is to track transgenic cell fate of specific cells during regeneration by tissue grafting (transplants). Tissue grafting are classical experiments used in the newts, but have never been tested using two promoter reporters transgenic tissue. Unlike dyes to tag tissue, transgenic reporter will not fade out with time. In an earlier experiment using Axolotl, transgenic EGFP embryo tissue from the limb field (Sobkow et al., 2006) was transplanted to wild type embryos, and this EGFP tissue was tracked until later mature stages to examine where that tissue differentiated into muscle of the limbs. In another experiment, also using Axolotl, tissue transplants were done in regeneration limbs to examine the fate of blastema derived tissue, authors concluded that regenerating cells were able to memorize where they came from during regeneration (Kragl, et al, 2009). Transgenic tissue transplant have not been published in *Cynops pyrrhogaster*. Using transgenic eye fields one can easy grafted into the wild type embryos to examine tissue interactions, and cell fate of RPE cells, even examine if the cell source of RPE embryonic tissue is the same during retinal regeneration of adult animals..

The RPE layer is heavily pigmented making it a very difficult to collect single cells for transcriptome analysis or single qPCR. During early regeneration RPE derived cells can slowly be monitor because they contain a melanin, but gradually in later stages of retinal regeneration they become difficult to detect since melanin decreases. Therefore using these two promoter/reporter transgenic lines one can monitor RPE cells or isolate them by selecting mcherry-positive cells after the removal of the neural retina.

6.2.2 CreER^{T2} loxP system

The CreER^{T2} loxP system is currently one of the standard genetic tools for vertebrate gene manipulation. This study only examined Cre as a deletion recombination event. This event was used to activate a reporter gene only, following the removal of a floxed reporter gene. This study set the ground work for the Cre transgenic newt. In theory the other two events inversion and translocation (homologous recombination) can also work, and can be studied in the future experiments. More genomic information in the newt is needed to test Cre translocation, since the positioning of loxP sites is essential.

6.3 Limitations to Consider

Newt transgenesis by *I-SceI* under the current situation is the best method for the newt. It is important to note that this method inserts transgenes at random, and therefore has limitations. In zebra fish using *I-SceI* led to approximately 4 to 8 copies of the transgene insert into the genome, nonetheless its a huge improvement from previous transgenic methods (Thermes, 2001). Random transgene insertion as described above, lead to positional effect, transgene expression variation or off target expression. Although insulators can reduce positional effect, under the current state positional effect is unstoppable by *I-SceI*. Future transgenesis in the newt requires a single transgene insertion in a safe genomic site that will not have an effect on the transgene expression. Because the newt genome is not sequenced, several considerations can be taken to circumvent positional effect. (1) Establish F1 generations by crossing with the wild type and selecting good candidates. I am currently working on generating F1 transgenic stable lines containing two promoter two reporter transgene. (2) Reducing the plasmid DNA/egg concentration to a minimum effective concentration will improve positional effect by minimising the insertion copies, in this system.

Other methods that introduce transgenes into a host genome require a safe place to insert into the genome to avoid side effects or disrupting gene function. This requires sequencing data, most model organisms such as the chicken, frog, mouse, and fish currently have their genomes sequenced and are available for these methods. Ideally any system that inserts transgenes in a site specific manner without positional effect or altering the normal expression would be preferred. In the case of the newt, the genome is not sequenced. I would use to examples to examine this point further.

TALEN (transcription activator-like effector nuclease) are engineered restriction enzymes that connect to TAL effector DNA binding domain to cut a targeted DNA. This system cuts the genome by a double-stranded break. Donor DNA can be introduced into this site specific, by homologous recombination or non homologous end joining (Grunwald, 2013).This system is very powerful for gene editing and gene manipulation, particularly for loss of function studies, gene knockout (Grunwald, 2013). This is relatively new, and has many parameters unexplored as, nonetheless it has shown tremendous applications in the fish (Grunwald, 2013.) This system requires sequencing information to work. Without the newt genome, target sites are limited.

Tol2 transgenesis is a system, that uses the transposons from the hAT family to insert a single copy of a transgene. Transposons are repeated sequences found in plant and animal that move from one locus in the genome to another (Kawakami et al., 2002). Conserved transposon elements and sequences have been identified (Kawakami et al., 2002) (Kawakami, 2007). This single copy insertion works by a cut and paste mechanism. It has been tested in the zebrafish, Tol2 elements are not found in the zebrafish genome, therefore this system can be used. Again the newt does not have the genome sequence, and application of this system before proper sequencing data is very cloudy in the newt.

Both systems have future promise in the newt, and regenerative studies will definitely

benefit from them in the road ahead.

6.4 Ongoing Research

Gene expression during newt retinal regeneration has been observed (Chiba and Mitashov, 2007), but it is important to examine the functional role of these genes. Interestingly, proto-oncogenes have been observed in retinal regeneration of adult newt such as FGF2, FGFR-1/2, MEK1/2, ERK1/2, Hes-1, Notch-1, and Musashi-1 as well as retinal transcription factors and stem cell markers such as Pax6 and Chx10 are assumed to be involved in the retinal regeneration (Chiba et al., 2006; Nakamura and Chiba, 2007; Susaki and Chiba, 2007; Chiba and Mitashov, 2007; Kaneko and Chiba, 2009).

Pax6 the master control gene of the eye, is up-regulated in early retinal regeneration, (**Figure 2)**.To take full advantage of these established transgenic techniques, new conditional transgenes were designed. This time implementing RNAi (interference) to knockdown Pax6 expression in the RPE layer. RNAi is another genetic tool never used in the newt, which I am currently examining in the RPE layer. Pax6 is a good candidate to test, for an initial gene knockdown because it is highly conserved among vertebrates and its a stem cell marker. (**Figure 32)**, depicts this system, it conditional activates Pax6 RNAi in the RPE layer using $CreER^{T2}$. This system will allow site-specific gene knockdown during retinal regeneration. This system can be used as a template, and target gene can be knockdown by RNAi.

Although Pax6 is known for its role of eye development, it is unclear what functions it plays in retinal regeneration. For example, we don't know the down stream genes Pax6 controls during retinal regeneration. Using this transgenic conditional knock down system

we expect to find novel genes that function down stream of Pax6. With the aid of a comprehensive transcriptome analysis pipeline we can identify unique genes from Pax6 -/- and Pax6+/+ RPE derived cells by comparing genes in their activity during regeneration. It is also unknown if Pax6 plays a role in RPE transdifferentiation or if other genes are involved; here I wish to examine this in future research. This transgenic system is unique and will improve the current gene manipulation technologies available and it can be used as a template to study future newt body-part regeneration.

Overall these transgenic techniques are not limited to retinal regeneration, as I mentioned in the beginning newts can regenerate jaws, spinal cord, limbs, retina, and sections of the heart (Reyer, 1954; Oberpriller and Oberpriller, 1974; Ghosh and Ferretti, 1994; Mitashov, 1996; Brockes and Kumar, 2002). Hence researchers interested in these specific tissues can also these techniques to study specific genes in specific tissue during newt regeneration.

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TABLES

Table 1
Optimization of *I-SceI* and Single Transgene Co-injection

I-SceI (U/egg)	DNA (ng/egg)	No. cleavaged eggs	No. Survivors at swimming larvae stage	EGFP expression patterns			
				Uniform		Mosaic	ND
				strong	moderate Weak		
0	0.4	107	40	0(0%)	1(2.5%)	23(57.5%)	16(40%)
0.001	0.08	109	24	0(0%)	10(41.7%)	7(29.2%)	7(29.2%)
0.001	0.4	108	32	4(12.5%)	12(37.5%)	9(28.1%)	9(28.1%)
0.001	0.8	106	37	6(16.2%)	16(43.2%)	11(29.7%)	11(29.7%)
0.004	0.4	68	24	6(25.0%)	11(45.8%)	6(25.0%)	1(4.2%)
0.004	0.8	75	17	*5(29.4%)	10(58.8%)	2(11.8%)	0(0%)

*Every larva showed very strong green fluorescence. Injection volume: 4 nl/egg; DNA- *I-SceI* incubation time: 40 min. ND: no detection.

Table 2. Tentative Unique Vertebrate Transcription Factor Binding Sites in the Newt RPE65 Promoter Region (-560bp to 0bp) containing Core Similarity = 1, Matrix Similarity ≥ 0.85

TF Family	TF Matrix	Core Sim.	Matrix Sim.	Matrix Information (Tissue)	Newt Promoter Sequence CAPS = Core 4/5bps (+/- strand)
V$EBOX	c-MYC/MAX_.01	1	0.985	c-Myc/Max Heterodimer	aaacACGTGttg (-)
	c-MYC/MAX_.02	1	0.965	(Pluripotent)	gaaaCACGTgttgg (+)
V$OSRF	OSR.01	1	0.934	Odd-skipped related factor 1 (Embryonic Structures)	ttactGTAGcaga (-)
V$ZICF	ZIC2.02	1	0.912	Zic family member 2 (Embryonic Structures, Brain, Central Nervous System, Nervous System, Neurons)	cgtccCAGCtggtgc (-)
V$SIXF	SIX1.01	1	0.874	Sine Oculis Homeobox 1 (Brain, Central Nervous System, Ear, Embryonic Structures, Endocrine System, Eye, Kidney, Muscle, Skeletal Muscles, Nervous System Pituitary Gland, Urogenital System)	atttgagTATCaaaa (-)

$ Represent Transcription factor family format based on TRANSFAC file data

Table 3

Promoter Activity in Four Transgenic Lines Containing Two Promoter Two Reporters. Promoters (pRPE65-mCherry and pCAGG`s-EGFP) were Positioned in the Same or Opposite Direction, with or without the Chicken HS4 Insulator.

Transgene Promoter Direction --> 5` to 3`	Total Eggs Inj. n=4	Blastula Embryos					
		Survival (%)	mCherry+ EGFP + (%)	EGFP + (%)	mCherry+ EGFP- (%)	M/ND (%)	Survival Stages 55 (%)
pRPE65--> pCAGG`s--> HS4*	220	133 (60)	9 (7)	37 (28)	0	87 (65)	81 (61)
pRPE65--> pCAGG`s-->	200	103 (51)	11 (11)	18 (17)	0	74 (71)	52 (50)
<--pRPE65 pCAGG`s--> HS4*	160	75 (47)	18 (24)	9(12)	0	48 (64)	40 (53)
<--pRPE65 pCAGG`s-->	170	71 (42)	19 (26)	6 (8)	0	46 (64)	33 (46)

* Transgenes contained 3 copies of the HS4 2X core, as shown in Figure 21C
n, is the number of trials
M/ND, Mosaic or No Detection of the Internal Control pCAGG`s-EGFP
✢ Survivorship calculated from surviving blastula embryos

Table 4

Newt pRPE65 Promoter Activity in the Neural Retina, Lens, and Iris of 4 Transgenic Lines at Metamorphosis.

Promoter --> 5` to 3` Direction	Samples	RPE L (%)	ONL (%)	INL (%)	GCL (%)	Lens (%)	Iris (%)
pRPE65--> pCAGG`s--> HS4*	7	7 (100)	2(29)	0 (0)	0 (0)	0 (0)	0 (0)
pRPE65--> pCAGG`s-->	6	6 (100)	1 (17)	0 (0)	0 (0)	2 (33)	1 (17)
<--pRPE65 pCAGG`s--> HS4*	6	6 (100)	3 (50)	2 (33)	1 (17)	2 (33)	0 (0)
<--pRPE65 pCAGG`s-->	8	8(100)	3 (38)	2 (25)	2 (25)	2 (25)	1 (13)

* Transgenes contained 3 copies of the HS4 2X core, as shown in Figure 21C

Table 5

Optimization of Two DNA Construct Microinjection Conditions for Generating CreER Newts at F0 Generation.

DNA* pg/egg	Eggs Injected	Blastula Embryos				
		Survival (%)	Cyan+ / YFP+			
			ND	mCherry +	mCherry -	Survival to Stage 55 mCherry- (%)
400	229	32 (14)	29	1	2	0
240	123	20 (16)	16	1	3	0
200	283	85 (30)	74	2	9	1(1)
160	75	19 (25)	16	0	3	1 (5)
100	128	48 (37.5)	44	0	4	3 (6)
80	48	27 (56)	23	0	4	3 (11)
40	111	67 (60)	65	0	2	2(3)
20	27	27 (100)	26	0	1	1 (4)

* pg is the total mass of two transgenes. DNA ratio was kept at 1:1. Trangenes used are depicted in Figure 28A
Cyan+/YFP+ count was based on moderate or strong fluorescent expression
Injection volume was fixed 1-2 nanoliter/ egg
I-SceI enzymes concentration was fixed at 1X10

Table 6
Pharmacological Treatment of Larvae with 4-Hydroxytamoxifen (4-OHT).

4-OHT (μM)	Solvent (v/v %)	Samples	Detection of mCherry+ / Larva				Survival (%)
			3hrs	6hrs	12hrs	24hrs	
100	DMSO 1%	3	0	0	0	0	(0)
50	DMSO 1%	3	0	0	0	0	(0)
25	DMSO 1%	4	0	3/4	3/3	2/2	2 (50)
10	DMSO 1%	5	0/5	4/5	4/5	4/5	5 (100)
5	DMSO 1%	4	0/4	0/4	2/4	3/4	4 (100)
1*	DMSO 1%	3	0/3	0/3	0/3	0/3	3 (100)
0	DMSO 2%	6	0/6	0/6	0/6	0/6	6 (100)
0	DMSO 1%	9	0/9	0/9	0/9	0/9	9 (100)
0	Ethanol 2%	6	0/6	0/6	0/6	0/6	6 (100)
0	Ethanol 1%	6	0/6	0/6	0/6	0/6	6 (100)

Swimming larvae (from stages 50), were submerged in 0.1X Holt with the above solvent and 4-OHT concentration
✢ Animals survived 24hr treatment with side-effects (abnormal tail bending, bloating, or stopped eating)
* Concentrations < 1μM, required longer than 24hrs, approximately a 5-days to detect mCherry

FIGURES

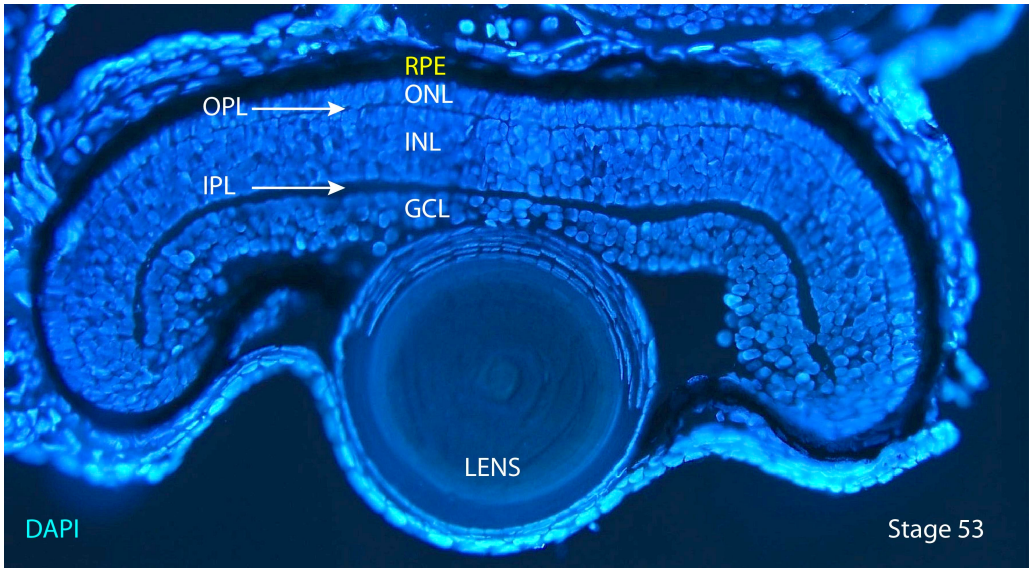

Figure 1. Retinal layers of the Newt Larva at Stage 53. The target tissue for this study is the RPE layer (retinal pigment epithelium), the primary cell source for retinal regeneration in the newt, shown in yellow. Note RPE layer is highly pigmented with melanin. ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer.

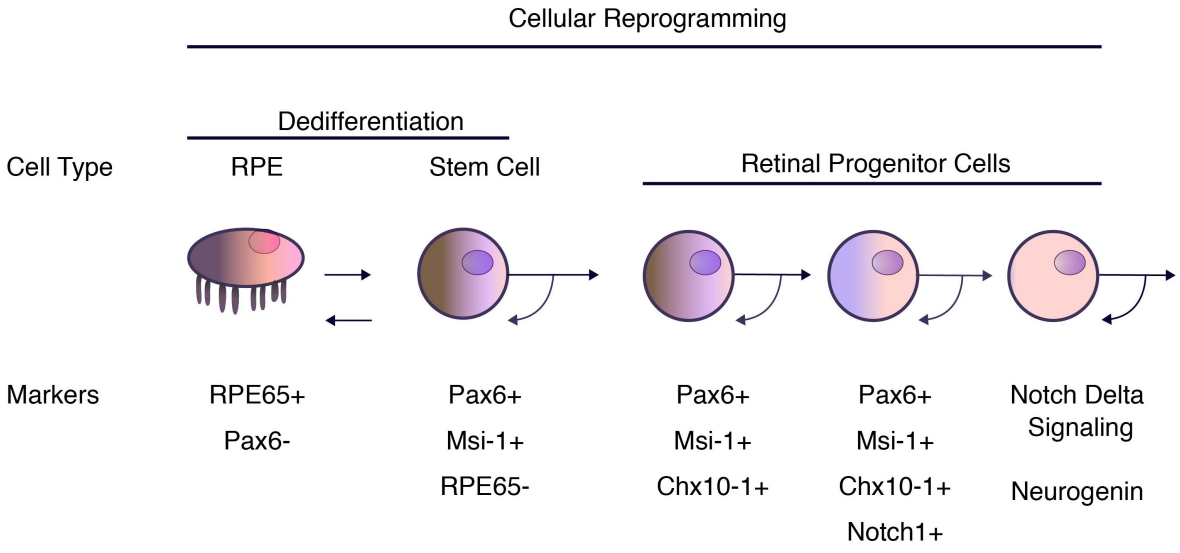

Figure 2. Current Working Hypothesis of Early Retinal Regeneration in the Newt as suggested by (Mitashov and Chiba, 2007).

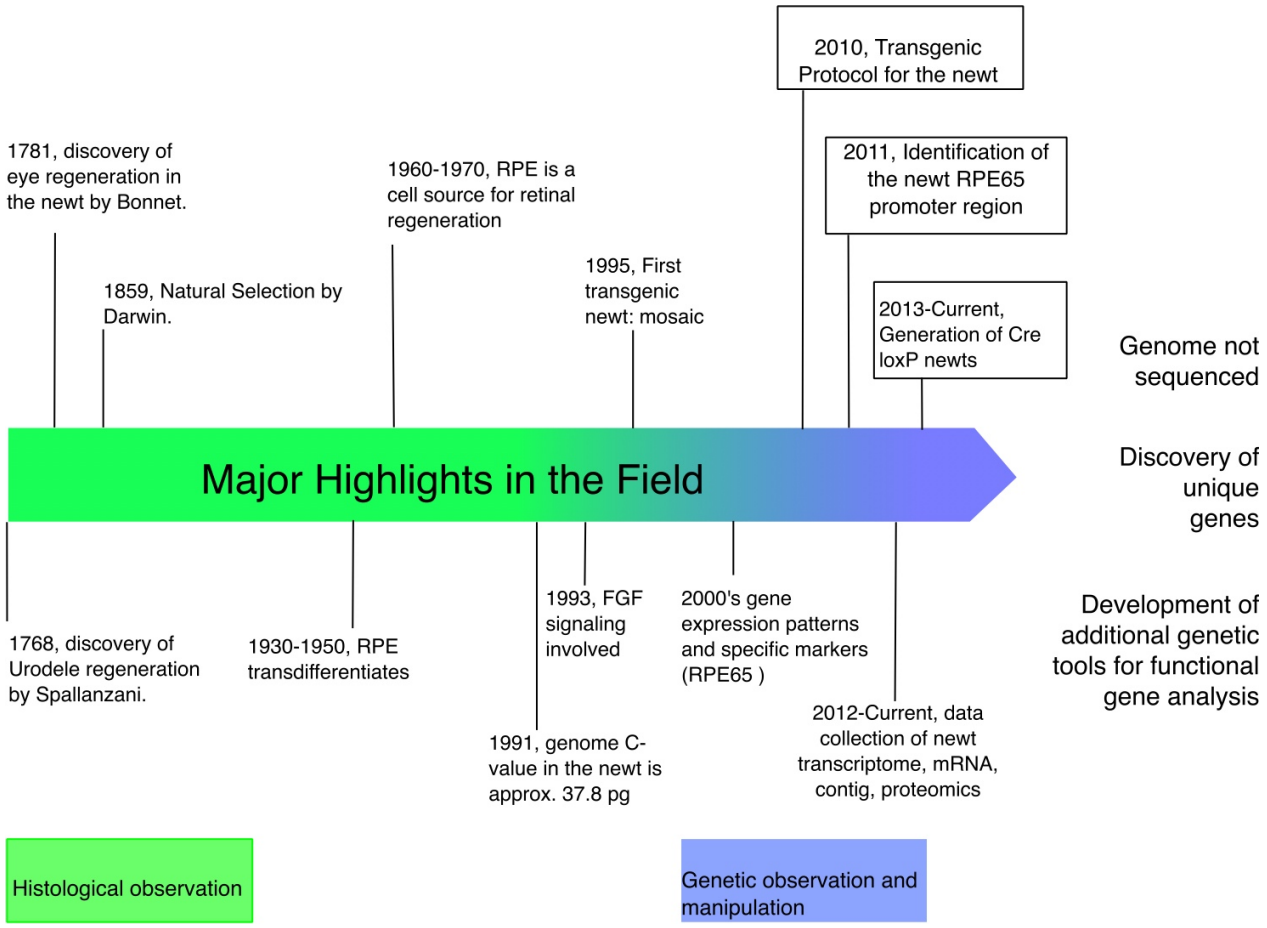

Figure 3. Current Direction and Major Highlights in the Field of Newt Retinal Regeneration. Boxes indicate the contribution of this study to the field. The birth of this field stretches back before the time of Darwin.

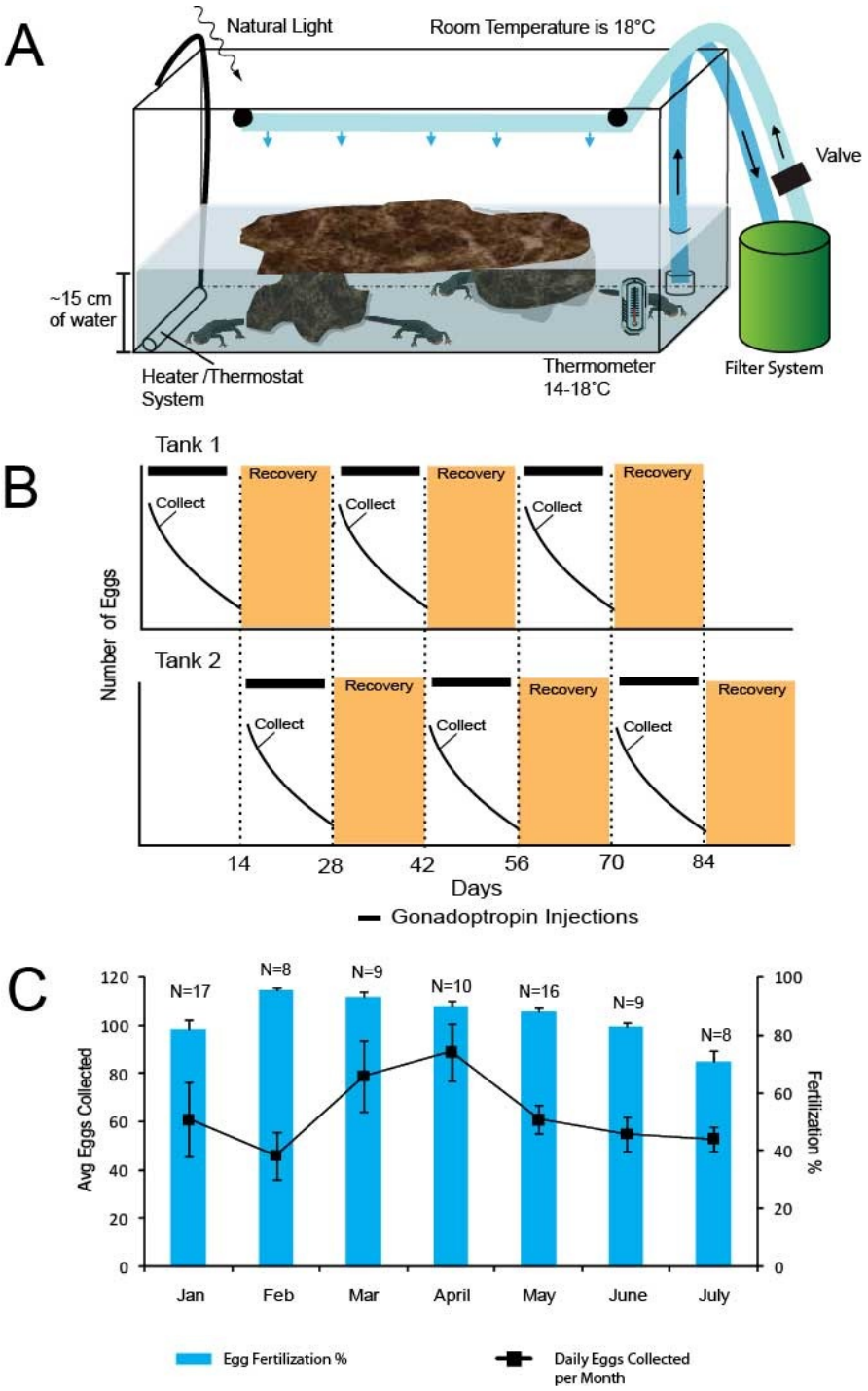

Figure 4. Newt Fertilized Egg Collection Strategy. (A) Two Mating Tank setup (only one shown here), used to obtain fertilized eggs of *Cynops pyrrhogaster*. (B) Alternating recovering period and collection period, using the two tank system during 14 day period intervals. (C) Fertilization percentage and average eggs collected per day per month during Jan to July using two tank system.

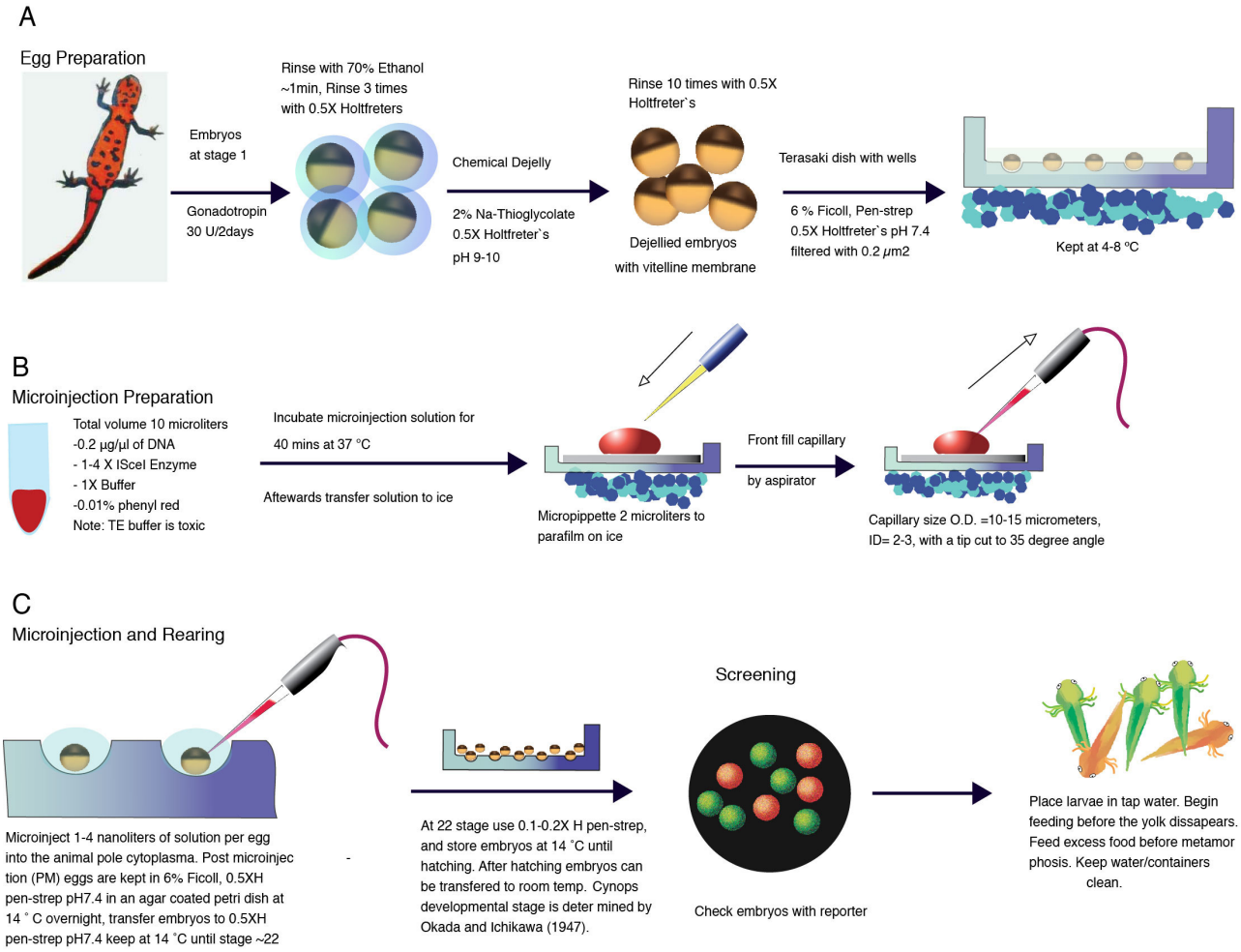

Figure 5. Follow Chart of Methods for Newt Trasngenesis. (A) Egg preparation. (B) Microinjection mix and needle preparation. DNA concentration varies depending on transgene used, working concentration should be tested empirically (C) Microinjection and rearing (culture conditions) .

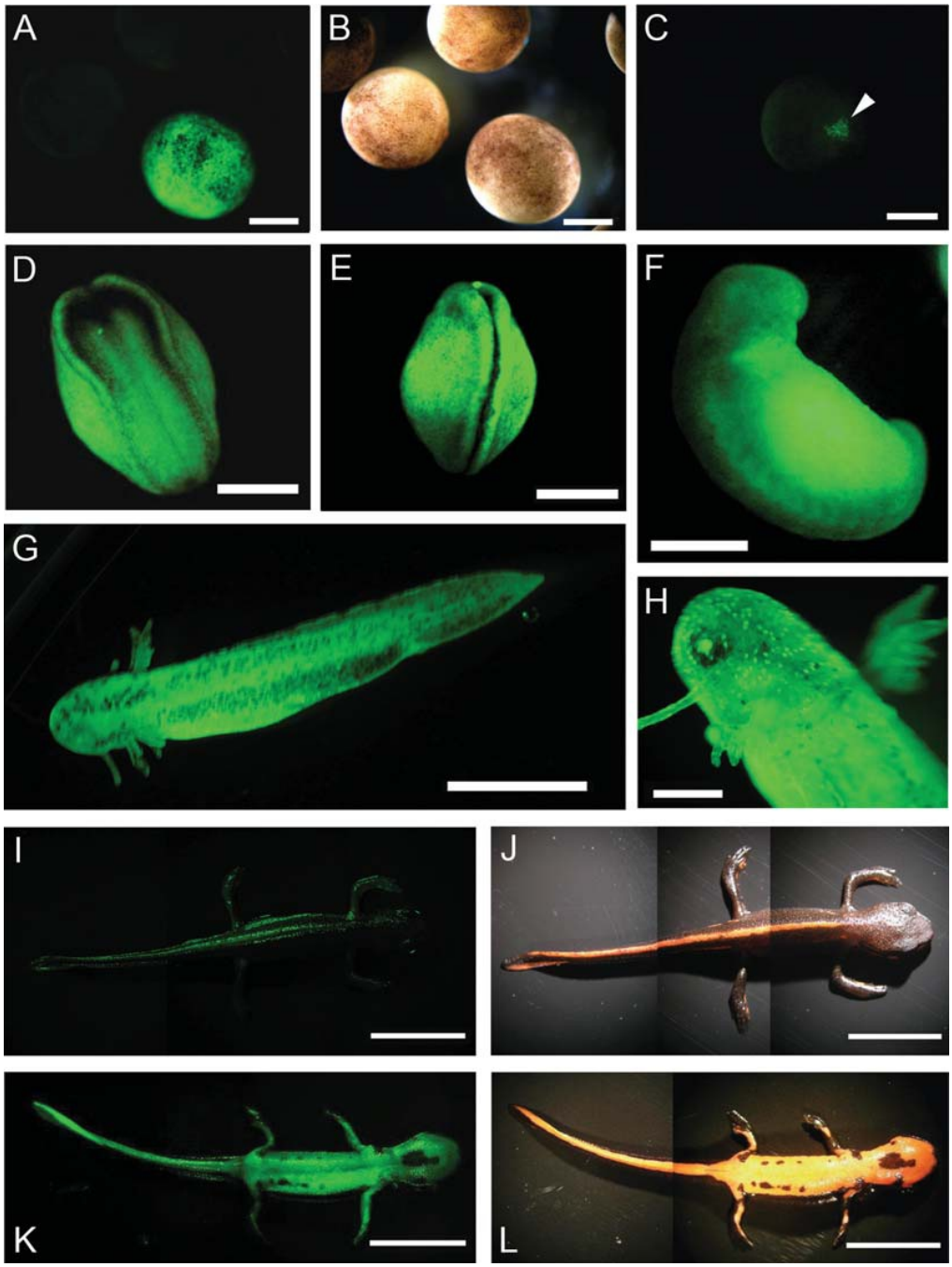

Figure 6. Expression of Transgenic Newt from Early Development to Metamorphosis. Transgenic newts generated by the *I-SceI* method with pCAGGs-EGFP(Sce) construct. A, D-G: Embryos showing an intense green fluorescence evenly at blastula (A), neurula (D, neural plate stage; E, neural tube stage), tail-bud (F), and swimming larvae stage (G). B: Bright-light image of the same field in A. C: A blastula embryo showing a mosaic expression pattern (arrowhead). H: A magnified view of the head region of the larva in G. I-L: A juvenile newt after metamorphosis (anesthetized). Dorsal (I, J) and ventral (K, L) views are shown. I, K: Fluorescence image; J, L: Bright-light image. Scale bars = 1mm (A-F), 3 mm (G), 1.2 mm (H), 1 cm (I-L).

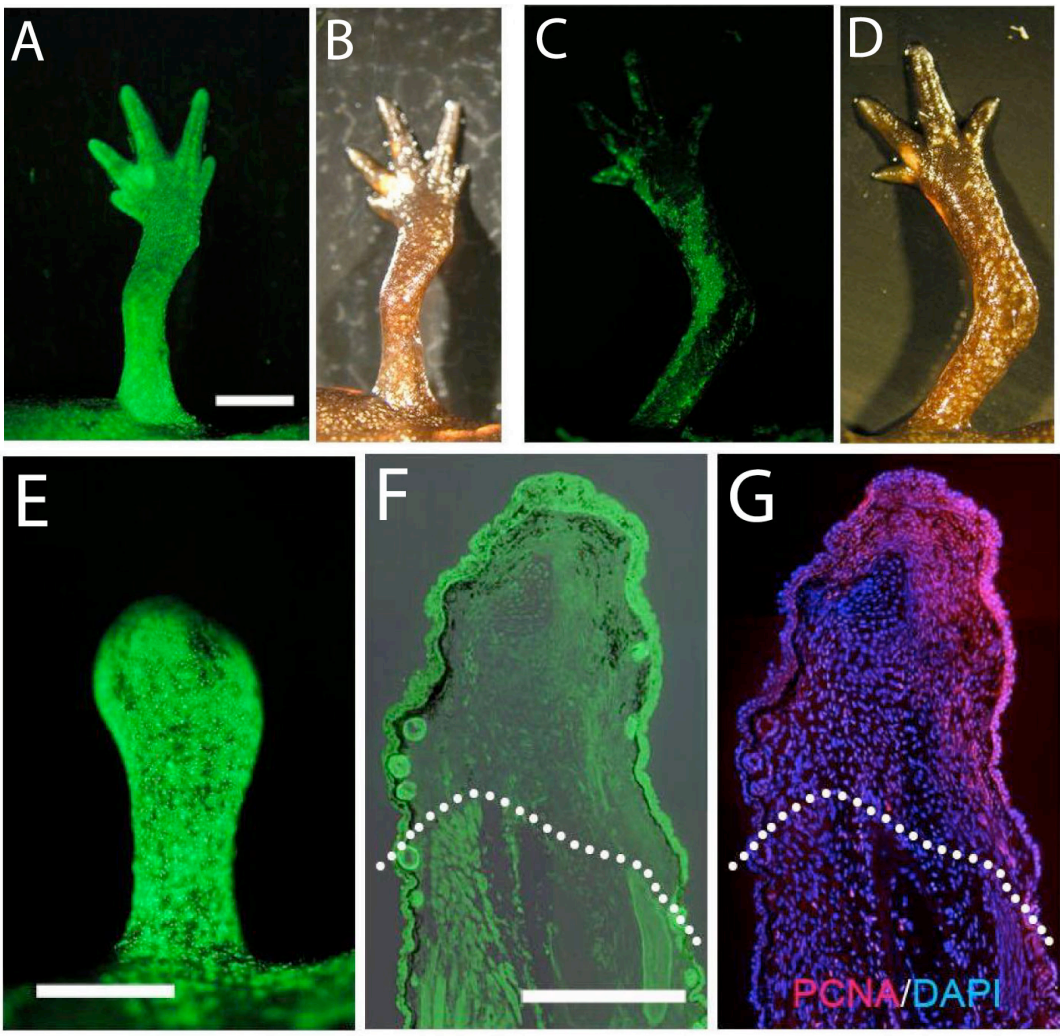

Figure 7. Expression of transgenic newt during regeneration.
A-D Limbs of metamorphosed juveniles (4-5 months) classified as 'Uniform' and 'Mosaic' at swimming larvae stage (A, B, fluorescence and bright light images of a hind limb of the 'Uniform' juvenile; C, D, those of a forelimb of the 'Mosaic' juvenile). (E) A regenerating limb (34 days after amputation) of a metamorphosed juvenile showing 'Uniform' expression pattern. (F) A dorsoventral section of the regenerating limb. The right-hand side of the panel is the ventral side. The dotted line shows the site of amputation. (G) PCNA-immunohistochemistry with the same section, visualizing many proliferating cells (red) in the regenerating tip of the limb. DAPI counterstaining of nuclei is shown in blue. Scale bars in A- E : 2 mm; F, G: 200 μm.

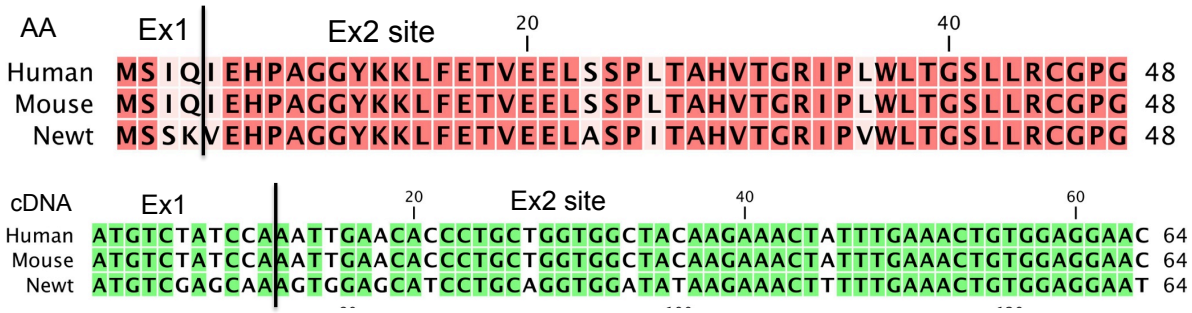

**Figure 8. RPE65 Amino acid and cDNA Alignment of the Human, Mouse, and Newt (showing only the 5` end).** The pink boxes represent conserved amino acids among the human, mouse, and newt on the 5` end. The green boxes represent conserved cDNA among the human, mouse, and newt on the 5` end. The vertical bar indicates the gap between exon1 and exon2. All gene specific reverse primers were designed to the left of the vertical bar to avoid intron crossing.

Primary PCR (AP1/GSRP1)

Cycles	Temp.	Time
7	94°C	25 Sec
	72°C	3 min
32	94°C	25 Sec
	68°C	3 min

Secondary PCR (AP2/GSRP1)

Cycle	Temp.	Time
5	94°C	25 Sec
	72°C	3 min
25	94°C	25 Sec
	68°C	3 min

Figure 9. Primary and Secondary (Nested) PCR Conditions for Genome Walking used for Newt RPE65 promoter Identification. Two step, touchdown PCR was necessary for each PCR run.

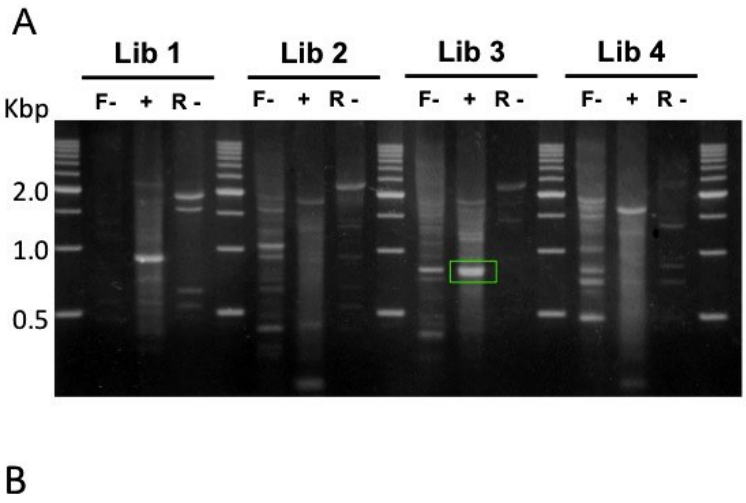

Figure 10. Identification of the Newt RPE65 Promoter Regions using Genome Walking (-560bp upstream). (A) Secondary (nested) PCR products using 4 genome walker libraries, greens box shows positive clone, which contained a matching 5` UTR region of the known newt RPE65 mRNA. Note several positive bands are present in Libraries 1-4, but after sequencing those bands, they did not contain RPE65 5` untranslated region, only the positive clone in Lib 3, marked in green contained the expected RPE65 5`UTR. (B) The newt RPE65 upstream -560bp promoter region. F-: Only forward primer was used. R-: Only reverse primer was used. +: Both Forward and Reverse Primers were used. Yellow Box: cDNA, Cyan Box: 5`UTR.

Figure 11. Clustal W Omega Alignment of RPE65 Promoter Region (-560 to 0 bp) Among Eight Species . Note reference -(minus) numbers were added by the alignment software during the addition of gap, it does not represent the promoter -560bp distance. Defaults alignment conditions were used.

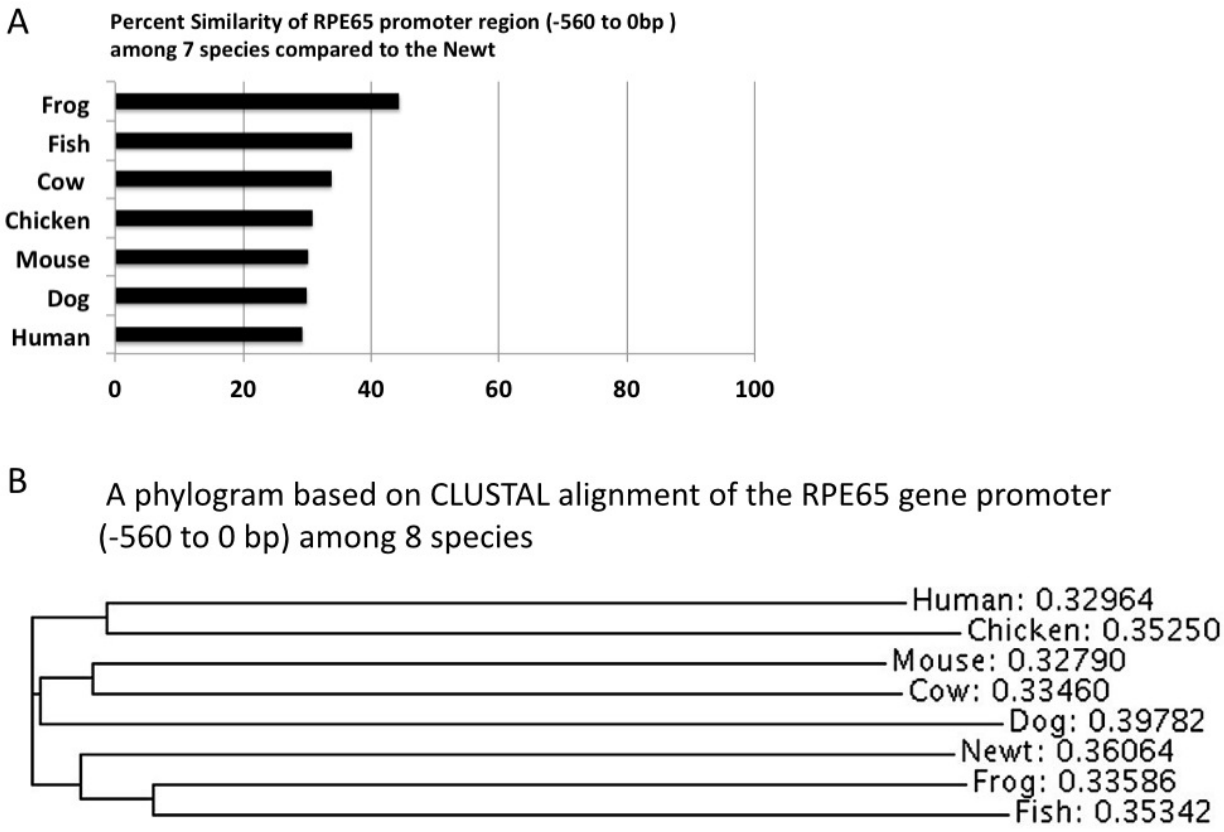

Figure 12. Comparison of the Newt RPE65 Promoter(-560 to 0 bp) Among Seven Species. (A) Percent similarity of RPE65 promoter region (-560bp to 0bp) among 7 species compared to the newt. (B) A phylogram based on clustal alignment (Figure 11) of the RPE65 promoter region (-560bp to 0bp) among 8 species. The distance values show the number of substitutions as a proportion of the length of the alignment (excluding gaps).

Figure 13. RPE65 Promoter T-Coffee Alignment (-560bp to 0bp) Indicating the Distribution of Tentative Transcription Factor Binding Sites Among 8 species . Red areas indicate high tentative transcription factor binding sites, blue-green indicated poor areas containing transcription factor binding sites.

A

RPE65 Promoter	Upstream Location	Conserved Box
Newt	-467bp	AGG-GGGATTGGCCCGCG
Consensus		**AGG GGGA TGGCC GCG**
Human	-256bp	AGGTGGGAGTGGCCAGCG
Newt	-81bp	GGATTTAGA
Consensus		**GGATTTAGA**
Human	-37bp	GGATTTAGA
Newt	-356bp	TAAATTGAATTA
Consensus		**TAAAT GAATTA**
Mouse	-339bp	TAAAT-GAATTA
Newt	-323bp	TTATGTAAA
Consensus		**TTATGTAAA**
Mouse	-160bp	TTATGTAAA

B

Figure 14. Transcription Factor Binding Sites of the Newt RPE65 Promoter have Translocated more Upstream the 5` end Compared to the Mouse and Human . (A) Identification of conserved boxes in the newt RPE65 promoter using Promoter Wise. **(B)** Black boxes represent conserved TFBS in all 3 species. Red boxes represent mammalian TFBS identified by DNA foot printing (Nicoletti et al., 1998). The blue boxes represent similar mammalian TFBS (sequence) found in the newt promoter.

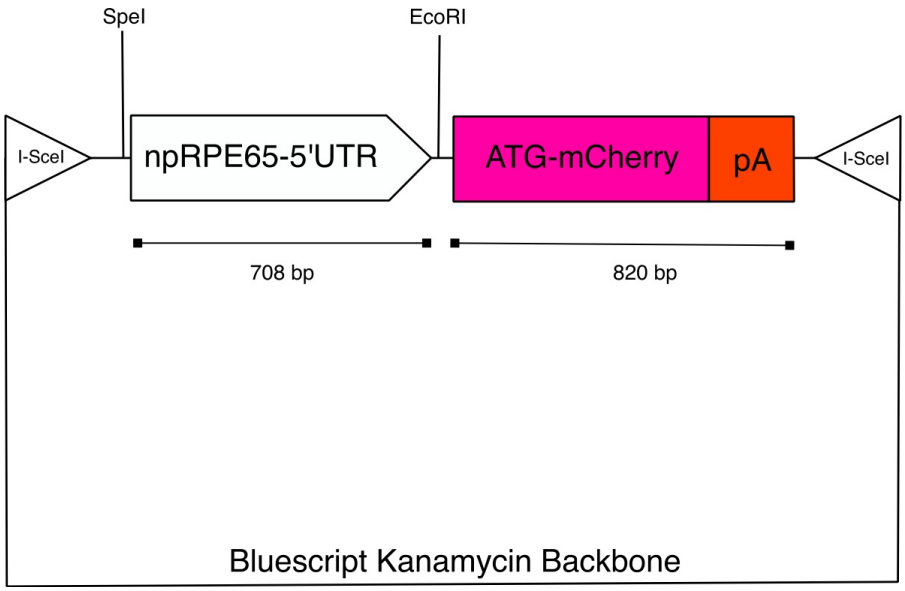

Figure 15. Newt RPE65 Promoter Driving mCherry Transgene Construct. The newt RPE65 promoter region and 5`UTR were inserted into an mCherry reporter construct. Promoter and reporter were floxed by *I-SceI* sites.

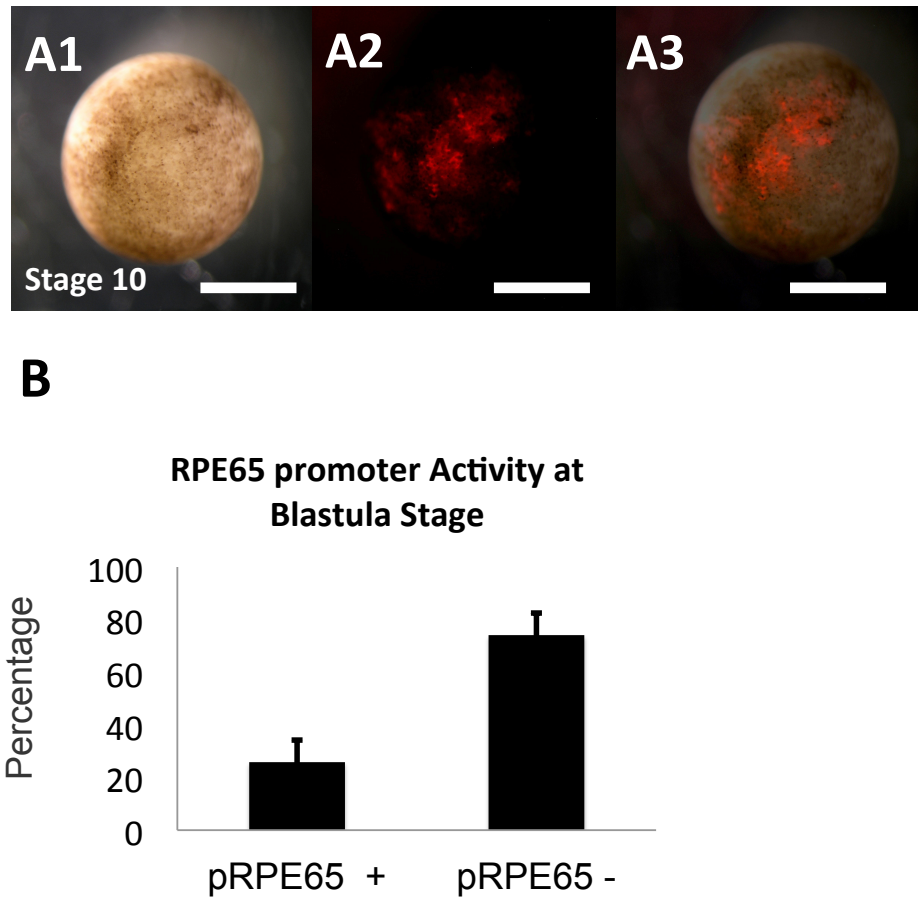

Figure 16. Newt RPE65 Promoter Activity in F0 Transgenic Blastula Embryos. The newt RPE65 promoter driving mCherry (A1) Light image. (A2) mCherry. (A3) Merge of A1 and A2. (B) Percentage of blastula embryos with positive newt RPE65 promoter activity. pRPE65 (-) represented embryos with no detection of mCherry. Scale bar 1mm.

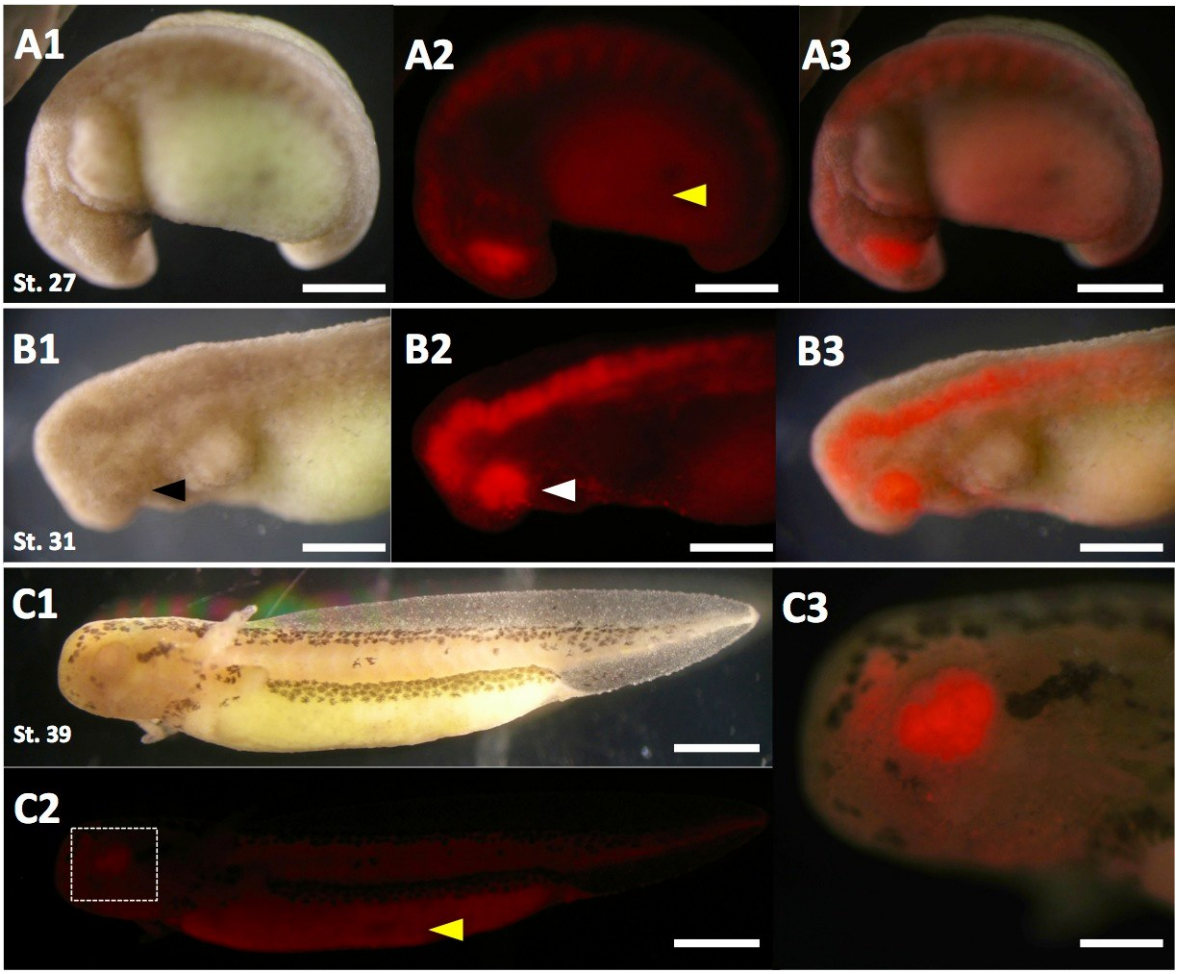

Figure 17. Newt RPE65 Promoter Activity During Development of F0 Transgenic Embryos. Transgenic embryos with expression pattern in Figure 16, were monitored. The newt RPE65 promoter driving mCherry. (A1) Light image stage 27. (A2) mCherry fluorescences of A1. (A3) Merge of A1 and A2. (B1) Light image stage 31. (B2) mCherry fluorescences of B1. (B3) Merge of B1 and B2. (C1) Light image stage 39. (C2) mCherry fluorescences of C1. (C3) Merge of C1 and C2 show a headshot. White and black arrows pointing to the developing eye without melanin pigmentation. Yellow arrows pointing at the embryo yolk, a source of auto-fluorescence. Scale bar: A-C 1mm.

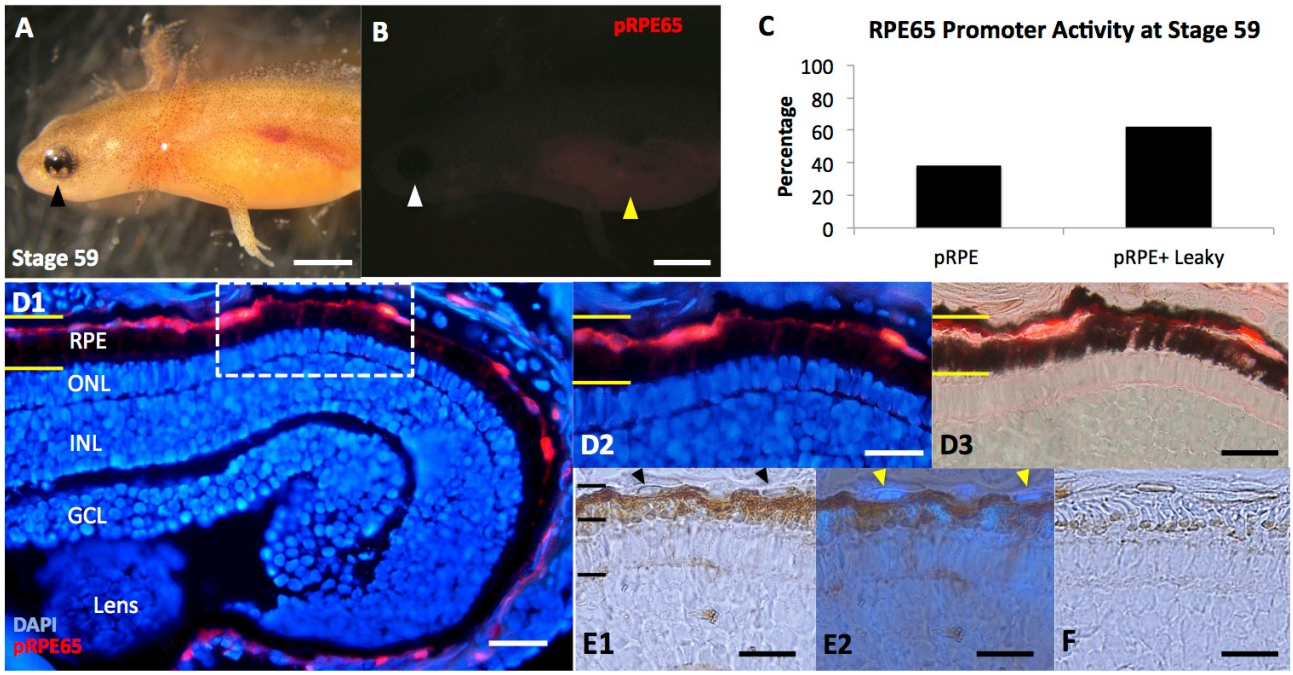

Figure 18. Newt RPE65 Promoter Activity at Stage 59 of F0 Transgenic Larvae. (A) Anterior body shot, light image at stage 59. (B) mCherry fluorescences of A. (C) Percentage of transgenic larvae expressing positive RPE65 promoter activity as shown in D1. pRPE + leaky expression are shown in Figure 19. (D1) Newt pRPE65 promoter activity driving mCherry in the RPE layer, photo merged with DAPI. Yellow double bars indicate RPE layer margins. (D2) is a magnification of D1. (D3) is a magnification of D1, showing a light image merged with mCherry. Double yellow bars indicate RPE layer margins. (E1) Immunohistochemistry with DsRed (mCherry) polyclonal primary antibody, with DAB immunoreactivity staining shown in brown. Samples E1, E2 and F were bleached to remove pigmentation in the RPE. Black arrows pointing to RPE cell nuclei. (E2) Showing E1 merged with DAPI, yellow arrows pointing at RPE cell nuclei. (F) Negative control without DsRed (mCherry) primary antibody. Scale bar: A and B 0.5cm, D1 400µm, D2 -F 100µm

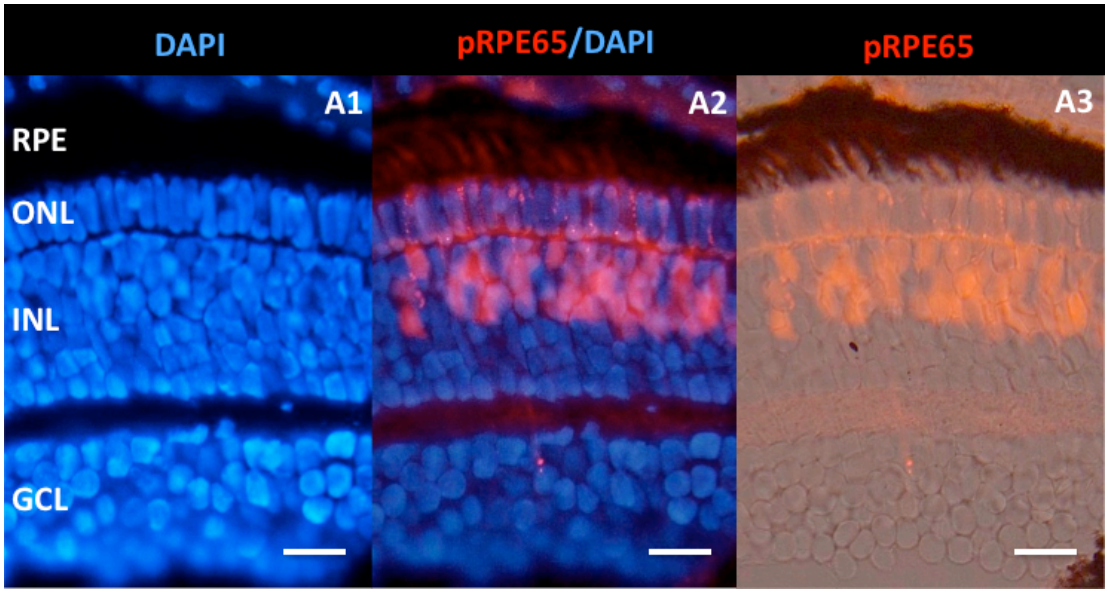

Figure 19. Newt RPE65 Promoter Leaky Expression (Off Target) at Stage 59 of F0 Transgenic Larvae. In some cases transgenic larvae expressed mCherry in the Outer nuclear layer and Inner nuclear layer of the retina. (A1) DAPI staining. (A2) Merge between pRPE65-mcherry and DAPI. (A3) Light image merged with mCherry. Scale bar: 100µm.

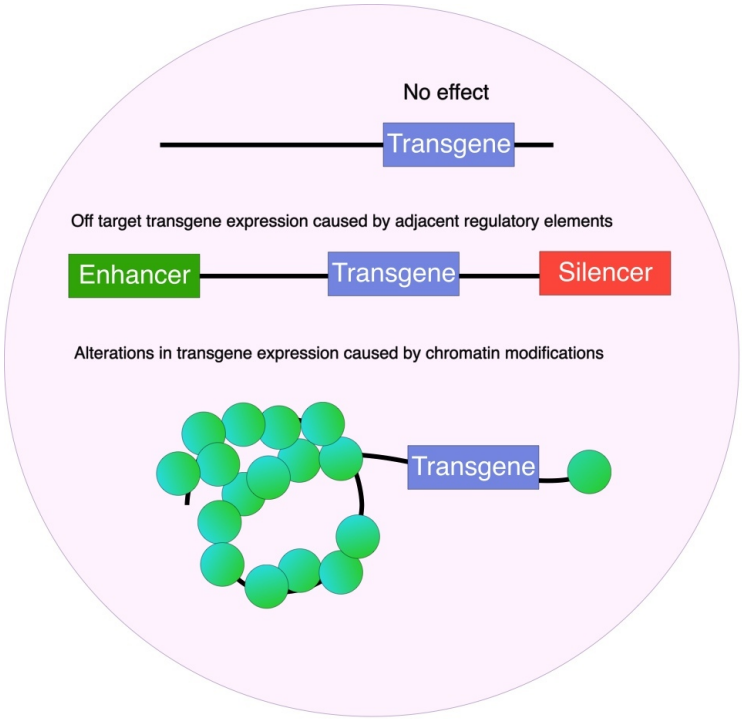

Figure 20. Illustration of Positional Effect. The Location of an Inserted Transgene Plays a Major Role in Transgene Expression. Neighbouring regulatory elements next to an inserted transgene, will lead to variations of transgene expression. Chromatin changes can also affect transgene expression.

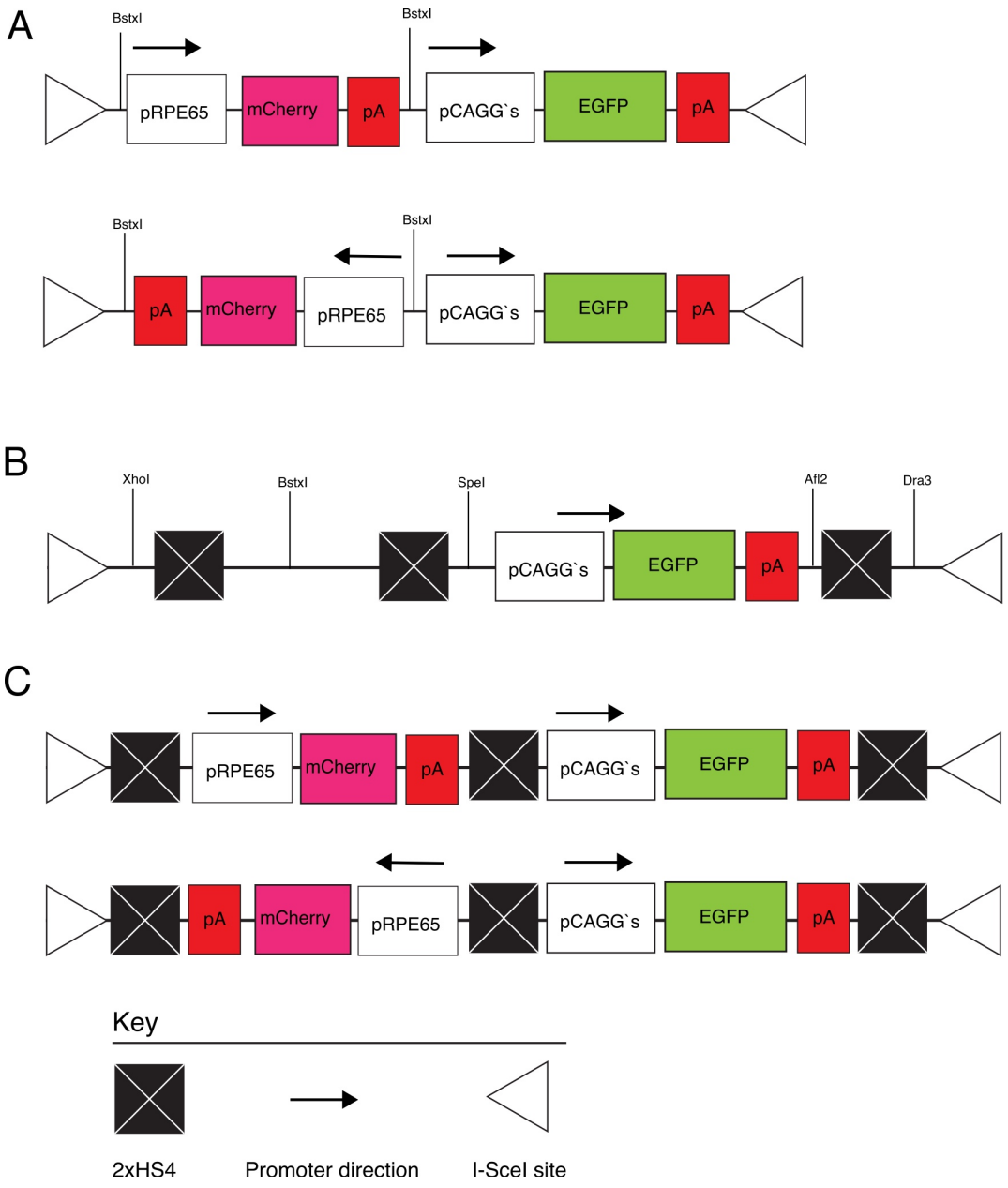

Figure 21. Two Promoter/ Two Reporter Transgene Constructs with or without HS4 Insulators. (A) The newt RPE65 promoter and mCherry reporter were inserted into a pCAGG`s-EGFP cassette vector. RPE65 promoter was placed in sense or antisense. (B) Three copies of HS4 (2X) core were inserted into the *XhoI/BstxI, BstxI/SpeI and Afl2/Dra3* restriction enzymes sites. (C) The newt RPE65 promoter driving mCherry was inserted in the construct B at the *BstxI* site, generating two promoter/two reporter transgene construct, RPE65 promoter was positioned in sense or antisense.

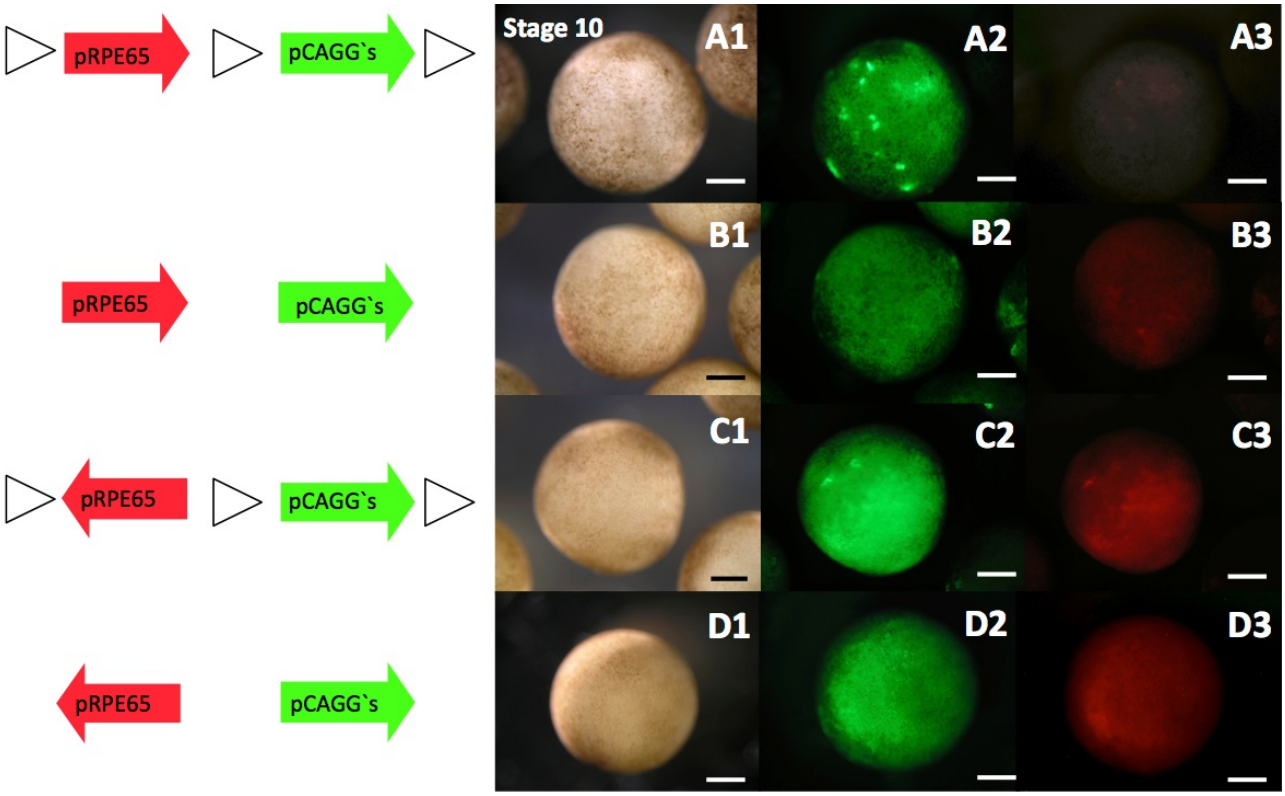

Figure 22. The Chicken HS4 Insulator Protects the Newt RPE65 Promoter from Leaky Expression During Early Development. Two promoter/two reporter F0 transgenic lines were examined during blastula stage. (A1,B1,C1,D1) Light image of Blastula embryos. (A2, B2,C2,D2) EGFP expression used as an internal control. (A3,B3,C3,D3) mCherry driven by the RPE65 promoter. Arrows on the left indicate promoter direction. Triangles represent HS4(2X) insulator. Red arrows indicate RPE65 driving mcherry. Green arrows indicate pCAGG`S driving EGFP.

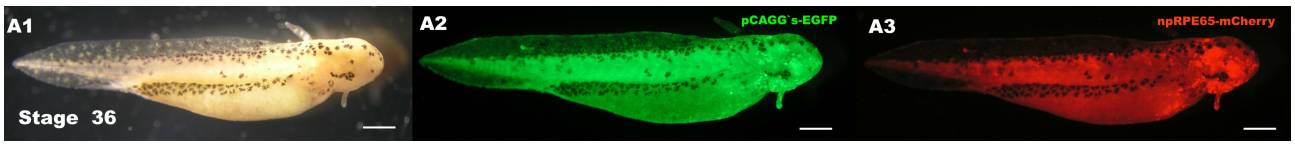

Figure 23. Example of Severe Off Target (Leaky) Expression Pattern without Insulation, in a Two Promoter Two Reporter Transgenic Larva. Two promoter/two reporter F0 transgenic larva, containing promoters in the same direction, without the chicken HS4 insulator. Larva is at stage 36. (A1) Light image. (A2) pCAGG`s-driving EGFP expression. (A3) The newt RPE65 promoter driving mCherry expression. Scale bar 1mm

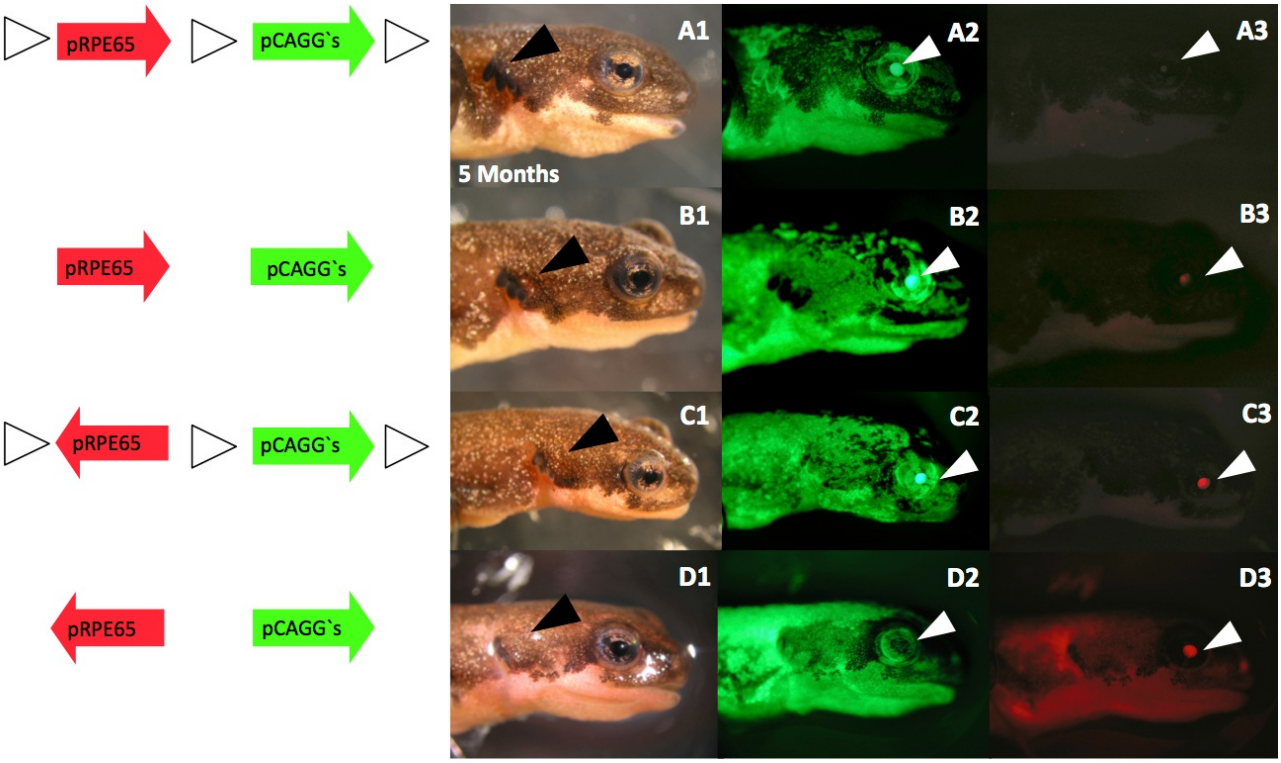

Figure 24. The Chicken HS4 Insulator Protects the Newt RPE65 Promoter from Leaky Expression During Metamorphosis. Two promoter/two reporter F0 transgenic lines were examined during Metamorphosis. (A1,B1,C1,D1) Light image of metamorphosing newts. (A2, B2,C2,D2) EGFP expression used as an internal control. (A3,B3,C3,D3) mCherry driven by the RPE65 promoter. Arrows on the left indicate promoter direction. Triangles represent HS4(2X) insulator. Red arrows indicate RPE65 driving mcherry. Green arrows indicate pCAGG`S driving EGFP. Black arrows on panels (A1,B1,C1, D1) indicating retracting gills. White arrows in panels (A2-D2) and (A3-D3) expressing pattern in the eye.

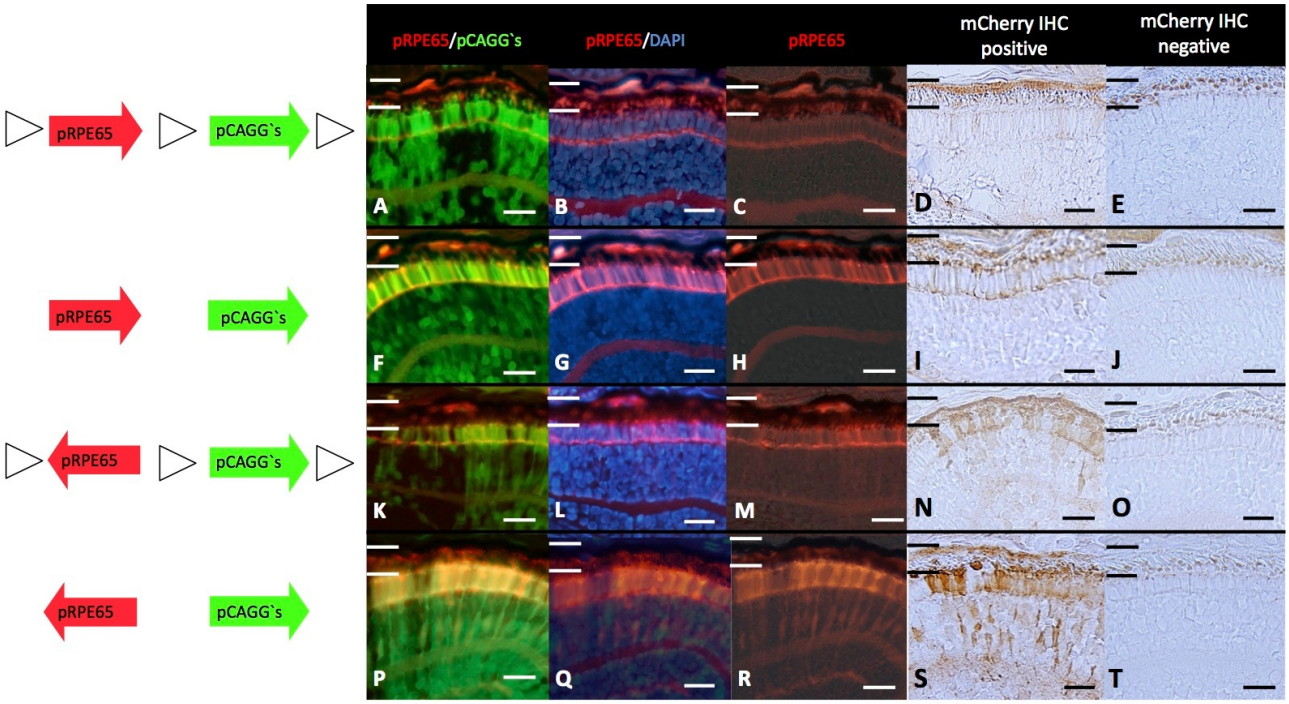

Figure 25. The Chicken HS4 Insulator Protects the Newt RPE65 Promoter from Leaky Expression in the Retina of Metamorphosed Newts. Retinal expression pattern of two promoter/two reporter F0 transgenic lines. (A, F, K, P) mCherry merged with EGFP. (B, G, L, Q) mCherry merged with DAPI. (C, H, M, R) mCherry merged with light image. Arrows on the left indicate promoter direction. (D, I, N, S) Immunohistochemistry sections treated with DsRed polyclonal (mCherry) primary antibody, immunoreactivity with DAB staining. (E, J, O, T) Negative control sections without primary antibody DsRed. Double white and black bars indicate the RPE layer margins. Arrows on the left indicate promoter direction. Red arrows indicate RPE65 driving mcherry. Green arrows indicate pCAGG`S driving EGFP. Triangles represent HS4(2X) insulator.

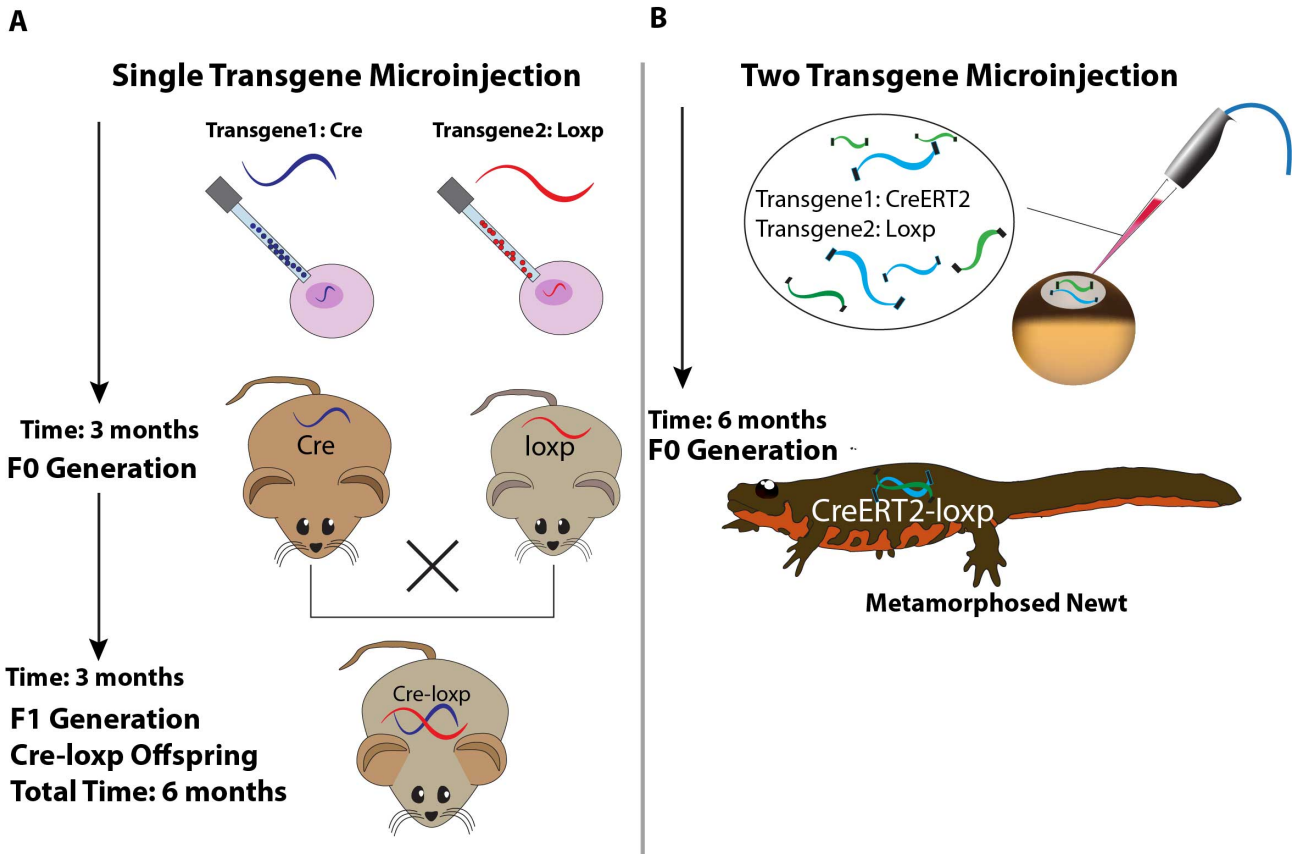

Figure 26. Comparison of Conventional Methods used to Generate Cre loxP Animals Compared to this Studies Suggested Method to Generate Transgenic CreERT2 loxP newts. . (A) An example of mouse techniques to generate Cre loxP F1 line, estimated time 6 months, to adulthood. Average generation time of the mouse is 3 months, to adulthood. Standard techniques microinject Cre and loxP separately to generate to transgenic lines. (B) Two transgene microinjection technique for the newt, transgenes are simultaneously introduced. CreERT2 loxP are generated at the F0 generation, time taken approximately 6 months to metamorphose.

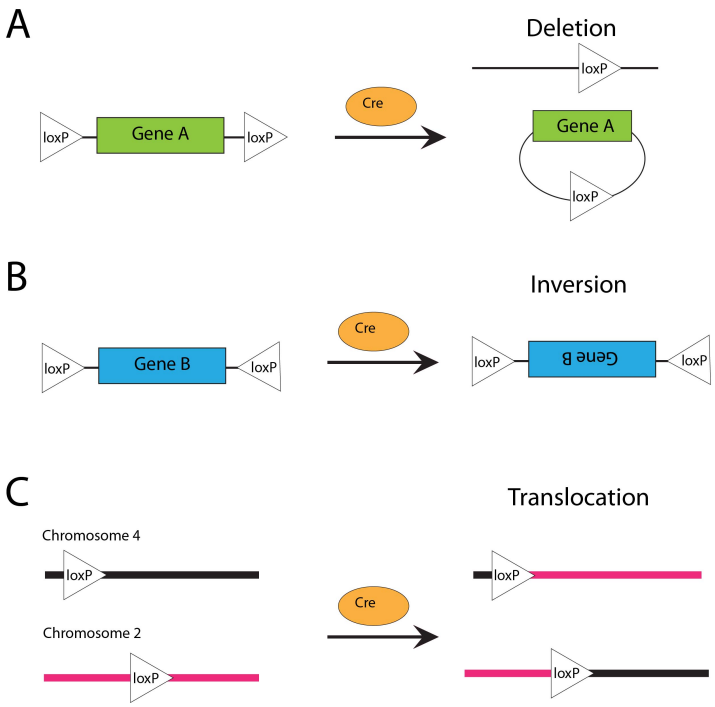

Figure 27. Cre Recombinase Events by Altering loxP Orientation. (A) Gene floxed by loxP in the same direction, leads to deletion. (B) Gene floxed by loxP sites direction towards each other, leads to an inversion event. (C) loxP sites positioned on separate chromosomes, leads to loci translocation (homologous recombination). This study only examined the deletion event, by floxing a transgene.

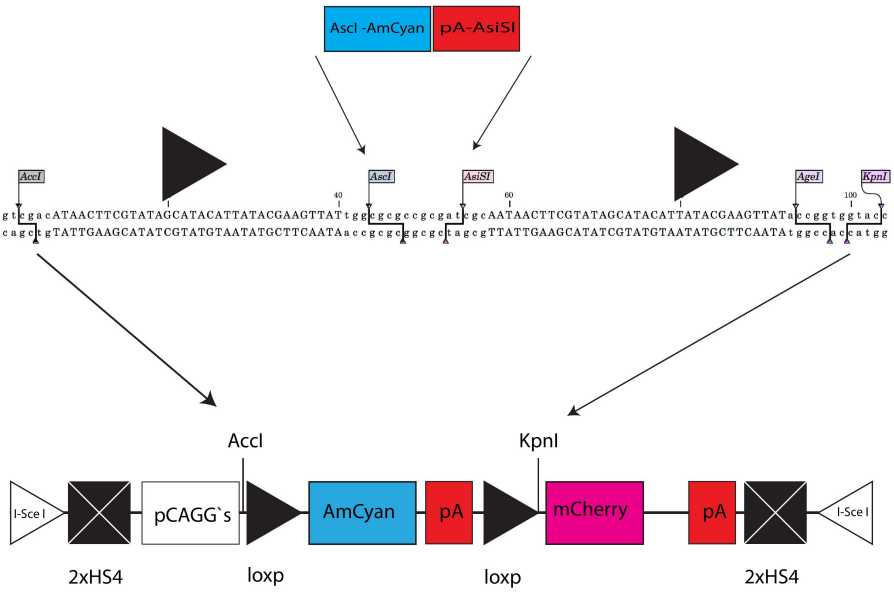

Figure 28. Design of loxP Sites, for CreER^{T2} Reporter Deletion System. Two loxP sites were inserted into a pCAGG`s-mCherry vector containing HS4 insulators. LoxP sites were introduced at the *AccI/KpnI* site. AscI-AmCyan-pA-AsiSI was introduced into *AscI/AsiSI* positioned between the loxP sites.

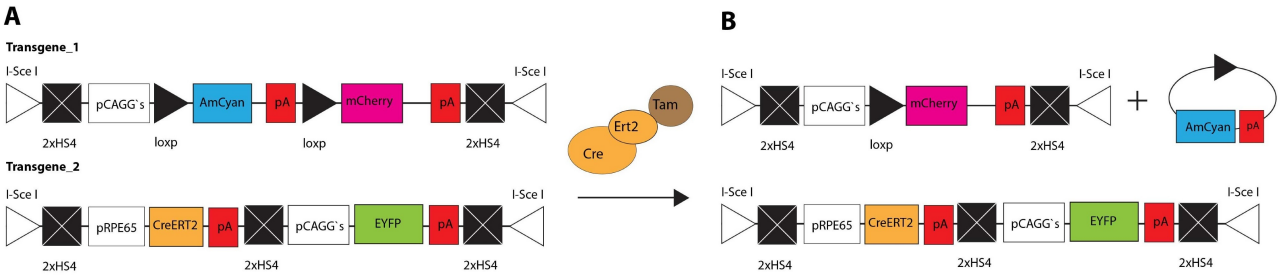

Figure 29. Transgene Design for CreER^{T2} loxP. Two transgenes (1 and 2) were used for this system. (A) CreER^{T2} loxP system is inactive. (B) System is activated by OH-Tamoxifen. Upon activation, CreERT2 binds to Tamoxifen and enters the cell nucleus. Inside the cell nucleus CreERT2 excises Amcyan reporter along with 3` loxP site. This experiment only examined deletion of a floxed reporter AmCyan

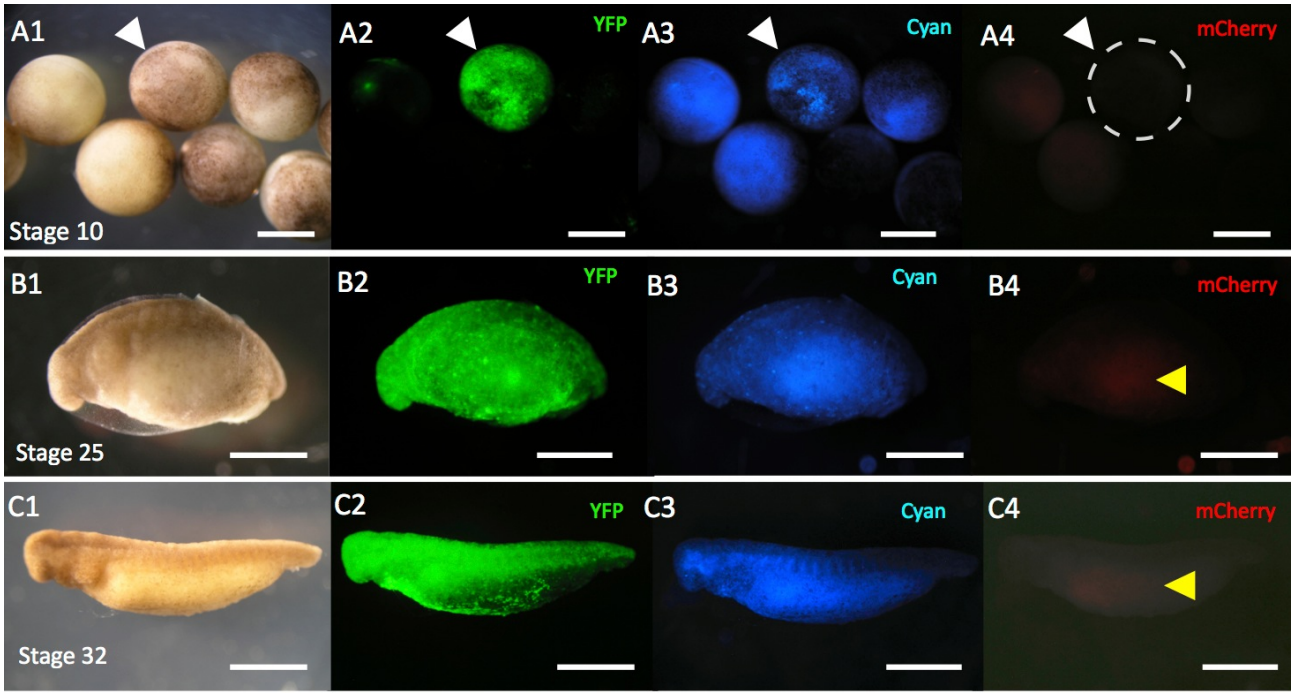

Figure 30. Generation of CreERT2 lopxP Newts by Two Construct Microinjection. Transgenes from Figure 29A were coinjected with *I-SceI* into the one cell stage of the newt.
(A1) Light image of blastula embryo at stage 10. (A2) YFP fluorescence of a positive blastula embryo. (A3) Cyan fluorescence of a positive blastula embryo. (A4) Negative mCherry fluorescence of the same embryo A1-3. White arrows indicating a blastula embryo containing both transgenes with mcherry expression. Note not all YFP/Cyan blastula express both genes. (B1) Light image embryo at stage 25. (B2) YFP fluorescence of a positive tailbud embryo. (B3) Cyan fluorescence of a positive tailbud embryo. (B4) Negative mCherry fluorescence of the same embryo B1-3. (C1) Light image embryo at stage 32. (C2) YFP fluorescence of a positive embryo. (C3) Cyan fluorescence of a positive embryo. (A4) Negative mCherry fluorescence of the same embryo C1-3. Yellow arrow indicate auto-fluorescences from the yolk.

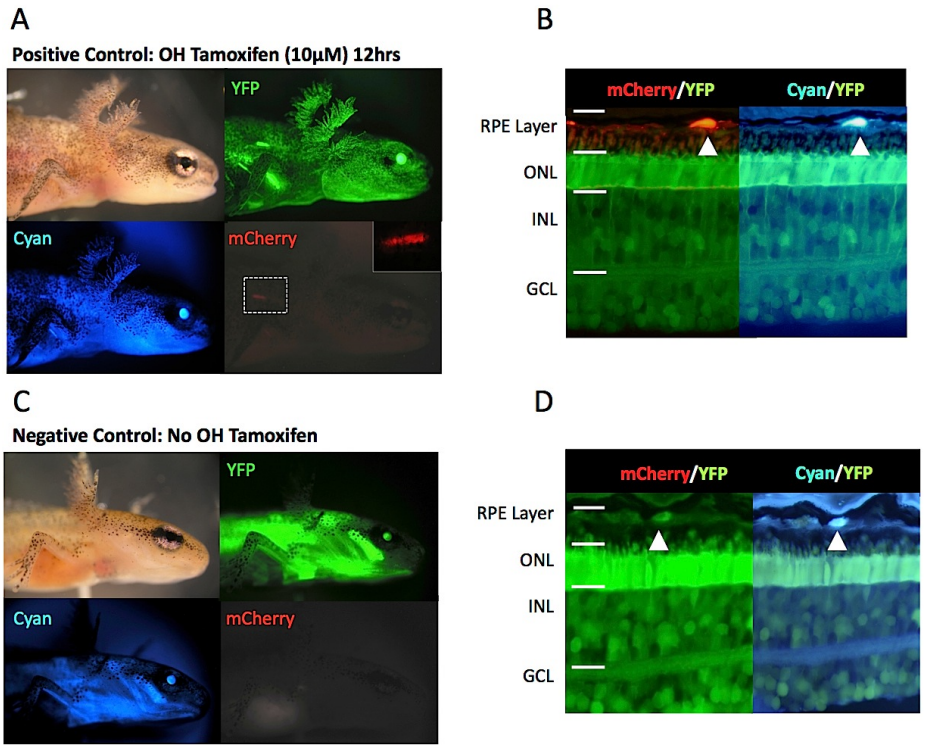

Figure 31. Activation of CreERT2 loxP Recombination in the RPE layer of F0 Transgenic Newts. (A) Newt larva treated with 10µM of OH-Tamoxifen, examined at 12hrs post treatment. The dotted box indicating some of target tissue expressing mcherry with Tamoxifen. (B) Retinal section of the sameness animal in A. RPE layer is expressing mcherry. The Outer nuclear, inner nuclear and ganglion cell layer are not expressing mcherry. Sample was immediately fixed following OH-Tamoxifen treatment, excised Cyan protein can be detected up to 36hrs until degradation.(C) Negative control larva not treated with OH-Tamoxifen. (D) Retinal section of larva C, retinal layers and RPE layer did not express mcherry.

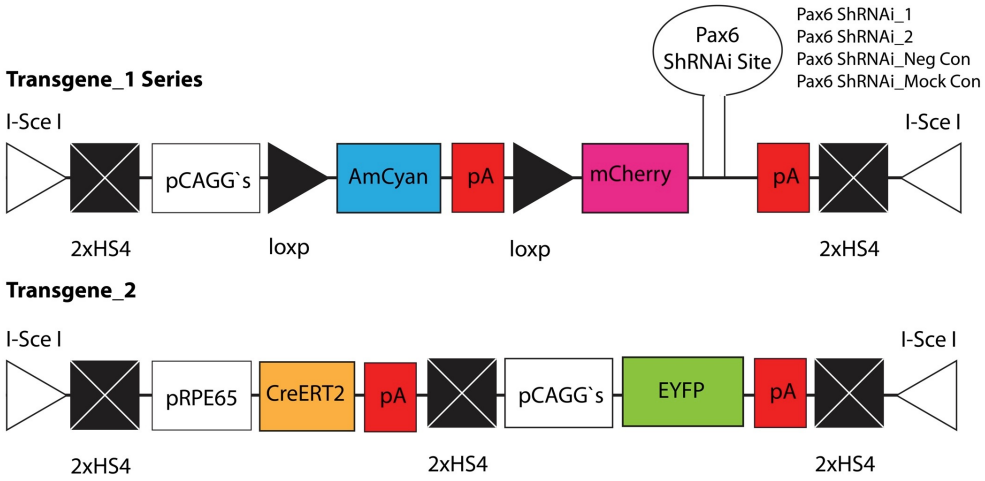

Figure 32. On Going Research to Knock Down Pax6 Expression During Early Newt Retinal Regeneration. This strategy uses a Conditional Site-Specific (RPE layer) RNAi Silencing System. Pax6 shRNAi (short hairpin RNA interference) were inserted into the loxP reporter construct, between mcherry and pA. Upon activation of CreERT2 loxP system, Amcyan will be cut, activating mcherry and Pax6 ShRNAi. Thus RPE cells or early derived cells will express mcherry indicating Pax6 shRNAi is active.