

The *Tg(ccnb1:mcherry)* transgenic zebrafish line: using a novel red-fluorescent protein to label proliferating cells during retinal development and regeneration

By Rachel Diehl

Honors Thesis, Neuroscience

University of Michigan, Ann Arbor

April, 2009

Advisor: Dr. Pamela Raymond

Readers: Dr. Richard Hume

Dr. John Schiefelbein

Table of Contents:

Page

Abstract.....1

Introduction3

Methods.....7

Results.....12

Discussion.....21

Acknowledgements.....24

References..... 25

Figures..... 28

Abstract:

Zebrafish have proven to be a powerful model organism for studying retinal patterning and regeneration. Generation of transgenic lines has become a popular method for understanding the function of various genes in regeneration and better understanding these processes. The *Tg(ccnb1:mcherry)* zebrafish transgenic line, allele name mi2009, was designed to label retinal progenitor cells by utilizing the promoter from the *cyclin B1* (*ccnb1*) gene in order to label all proliferating cells. mCherry reporter was used as the reporter because of its unique red fluorescence. The line was generated through microinjection of a specifically engineered construct into early stage embryos. To create the *Tg(ccnb1:mcherry)* construct, a 1.5kb promoter region of the zebrafish *cyclin B1* gene was cloned using wild-type genomic DNA and a set of primers previously utilized in the laboratory of Dr. David Hyde. This promoter was ligated into a *Tol2* backbone vector containing mCherry reporter, due to the high germline transmission efficiency of *Tol2* transposon systems (Kawakami, 2000). The identity of the construct was confirmed using restriction enzyme digestion and through sequencing at the University of Michigan DNA Sequencing Core. Microinjected fish were screened for transient mCherry fluorescence, and these fish were raised and screened to determine which ones had incorporated the transgene into their germline. Offspring from one founder fish were subsequently sectioned and immunocytochemistry (ICC) was performed. PCNA stains provided controls for labels on proliferating cells. The mCherry expression pattern revealed broad labeling of proliferating cells within the retina. However, co-localization between PCNA and mCherry fluorescence in the brain confirmed the expected pattern of mCherry expression. The broad labeling in the retina is attributed to the insertion site of the *ccnb1:mcherry* transgene, which is variable due to the *Tol2* transposon system employed. Identification of further founder fish will allow for additional examination of this expression pattern. The mCherry fluorescent reporter utilized in the

Tg(ccnb1:mcherry) line is particularly desirable because it allows for convenient visualization of regenerating cells in the retina without performing time consuming procedures such as ICC. Moreover, many transgenic lines are made with GFP reporters, so the unique fluorescence of mCherry is convenient for crossing with GFP reporter lines. Thus far, the *Tg(ccnb1:mcherry)* line has been crossed to the *Tg(gfap:gfp)* transgenic line (mi2002 allele name), in which all müller glial cells are labeled with the GFP reporter, which is driven by glial fibrillary acidic protein (GFAP) regulatory elements (Bernardos, 2006). This cross has allowed the visualization of both müller glial cells and regenerating cells at the same time. Müller glia are known to be one of two cell subsets in which neurogenesis persists in the adult retina, and this allows the unique opportunity to show double labeling of mCherry and GFP on those müller glia that are also in the process of dedifferentiating and regenerating. Thus, this transgenic line will serve as an excellent tool to study neurogenesis in the zebrafish retina.

Introduction:

Zebrafish (*Danio rerio*) have recently been recognized as an especially strong model organism for the study of developmental processes. Zebrafish possess several unique characteristics that make them both particularly useful and relevant model systems. They develop rapidly, reaching sexual maturity within three months, and breed frequently and reliably, producing large clutches of transparent embryos that can easily be manipulated. External fertilization and development of these embryos allow for easy accessibility and study of the zebrafish at early developmental stages. The transparency of embryos is also useful because it allows visualization of neural structures and mutational screening without harming embryos (Glass, 2004).

A well developed visual system that is highly homologous to other vertebrates, including humans, makes the zebrafish retina a very rewarding area of study (Bilotta, 2001). The zebrafish retina is proportionately large and develops rapidly (Neumann, 2001). It provides a unique and isolated area of nervous system development apart from the central nervous system, making it of particular interest. Also of interest is the unique timeline of retinal anatomy development, which is not completed until three days after hatching (Bilotta, 2001). This allows the unique opportunity to observe initial normal neurogenesis of the retina during the first few days of life (Glass, 2004). Understanding the process of regeneration and differentiation that proceeds in normal retinal development is critical to understanding the problems that occur during development when genes affecting this patterning and regeneration have been mutated or blocked.

Retinal Anatomy

Vertebrates, including zebrafish, have a retina comprised of seven major types of cell that are arranged into a defined pattern consisting of three main layers (Neumann, 2001). This

patterning process is carefully orchestrated, and occurs not only in initial retinal development, but also in regeneration of the retina after cell ablation (Neumann, 2001). This process of retinal formation begins with a sheet of neuroepithelial cells that differentiate to form the cell types of the retina (Hitchcock, 2004). This process occurs when multipotent cells that comprise the neuroepithelial sheet exit the cell cycle, becoming neurogenic progenitor cells. These neurogenic progenitor cells then differentiate into all retinal cell types, and form the laminar pattern of the retina by three days post-fertilization (3dpf) (Neumann, 2001).

The retina can be organized into three major nuclear layers and two smaller plexiform layers. Beginning with the outermost layer, the outer nuclear layer (ONL) consists of the cell bodies of the rods and cones; the inner nuclear layer (INL) the cell bodies of the horizontal, bipolar and amacrine cells; and the ganglion cell layer ganglion cell bodies (Neumann, 2001).

Regeneration

Previous research has demonstrated that zebrafish can regenerate a number of their somatic tissues, including all cell types of the retina (Hitchcock, 2004). This makes zebrafish a unique tool for studying regeneration. The capacity of the retina to regenerate is due to two main sources, the circumferential marginal zone (CMZ) and müller glia (Hitchcock, 2004). The CMZ is located between the retina and the iris and is comprised of a population of undifferentiated cells that are known to give rise to all retinal cell types, while the müller glia are found in the ganglion cell layer, and regeneration is the result of dedifferentiation of these cells (Hitchcock, 2004).

Recent studies have shown through microarray and RT-PCR that numerous proteins are upregulated during regeneration, indicating that they play a critical role in these processes (Barton, 2008). Cyclin B1 is found to be upregulated with significantly higher levels when cells

are undergoing the G2/M transition that is crucial to regeneration (Hyslop, 2004). Therefore, the *cyclin B1* promoter has been identified as an excellent candidate to regulate the expression of the mCherry fluorescent reporter as a marker expressed during retinal regeneration.

Cyclin B1

The cyclin family is known to have many functions in the cell cycle and, as this family of regulatory proteins is studied, new processes continue to be elucidated. Since Hunt's discovery of Cyclin B1 in 1982, comprehension of the processes and methods concerning cyclins has been constantly evolving. An observation made in the discovery of cyclin, however, is still crucial to our understanding of its actions. Hunt found cyclin levels to be the lowest during interphase and highest between G2 to Mitosis (Evans, 1983). Cyclin B1 is now known as a critical regulatory element in the transition between G2 and meiosis or mitosis (Hyslop, 2004). This transition is a critical regulatory step in the regenerative process, as regenerative cells must proceed through the cell cycle. This expression of Cyclin B1 in all regenerating cells makes it an excellent candidate to be used in generating a transgenic line with the goal of labeling all proliferating cells.

The Regulation of Cyclin B1 in Regeneration

The powerful role of Cyclin B1 in the cell cycle makes it the subject of strict regulation. Many aspects of this regulation have recently been elucidated; however, there are still many more processes that are not well understood. It is important to understand at what levels Cyclin B1 is regulated so that the function of the *ccnb1:mcherry* transgene is fully understood. Any transcriptional regulation to which Cyclin B1 is subjected will affect the expression pattern in the *Tg(ccnb1:mcherry)* transgenic fish, because the *mcherry* reporter gene is driven by the *ccnb1* promoter. This promoter, although it is not driving the *ccnb1* gene in this instance, is still subject to the same regulation.

The *ccnb1* promoter has been shown to be regulated at both the transcriptional and translational levels, and its expression has also been shown to be influenced by ubiquitination. Recent studies suggest that transcriptional factors regulate Cyclin B1 levels (Ito, 2000). These studies have found that the promoter contains evolutionarily conserved motifs, including an E-box and CCAAT box, where regulation is likely to occur. Also, multiple *cis*-elements and *trans*-factors have been identified during G2/M specific activation of the *ccnb1* promoter (Kirkpatrick, 2006).

Being a key regulator in the transition between G2 and meiosis or mitosis affords Cyclin B1 an important role in regenerative processes. Microarray and quantitative real time polymerase chain reaction experiments performed on damaged zebrafish retinal tissue revealed that Cyclin B1 mRNA is upregulated during the proliferative stages of retinal regeneration (Kassen, 2008). This upregulation has identified Cyclin B1 as a probable candidate to regulate the expression of a marker in retinal progenitor cells during retinal degeneration and regeneration (Kassen, 2008).

Conclusions

The aforementioned research demonstrates the crucial role of Cyclin B1 in regulation of cell cycle progression. As an essential gatekeeper of the G2 to M transition, Cyclin B1 can be considered an appropriate marker for proliferating cells. The well understood regulation of the *ccnb1* promoter makes it an excellent candidate to drive the expression of mCherry reporter and for use as a genetic tool to better understand neurogenesis in zebrafish.

Methods:

Zebrafish care

Adult zebrafish were raised under normal facility conditions (14 h light, 10 h dark). Zebrafish embryos were raised at 28.5° C in E3 embryo media and staged according to hours post-fertilization.

Generation of transgenic zebrafish

Wild-type genomic DNA was used to isolate the 1.5 kb region of the zebrafish *ccnb1* promoter identified by the Hyde lab. Specific primers (Forward primer: 5'ctcgaggagtgcgaattgcagcaagtgtg3' and reverse primer: 5'ggatccctcgtggtttattgctgtgactc3') were identified that contained cut sites for restriction enzymes Xho1 (forward primer) and BamH1 (reverse primer). The promoter sequence was PCR amplified using the Qiagen spin miniprep kit (Maryland) and purified using the Roche high pure PCR product purification kit (Indianapolis, IN). Identity of PCR products were confirmed by the University of Michigan Sequencing Core. The Tol2 backbone of the construct that contained the mCherry reporter was also isolated through restriction enzyme digest with Xho1 and BamH1. After enzyme digestion (50 µl system), products were run on a gel, and gel extraction using the QIAquick Gel Extraction kit was performed.

The backbone and insert were ligated using a 2:1 ratio of insert to backbone at 16°C. Transformation of competent cells with the ligation reaction was performed to confirm the desired construct (Figure 1). The ligation product was transformed into *Escherichia coli* bacteria, plated on media with Ampicillin antibiotic and grown overnight at 37°C. The Tol2 vector has an Ampicillin-resistance gene so only colonies with the inserted vector grew. Some well-isolated colonies were inoculated and a liquid culture was created using Luria Broth and Ampicillin and

grown overnight at 37°C. Miniprep was performed the following day to test for presence of the Tol2 vector insert and to confirm its identity. Miniprep products were subjected to enzyme digest, and the product run on an agarose gel to confirm the correct identity.

mRNA preparation

mMessage mMachine High Yield Capped RNA Transcription Kit reagents and protocol (Applied Biosystems) were used to generate mRNA. Identity of plasmid pT3TsTol2 (glycerol stock 329 AB-5) was confirmed with an enzyme digest. Restriction enzymes BglII and SpeI were used to cut the plasmid into two fragments. Roche buffer H was used because it had the highest activity levels for both restriction enzymes. Circular mRNA was generated and linearized using 30µl plasmid, XbI restriction enzyme and Roche buffer H. Proteinase K treatment was done at 50°C for 30 minutes, followed by a phenol chloroform extraction and a capped transcription reaction. The resultant mRNA product was measured into 1.1µl aliquots and stored at -80°C until the time of microinjection.

Microinjection Protocol

Pairs of either TL-WT breeders or a combination of TL-WT male and mi2002 female parental breeder fish were set up the evening prior to injection. For generation of the single transgenic line, TL-WT provided strong background. Because of future goals to generate a double transgenic line, injecting embryos from mi2002 x TL-WT crosses was desirable. Heterozygote females were bred to TL-WT males due to difficulty identifying any male carriers, or female homozygote mi2002 carriers. Each pair was fed and then put into an individual tank, with the male and female separated by a clear, plastic divider. Fish were left overnight, and, the following morning between 8am (lights on) and 8:30am the dividers were pulled. Fish were then allowed to mate and checked at 20 minute intervals, with any embryos being collected

immediately. Embryos were kept on ice to slow development slightly, until injection was performed. Embryos were held in place in a 5% agar gel tray, and microinjected between the 1 and 4 cell stage. According to protocol established for the Tol2 system by the Kawakami Lab, 25 ng/ μ l was determined as the ideal concentration of injection solution for DNA and mRNA, with an injection volume of 1nl (Kawakami, 2000). Therefore 1 μ l DNA and 1 μ l mRNA were added to a 10 μ l system to create the injection solution with this desired concentration, and 1-2nl injection solution were injected into each embryo (Kawakami, 2000). Injections were performed within 2 hours of preparing injection solution, so as to maintain the viability of the mRNA. Injection solution was prepared with RNase free reagents to preserve the transposase mRNA from degradation prior to microinjection. Embryos were treated with 10 μ l pronase the morning after microinjection, to allow for dechoriation.

Twelve sets of microinjections were performed, with each round of microinjections yielding approximately 10% mCherry-positive embryos. The embryos were raised to adulthood and at this time screened to determine which fish had integrated the construct into their germline cells.

Identifying Founder Fish

Fertilized eggs coinjected with DNA and transposase mRNA were screened at 24 hpf for mCherry fluorescence. mCherry positive fish were grown and at three months post-fertilization were mated to TL wild-type fish. F1 offspring were also examined 24 hpf for mCherry fluorescence. Founder fish were identified based on results of F1 screening. If the mCherry positive fish had the construct incorporated into its germ cells, this construct would be passed on to its offspring. Therefore, mCherry positive F1 fish indicated a founder parent. Approximately 10% of F1 fish received the construct from the founder parent.

Expression of mCherry fluorescence was the indicator of germline transmission of the gene. The cyclin promoter should drive the expression of mCherry in proliferating cells because cyclin proteins are regulatory elements for progression of cells through the cell cycle, which proliferating cells are taking part in. PCNA labeling to confirm the accuracy of the mCherry construct in labeling proliferating cells confirmed this labeling.

Fixation and Cryoprotection

mCherry positive embryos at 4dpf were fixed with a 4% paraformaldehyde solution at 4°C overnight. Cryoprotection was done to ensure the most accurate histology and to prevent damage due to subsequent freezing. This was done by infiltrating the tissue first with phosphate buffer with 5% sucrose solution, followed by solutions of 0.1M phosphate buffer/5% sucrose: 0.1M phosphate buffer/20% sucrose in the following proportions 1:1, 2:1, 1:1, 1:2. Finally, the tissue was infiltrated with 0.1M phosphate buffer with 20% sucrose overnight at 4°C on a rotator, completing the cryoprotection.

Embryos were placed in a 2:1 solution of 0.1M Phosphate buffer/20% sucrose: OCT solution, and frozen utilizing cooled 2-methylbutane. Blocks were frozen at -80°C until the time of cryosectioning. Cryosectioning was done on a Leica CM 3050 cryostat at -20°C, producing three-micron sections that were placed on slides and stored in the -80° freezer until the time of immunocytochemistry.

Immunocytochemistry

Slides containing sections of retinal tissue were taken from the -80° and allowed to thaw at room temperature. The slides were then rinsed with methanol in the -20° freezer, followed by PBS while on the rotator. From this point on, the slides were kept moist at all times. Slides were placed into a humid chamber and covered with blocking reagent at room temperature. The slides

were then plated with the PCNA primary antibody (1:1000), and incubated overnight at 4°C. The next morning, slides were washed with wash buffer on a rotator. Secondary antibody ms-cy5 (1:50) was then plated while within the humid chamber. Slides were washed with DAPI to allow for clear visualization of the nuclei. Slides were then stored at -20°C and examined for fluorescence indicating co-localization the same afternoon.

Fluorescence Microscopy

Fluorescence microscopy was done on Zeiss microscope using AxioVision software. The auto-exposure function was used to capture the images. The exposure time of the mCherry channel was 1-2 seconds.

Results:

I. Generating the Transgenic Line

*Isolating *ccnb1* promoter*

Dr. David Hyde's laboratory at Notre Dame successfully generated a transgenic line of zebrafish that carries a transgene that labels proliferating cells with EGFP. This transgene, *Tg(ccnb1:EGFP)*, is designed in a Tol2 vector with a promoter from the zebrafish *ccnb1* gene and EGFP as the reporter. The cyclin promoter drives the expression of EGFP in proliferating cells because cyclin proteins are regulatory elements for progression of cells through the cell cycle, in which proliferating cells are taking part. This was used as a starting point in the generation of the *Tg(ccnb1:mcherry)* line. Although Dr. Hyde's Lab sent a sample of *ccnb1:EGFP* DNA spotted onto filter paper, it was difficult to obtain an adequate amount of competent cell growth after transformations were performed.

Because of this difficulty, wildtype genomic DNA, rather than the DNA sent by the Hyde Lab, was used to isolate the desired *ccnb1* promoter through use of specific restriction enzyme cut sites utilizing the primers that the Hyde lab had also used. By utilizing the primers isolated by the Hyde Lab and these specific restriction enzyme cut sites, isolation of the *ccnb1* promoter from the wildtype zebrafish genome was possible.

Figure 2 shows an agarose gel confirming this result. The isolated product thought to be the *ccnb1* promoter was PCR amplified and purified, and run in the gel along with the known *ccnb1* primer product from the Hyde lab, which was also PCR amplified and purified. The resultant gel shows two bands at 1.4kb, which was the expected size of the primer based on University of Michigan Sequencing Core results, as well as sequencing done by the Hyde lab. The band seen on the left on the gel reflects the PCR product of the *ccnb1* product generated in

lab, and is much denser than the band on the right, which came from the Hyde lab construct. This difference in band width reflects the varying amounts of genomic material.

After confirming the identity of the isolated *ccnb1* promoter on the gel, the PCR product was also sent to the UM Sequencing Core. The forward alignment showed a 94% identity and the reverse alignment 100% identity to the database sequence for the *ccnb1* promoter. Although the forward primer showed only a 94% identity, this was attributed to artifacts during sequencing, not to any mismatched base pairs. This further confirmed the identity of the *ccnb1* insert isolated from the wildtype genomic DNA.

Isolating mCherry reporter

With the desired *ccnb1* promoter isolated, the next step was to isolate the mCherry reporter. This was done utilizing a glycerol stock (337AB) of the pmini Tol2-SWS2up3-2-mCherry (*Tg(SWS2:mCherry)*) already viable within the Raymond lab. pmini Tol2-SWS2up3-2-mCherry glycerol stock was grown on LB agar plates at 37°C overnight. Individual colonies were transferred to LB broth after overnight growth at 37°C. Figure 3 shows the result of an agarose gel confirming the identity of these *Tg(sws2:mCherry)* clones as correct. Restriction enzyme digest of the miniprep DNA resulted in two bands representing fragments of the expected sizes of 4.7kb and 3.2 kb. The 4.7 kb fragment was removed from the gel via gel extraction, and is the backbone for the construct.

This transgenic line is composed of an mCherry reporter driven by a *sws2* promoter in a Tol2 vector. This was an ideal starting point because we were able to remove the *sws2* promoter, leaving the reporter and backbone intact.

BLASTing the Sequence

Upon isolation of the *ccnb1* promoter from the genomic DNA, the NCBI Basic Alignment Search Tool (BLAST) was used to verify the identity of this DNA fragment. The DNA fragment isolated through restriction enzyme digest of genomic DNA with the primers used by the Hyde Lab was PCR amplified and then characterized. This was done to ensure its correct identity as well as to identify information about homology across different species. Sequencing resulted in a 1285 base pair sequence between the two primers. This was the expected length of the promoter based on information from the Hyde lab. Utilizing BLAST, the identity of this DNA fragment as the *ccnb1* promoter was confirmed with 100% identity match to the database sequence for *Danio rerio*.

Ligating ccnb1 promoter and mCherry reporter

The SWS2 promoter was excised using Xho1 and BamH1 restriction enzymes. Since both the *ccnb1* promoter and the *sws2* promoter were both excised with the same two restriction enzymes, directionality was maintained. This allowed for easy insertion and ligation of the *ccnb1* promoter in the same site where the SWS2 promoter was excised.

After the ligation reaction, it was necessary to confirm that the insertion of the *ccnb1* promoter had been inserted successfully. The ligation products were PCR amplified, digested with BamH1 and XhoI, and run on an agarose gel. Figure 4 shows the resulting banding of five colonies. While the fifth was ruled out because it was not the appropriate size (6.1 kb) the other four were sent to the UM Sequencing Core. The results showed that the first colony was a 99% identity match, the second colony was a 97% identity match, the third colony was a 100% identity match and the fourth colony was a 99% identity match. These results indicated a successful ligation between the backbone and promoter sequences. Only the ligation product with 100% identity match was used for injection solution to microinject into zebrafish embryos.

Determining an Appropriate Vector System

The transposon system was identified as a useful vector using the Tol2 transposon system. It was found to have a significantly higher transmission rate than traditional microinjection methods. This vector was utilized due to its high germline transmission rate and its ability to integrate into chromosomes of zebrafish germ cells (Kawakami, 2000). The germline transmission rate was of particular importance because of the future goals of establishing a stable transgenic line. Transposons are an important genetic tool because they allow for much more efficient transgenesis than DNA microinjection. Microinjection alone without utilizing a transposase system only allows a 5% germline transmission rate. With such a low germline transmission rate, it would take much longer to generate a stable transgenic line.

In vitro mRNA synthesis

In previous studies, pairing transposase mRNA with transposon DNA vector resulted in a 50% germline transmission rate, significantly higher than the 12.5% germline transmission rate using DNA vector alone (Kawakami, 2000). Transposase mRNA is essential in the Tol2 system for the high germline transmission frequency because of transposase's function in allowing the construct to insert itself multiple times into the genome. The transposase gene is removed from the Tol2 construct to prevent the gene from maintaining mobility within the genome, and the possibility of being excised after screening. Thus, utilizing transposase mRNA allows for transient transposase expression and limits activity to the first hour after microinjection because of the rapid degradation of mRNA. This ensures maximal insertion of the construct with minimal disruption of cell function.

To generate the transposase mRNA, it was first necessary to grow pT3TsTol2 clones *in vitro* which were used as a template. Figure 5 is an agarose gel, confirming the identity of this

clone. Figure 6 shows the final mRNA product, which was run on a gel to confirm identity and estimate concentration. Spectrometry readings were taken and indicated a final mRNA concentration of 2380.0 ng/ μ l.

II. Confirmation of transgene expression pattern

Tg(ccnb1:mcherry) embryos from the first founder fish showed very strong mCherry expression in the head and eye, as well as other somatic tissues at 24 hpf (Figure 7). Siblings not expressing mCherry fluorescence expressed some very dull autofluorescence, however there is a clear difference between mCherry positive and negative embryos (panel b).

To further characterize the expression pattern of mCherry in these embryos, immunocytochemistry was performed using PCNA primary antibody and ms-cy5 secondary antibody (figure 8). ICC was performed on embryos at 3dpf and 4dpf. These ages are ideal because by 3dpf, neurogenesis in the retina is nearly complete. It was observed by the Hyde lab that until 36hpf, PCNA and EGFP expression patterns were very broad due to a large number of proliferating cells (Kassen, 2008). By 72hpf, however, the signals were more confined and found in the CMZ. According to observations made by the Hyde lab, both 3dpf and 4dpf embryos should give a clear and strong co-localization between PCNA and fluorescent protein in the CMZ.

Two founder fish were identified from the TL-WT background injections, and expression patterns in both were examined. The first *ccnb1:mcherry* founder fish was also crossed into the *gfap:gfp* line, which labels müller glial cells. ICC was performed on retinal sections of these fish as well to examine the expression patterns in tandem. Images from ICC of the first founder fish can be found in figures 9-11.

Immunocytochemistry of Tg (ccnb1:mcherry) fish

Immunocytochemistry was performed using PCNA antibody to determine the extent of labeling on proliferating cells by the *ccnbl:mcherry* construct. Because the goal of the *ccnbl:mcherry* construct is to label all proliferating cells within the developing retina, and PCNA staining allows visualization of all dividing cells by labeling their nuclei, co-localization of the mCherry and PCNA markers demonstrates labeling of proliferative cells by the *ccnbl:mcherry* construct (Kassen, 2008).

Figure 8 shows ICC performed on 4dpf *Tg(ccnbl:mcherry)* fish. Fish were first screened for mCherry fluorescence. Panel a shows mCherry fluorescence, which is strongest in the ONL and part of the INL. There is very little, if any, mCherry signal visible in the CMZ and the signal is very weak. This expression pattern varies from that of PCNA (panel b), where the strongest fluorescent signal is seen in the CMZ (arrow). Panel c shows a DAPI PCNA overlay. DAPI allows for visualization of individual cell nuclei and helps to set a standard to determine where PCNA and mCherry fluorescence should be seen. While significant co-localization of the PCNA and mCherry fluorescence was expected, this is not evident (panel d). PCNA mCherry overlay shows that there is very slight co-localization of PCNA and mCherry in the CMZ, while there is no co-localization between the two signals in the ONL or INL where mCherry fluorescence is seen. This may be due to the short half life of PCNA in comparison to mCherry, which could account for the mCherry signal persisting in cells that are no longer proliferative and which PCNA no longer labels. Two founder fish were examined and similar expression patterns were preliminarily seen in both. Further testing is necessary to confirm the expression pattern in the embryos from the second founder fish. This difference in the expected expression pattern may be due to the site of construct insertion, or a problem in the promoter. Because the same promoter

sequence was used by the Hyde lab, and they saw significant co-localization between PCNA and EGFP, it is most likely that the problem lies within the insertion site.

Immunocytochemistry of mi2009xmi2002 cross

A major goal of the *ccnbl:mcherry* transgenic line is the potential to outcross to other transgenic lines with alternate fluorescent labels. The first application of this unique possibility to double label has been initiated and is ongoing. The *ccnbl:mcherry* founder fish were bred with *gfap:GFP* fish to produce double transgenic fish (figure 9,10,11). The *gfap:GFP* transgenic line, allele name mi2002, consists of glial fibrillary acidic protein regulatory elements driving the GFP reporter (Bernardos, 2006). Creating a line of *ccnbl:mcherry-gfap:GFP* transgenic fish will be particularly useful in the lab because it will allow further elucidation of the regenerative processes of müller glia.

Two different schemes were utilized in order to generate embryos that expressed both GFP and mCherry fluorescence. In the first scheme, *Tg(gfap:gfp)* fish were bred to TL-WT fish, and these embryos were microinjected with the *ccnbl:mcherry* transgene with the goal of labeling two different subsets of neural cells. This was done initially in order to eliminate the need to breed *Tg(gfap:gfp)* fish to *Tg(ccnbl:mcherry)* fish to generate the desired line carrying two transgenes. However, only 25% of TL-WT X *Tg(gfap:gfp)* embryos were GFP positive, and because of the time sensitive nature of microinjection, these embryos were not screened prior to injection. Therefore, a very small number of the *ccnbl:mcherry* fish were expected to be GFP positive, and an even smaller number to be both founder fish and GFP positive.

Due to the small number of *Tg(ccnbl:mcherry-gfap:gfp)* transgenic fish, a second scheme was used and *Tg(gfap:gfp)* fish were bred with *Tg(ccnbl:mcherry)* founder fish. This cross resulted in embryos that expressed both mCherry and GFP fluorescence. In order to isolate

Tg(ccnb1:mcherry-gfap:gfp) embryos, resultant embryos were screened first for mCherry fluorescence, followed by GFP fluorescence. Approximately 20% of the embryos showed mCherry fluorescence, while 40% of these embryos showed GFP fluorescence as well.

ICC was performed on 3dpf *gfap:GFP-ccnb1:mcherry* retinal sections to examine the expression pattern. These transgenic fish were acquired following the second scheme from above because the first *Tg(ccnb1:mcherry)* founder fish was not expressing GFP following the first scheme. Figures 9-11 show ICC performed on three different fish from the same founder, which all exhibited similar expression profiles. Figures 9-11 show ICC performed on sections of 3dpf *gfap:gfp-ccnb1:mcherry* embryos. Panel a shows expression of mCherry. Fluorescence levels were relatively equal to those of the single transgenic line when comparing fluorescence levels in the ONL, INL, and CMZ. Localization was not as specific to proliferating cells as was desired. The expression is seen throughout the ONL and the outer part of the INL. While it was expected that there would be labeling in the CMZ (dashed arrow), where a large number of cells are proliferative, this is not the case. More specific labeling is seen in the proliferating cells of the diencephalon (solid arrow), where neurogenesis continues after hatching. In panel c, PCNA expression is seen, with clear fluorescence in the CMZ (dashed arrow). There is also distinct PCNA labeling in the proliferative cells of the diencephalon (solid arrow). Panel e shows co-localization of PCNA and mCherry in the diencephalon. The significant co-localization seen in the proliferative cells of the diencephalon indicates the expression pattern of mCherry partially matches that of PCNA. Because a goal of the *Tg(ccnb1:mcherry)* line is to allow visualization of multiple fluorescent fields, the GFP channel is also shown (panel b). Panel b is a merge of DAPI, which labeled all nuclei, and GFP that labeled all müller glial cells showing specific labeling of the desired cell types. Panel d shows a merge of PCNA and GFP channels, which did not show

co-localization because the müller glial cells were no longer proliferative at the time of sectioning. Because there was no co-localization between PCNA and GFP, a merge between mCherry and GFP was not necessary. In the future, labeling of müller glial cells which are proliferative after lesion in the regenerating retina with both mCherry and GFP fluorescence is desired.

Although the mCherry expression pattern is broad, the PCNA mCherry merge shows that there is some co-localization in the CMZ. There is also significant co-localization between the two labels in the developing brain. Previous research by the Hyde Lab using the same promoter sequence showed co-localization of EGFP and PCNA in their *ccnb1:EGFP* transgenic line (Kassen, 2008). Research on the mouse retina has also shown significant co-localization of Cyclin B1 and PCNA (Barton, 2008). Because the expression pattern of PCNA is well understood, this co-localization further confirmed the role of Cyclin B1 in neurogenesis (Barton, 2008).

Discussion:

My work has established a new transgenic line of zebrafish carrying the transgene, *Tg(ccnb1:mCherry)*, allele name mi2009, although expression in any founder fish examined thus far has not been optimal. This transgene specifically labels all proliferating stem and progenitor cell populations in the zebrafish retina with the fluorescent protein mCherry. In this study, the co-localization of the mCherry reporter and the DAPI and PCNA stains, which labeled nuclei and proliferating cells in the zebrafish retina respectively, shows that the reporter is broadly expressed in proliferating cells within the retina.

Creating the transgenic line with an mCherry reporter serves as an important tool since it provides inherent labeling of all proliferating cells, allowing for easier visualization of these cells. Also, the mCherry marker is able to be visualized while the zebrafish is still alive whereas PCNA and Brdu markers are not only time consuming but also require fixation and subsequent immunolabeling.

mCherry is an excellent fluorescent protein to use in combination with other fluorescent proteins because the emission signal (610nm peak) is easily distinguishable from blue, cyan, green or yellow fluorescent protein variants commonly used as reporters (Larina, 2009). One of the major goals for generating the *Tg(ccnb1:mcherry)* transgenic line was the possibility of labeling two distinct cell populations. Founder *Tg(ccnb1:mcherry)* fish have been crossed with the mi2002 line of *gfap:gfp* transgenic fish with its glial cells labeled with GFP. By crossing the *Tg(ccnb1:mcherry)* line into the *Tg(gfap:gfp)* line, the effectiveness of mCherry as a reporter in conjunction with other widely used reporters such as GFP was shown. This cross of the *ccnb1:mcherry* transgenic line with the *gfap:gfp* is only one of many ways that the unique mCherry reporter will be used as a genetic tool.

Thus far, two independent *ccnb1:mcherry* founder fish have been identified with similar expression patterns that do not match the predicted result. ICC analyses show mCherry fluorescence that is not as bright or specific as desired. The co-localization was strong in the brain, but was considerably weaker in the retina. The reason for these two consistent yet undesirable expression patterns in the two founder fish identified is uncertain. The *ccnb1:mcherry* transgene insertion site could be the problem. Because of the unpredictable nature of the Tol2 transposase system, it is uncertain where in the genome the *ccnb1:mcherry* construct has been inserted. The broad expression pattern currently observed could likely be the result of the insertion site affecting the gene expression pattern. It is unlikely, however, that both independent insertions have occurred in locations that result in nearly identical expression patterns. Previous research confirms this, as the Hyde lab identified two independent *ccnb1:EGFP* founder fish when generating a similar transgenic line using the Tol2 transposon system, with both showing strong co-localization between EGFP and PCNA. Another possibility that should be considered for the unexpected mCherry expression pattern is that the promoter element is insufficient to effectively drive specific mCherry expression. This seems likely because both founder fish exhibit similar unexpected expression patterns, however, this promoter was used successfully by the Hyde lab to generate a transgenic line with this promoter driving EGFP expression. Also, the promoter element used in the construct was sequenced with the result matching the database result for the desired promoter. Both theories to explain the cause of the unexpected mCherry expression pattern have considerable flaws.

Having established the transgenic line, it will be important to utilize techniques such as immunocytochemical labeling, in situ hybridization, and cell apoptosis assays studies to further elucidate processes of retinal regeneration. Further labeling F1 embryos from additional founder

parents with PCNA and Brdu will allow for added confirmation of co-localization of cyclin B1 with the mCherry reporter and for more specific labeling. Also, to investigate regeneration patterns, retinal cells can be ablated using a light lesion. After waiting several days, examination of retinal sections should reveal cells in different stages of regeneration labeled by the *mcherry* promoter. This labeling will hopefully provide insight into the behavior of retinal stem cells during regeneration.

The possibilities of future work with the *Tg(ccnb1:mcherry)* transgenic line are exciting. The process of neurogenesis is still being elucidated and the *Tg(ccnb1:mcherry)* fish are a powerful tool for studying development, regeneration, and disease in the zebrafish retina.

Acknowledgements:

I would like to acknowledge Zhao Qin for all of her guidance during my experimentation as well as the editing of my thesis. I would also like thank my mentor Dr. Pamela Raymond for all of her help, as well as the members of the Raymond lab, including Linda Barthel, Dilip Pawar, Chen Kuang and Yuliang Ma.

References:

- Barton K, Levine E (2008) Expression Patterns and Cell Cycle Profiles of PCNA, MCM6, Cyclin D1, Cyclin A2, Cyclin B1, and Phosphorylated Histone H3 in the Developing Mouse Retina. *Dev Dyn* 237: 672-82.
- Bernardos RL, Raymond PA (2006) GFAP transgenic zebrafish. *Gene Expr Patterns* 8:1007-13.
- Bilotta J, Saszik S (2001) The zebrafish as a model visual system. *Int J Dev Neurosci* 19: 621-29.
- Campbell RE, Tour O, Palmer AE, Steinbach PA, Baird GS, Zacharias DA, Tsien RY (2002) A monomeric red fluorescent protein. *Proc Natl Acad Sci USA* 12:7877-82.
- Cao Q, Richter JD (2002) Dissolution of the maskin-eIF4E complex by cytoplasmic polyadenylation and poly(A)-binding protein controls cyclin B1 mRNA translation and oocyte maturation. *EMBO J* 21:3852-62.
- Clute P, Pines J (1999) Temporal and spatial control of cyclin B1 destruction in metaphase. *Nature Cell Biol* 1:82-7.
- Dervan PA, Magee HM, Buckley C, Carney DN (1992) Proliferating cell nuclear antigen counts in formalin-fixed paraffin-embedded tissue correlate with Ki-67 in fresh tissue. *Am J Clin Pathol* 97: 0-8.
- Evans T, Rosenthal ET, Youngblom J, Distel D, Hunt T (1983) Cyclin: A Protein Specified by Maternal mRNA in Sea Urchin Eggs That Is Destroyed at Each Cleavage Division. *Cell* 33: 389-96.
- Glass A, Dahm R (2004) The Zebrafish as a Model Organism for Eye Development. *Ophthalmic Research* 36:4-24.
- Groisman I, Huang Y, Mendez R, Cao Q, Theurkauf W, Richter JD (2000) CPEB, Maskin, and cyclin B1 mRNA at the mitotic apparatus: implications for local translational control of cell division. *Cell* 103:435-47.
- Hitchcock PF, Raymond PA (2004) The Teleost Retina as a Model for Developmental and Regeneration Biology. *Zebrafish* 1:257-71.
- Hyslop LA, Nixon VL, Levasseur M, Chapman F, Chiba K, McDougal A, Venables JP, Elliot DJ, Jones KT (2004) Ca²⁺-promoted cyclin B1 degradation in mouse oocytes requires

- the establishment of a metaphase arrest. *Dev Biol* 269:206-19.
- Ito M, 2000 Factors controlling cyclin B expression. *Plant Molec Biol* 43:677-90.
- Kassen SC, Thummel R, Burket CT, Campochiaro LA, Harding MJ, Hyde DR (2008) The *Tg(ccnb1:EGFP)* transgenic zebrafish line labels proliferating cells during retinal development and regeneration. *Mol Vis* 14:951-63.
- Kawakami K, Shima A, Kawakami N (2000) Identification of a functional transposase of the Tol2 element, an Ac-like element from the Japanese medaka fish, and its transposition in the zebrafish germ lineage. *Proc Natl Acad Sci USA* 97:11403-8.
- Kirkpatrick DS, Hataay NA, Hanna J, Elasser S, Rush J, Finley D, King RW, Gygi SP, (2006) Quantitative analysis of in vitro Ubiquitinated cyclin B1 reveals complex chain topology. *Nature Cell Biol* 8:700-10.
- Larina IV, Shen W, Kelly OG, Hadjantonakis A, Baron M, Dickinson M (2009). A Membrane Associated mCherry Fluorescent Reporter Line for Studying Vascular Remodeling and Cardiac Function During Murine Embryonic Development. *Anatomical Record* 292: 333-41.
- Malicki J (2000) Harnessing the power of forward genetics—analysis of neuronal diversity and patterning in the zebrafish retina. *Trends in Neuroscience* 23: 531-541.
- Merzlyak EM, Goedhart J, Shcherbo D, Bulina ME, Shcheglov AS, Fradkov AF, Gaintzeva A, Lukyanov KA, Lukyanov S, Gadella TW, Chudakov DM (2007) Bright monomeric red fluorescent protein with an extended fluorescence lifetime. *Nat Methods* 7:555-7.
- Neumann CJ (2001) Pattern formation in the zebrafish retina. *Semin Cell Dev Biol* 12: 485-90.
- Nixon, VL, Levasseur M, McDougall A, Jones KT (2002) Ca^{2+} oscillations promote APC/C dependent cyclin B1 degradation during metaphase arrest and completion of meiosis in fertilizing mouse eggs. *Curr Biol* 12:746-50.
- Otteson DC, Hitchcock PF (2003) Stem cells in the teleost retina: persistent neurogenesis and injury-induced regeneration. *Vision Res* 43:927-36.
- Qiu G, Ramachandra RK, Rexroad CE, Yao J, (2007) Molecular characterization and expression profiles of cyclin B1, b2 and Cdc2 kinase during oogenesis and spermatogenesis in rainbow trout (*Oncorhynchus mykiss*). *Anim Reprod Science* 105:209-25.

Shaner NC, Steinbach PA, Tsien RY (2005) A guide to choosing fluorescent proteins. *Nat Methods* 12: 905-09.

Stenkamp DL (2007) Neurogenesis in the fish retina. *Int Rev Cytol* 259: 173-224.

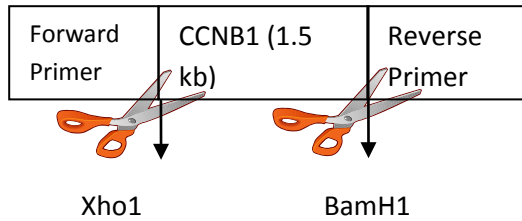
Takizawa CG, Morgan DO, (2000) Control of mitosis by changes in the subcellular location of cyclin-B1-Cdk1 and Cdc25C. *Curr Opin in Cell Biol* 12:658-65.

Tay J, Hodgman R, Richter JD, (2000) The control of cyclin B1 mRNA translation during mouse oocyte maturation. *Dev Biol* 221:1-9.

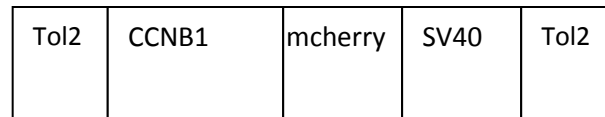
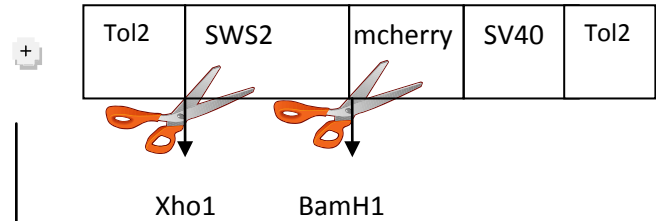
Wang W, Cladwell MC, Lin S, Furneaux H, Gorospe M, (2000) HuR regulates cyclin A and cyclin B1 mRNA stability during cell proliferation. *J EMBO* 19:2340-50.

Figures:

Wildtype genomic DNA



Donor Construct



Final Construct.

Figure 1: Schematic diagram of the steps taken to generate the desired construct, which was introduced into zebrafish embryos in order to produce the *Tg(ccnb1:mcherry)* line. 1.5kb *ccnb1* promoter was isolated from genomic DNA with the knowledge of specific primers (forward: 5'ctcgaggagtgcgaattgcagcaagtgtg3' and reverse: 5'ggatccctcgtggtttattgtgtgactc3') that contained restriction enzyme cut sites for Xho1 and BamH1, respectively. Backbone was prepared by excising the existing SWS2 promoter from donor construct, pmini Tol2-SWS2up3-2-mCherry. Backbone and insert were combined in a ligation reaction to yield the final construct.

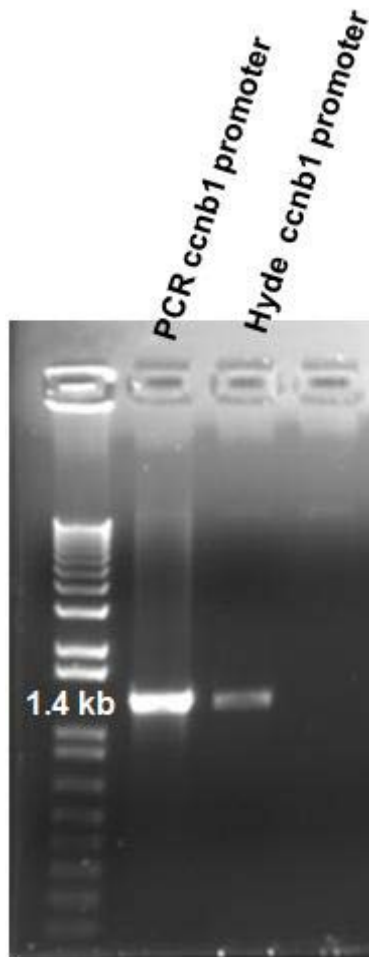


Figure 2: Successful transformation of competent cells with the *Tg(ccnb1:EGFP)* DNA from the Hyde lab resulted in the need to isolate the *ccnb1* promoter from genomic DNA. The isolated promoter element was run on a gel, along with a sample of known *ccnb1* promoter isolated from the Hyde lab sample. The gel shows both lanes at the same position, indicating they are the same size. The ladder on the left indicates their size at 1.4kb, the known size of the *ccnb1* promoter. Note the difference in thickness of the two bands, which reflects the relative concentrations. The faintness of the band on the right indicates a very low DNA concentration and necessitated the isolation of the *ccnb1* promoter from genomic DNA.

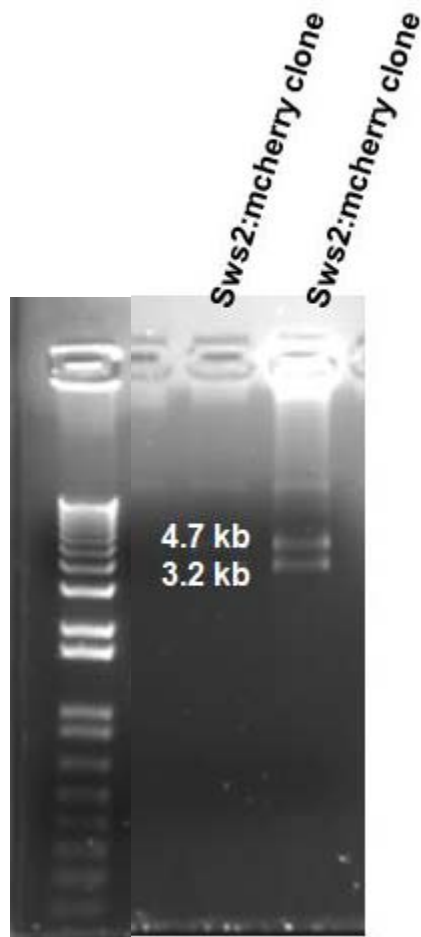


Figure 3: pmini Tol2-SWS2up3-2-mCherry clones were grown and used as a backbone for insertion of the *ccnb1* promoter. An enzyme digest using Xho1 and BamH1 restriction enzymes on the miniprep product revealed that both clones had two identical bands, at 4.7 and 3.2 kb. Based on the known sequence and restriction enzyme cut sites for the pmini Tol2-SWS2up3-2-mCherry construct, these bands confirmed the correct identity of the clones.

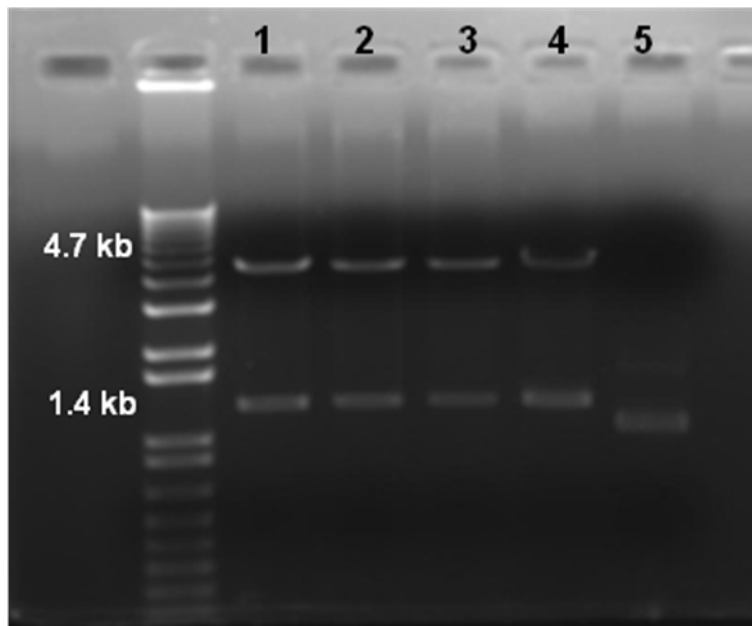


Figure 4: The ligation products were transformed into *E. coli* cells, and plated onto LB agar plates. Five isolated colonies were picked and grown in liquid broth. Miniprep was performed and enzyme digest using Xho1 and BamH1 revealed two bands at 4.7kb and 1.4kb for clones 1-4, indicating successful ligation reaction for these clones.

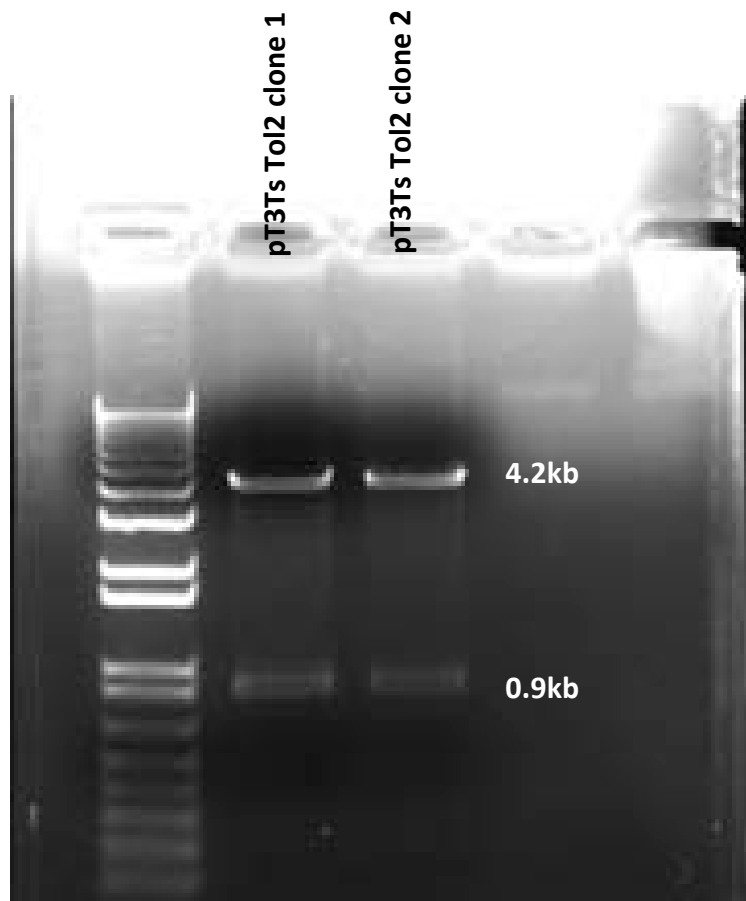


Figure 5: pT3TsTol2 clones were grown on LB agar plates. Two isolated clones were picked from the plate and grown in liquid broth overnight. Miniprep product from these clones was treated with restriction enzymes and run on agarose gel. The two clear bands at 4.2kb and 0.9 kb as expected confirm the identity of both clones.

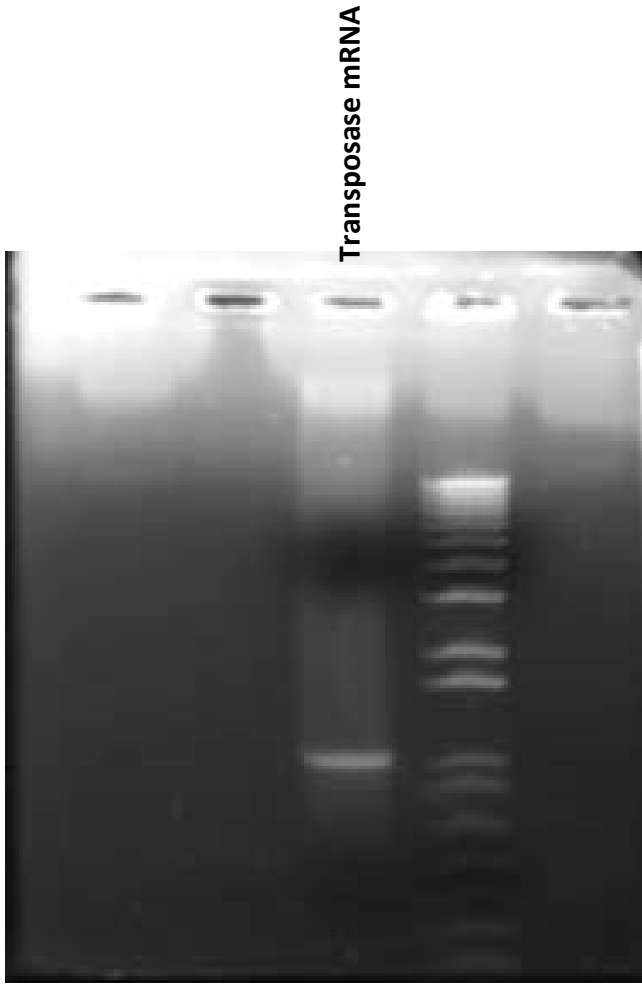


Figure 6: The final transposase mRNA product was run on an agarose gel to determine the product of the mMessage Machine protocol. The single, clear band indicates successful synthesis of transposase mRNA. This is confounded by a high spectrometer reading (2038 μ l/ng)

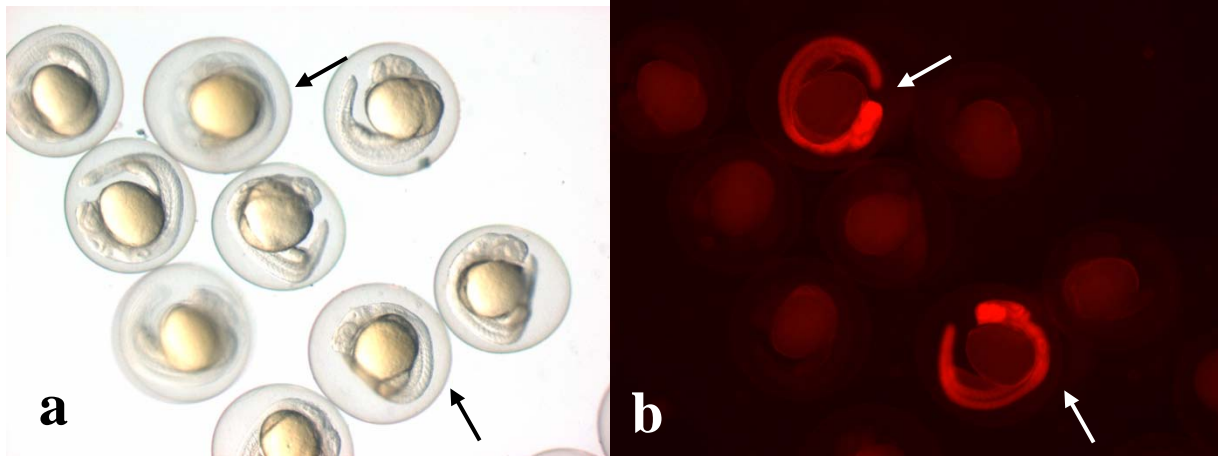


Figure 7: Images of 24hpf F1 embryos from second *Tg(ccnb1:mcherry)* founder fish taken at low magnification. Image on the right shows the strong mCherry expression in select F1 embryos. Image on right is a Phase-Contrast image of the same embryos allowing visualization of mCherry negative embryos in addition to mCherry positive embryos. Arrows indicate mCherry positive embryos.

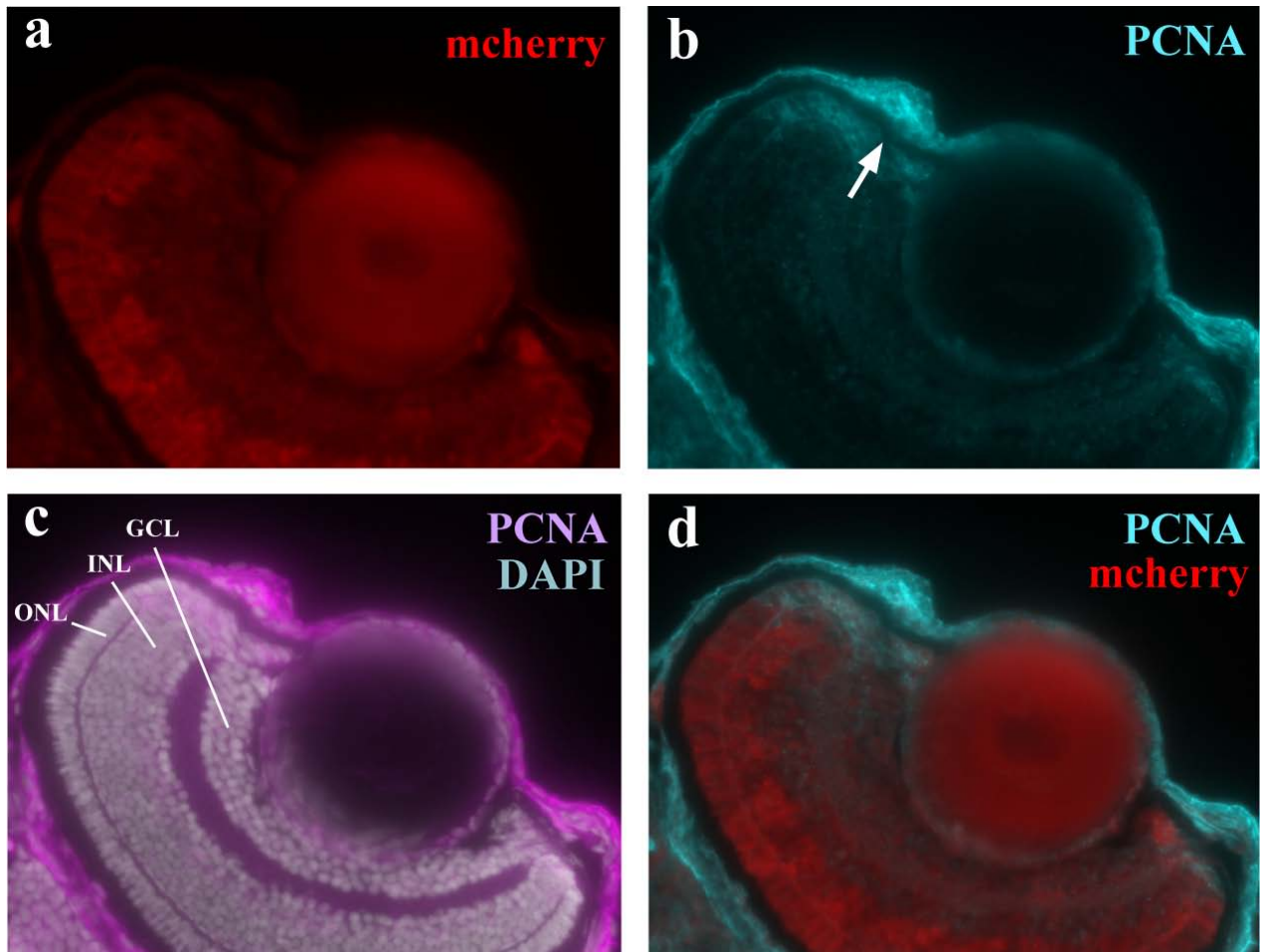


Figure 8: *Tg(ccnb1:mcherry)* fish 4dpf retinal sections, ICC. Panel a: mCherry, b:PCNA, c: DAPI, PCNA, d: PCNA, mCherry.

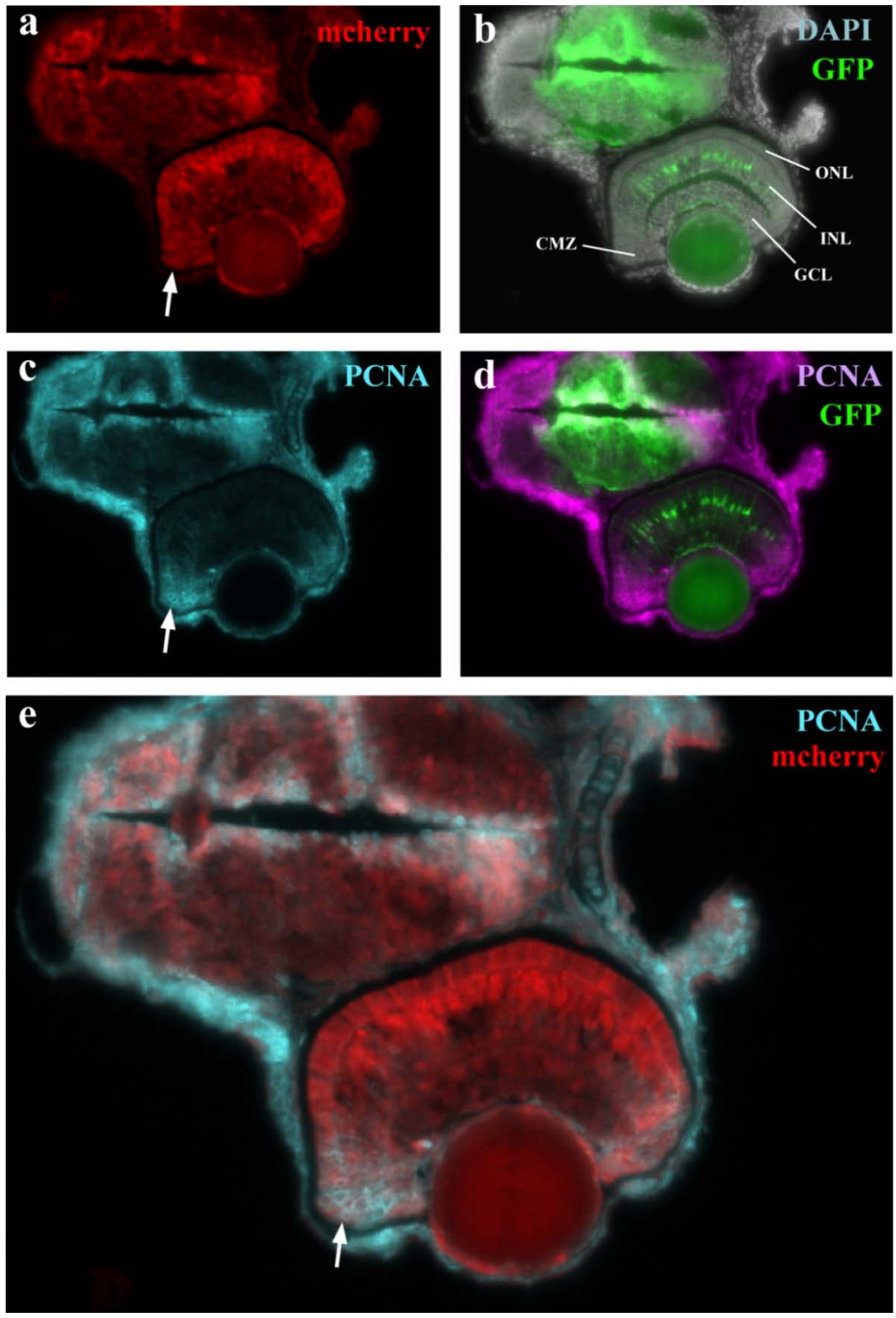


Figure 9: *gfap:EGFP-ccnb1:mcherry* embryos 3dpf retinal sections, ICC. Panel a: mCherry, b: DAPI, GFP merge, c:PCNA, d: PCNA, GFP merge, e: PCNA, mcherry merge. Arrows point to proliferating cells in the CMZ.

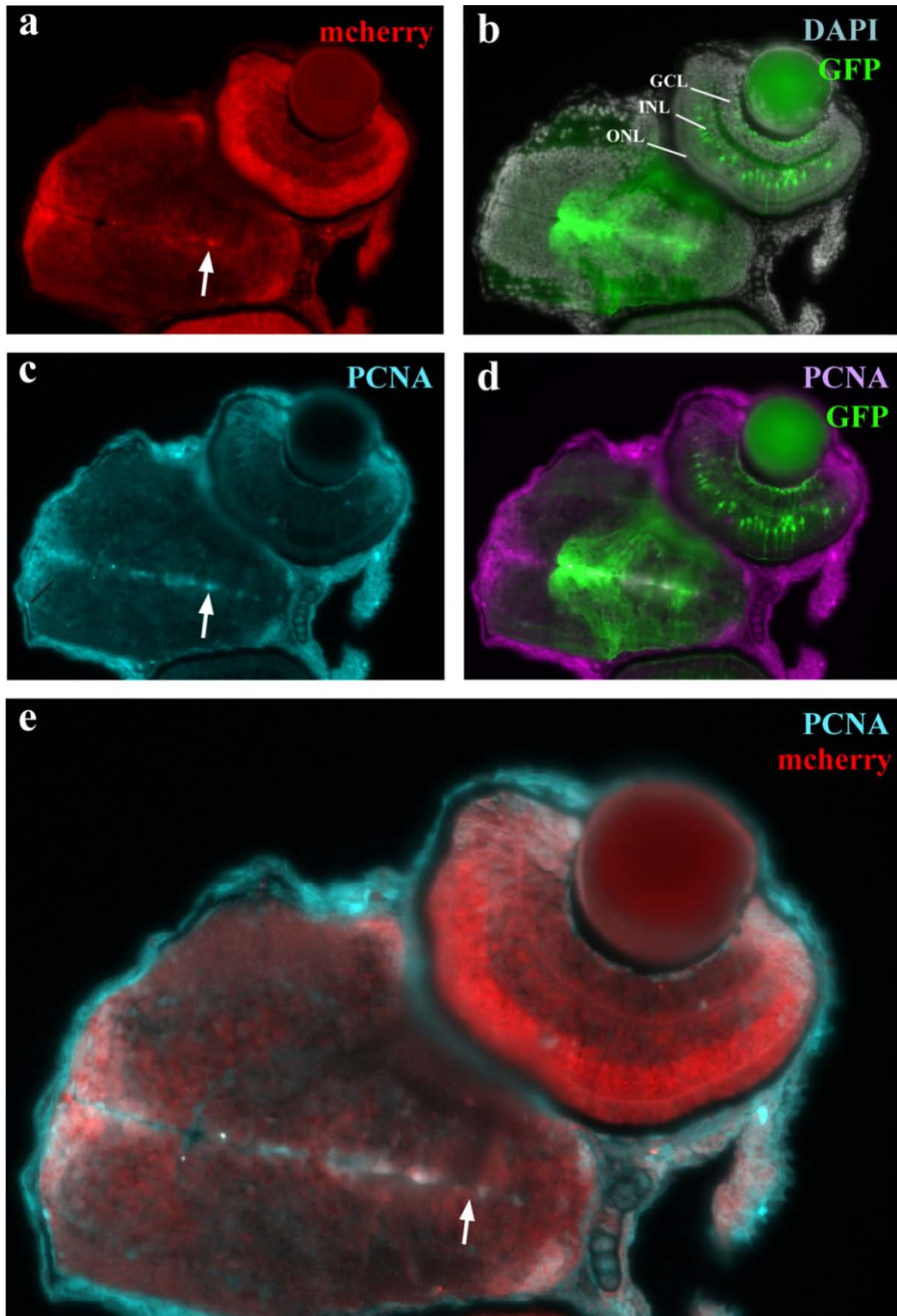


Figure 10: *gfap:EGFP-ccnb1:mcherry* embryos 3dpf ICC. Panel a: mCherry, b: DAPI, GFP merge, c:PCNA, d: PCNA, GFP merge, e: PCNA, mcherry merge. Arrows point to proliferating cells in the diencephalon.

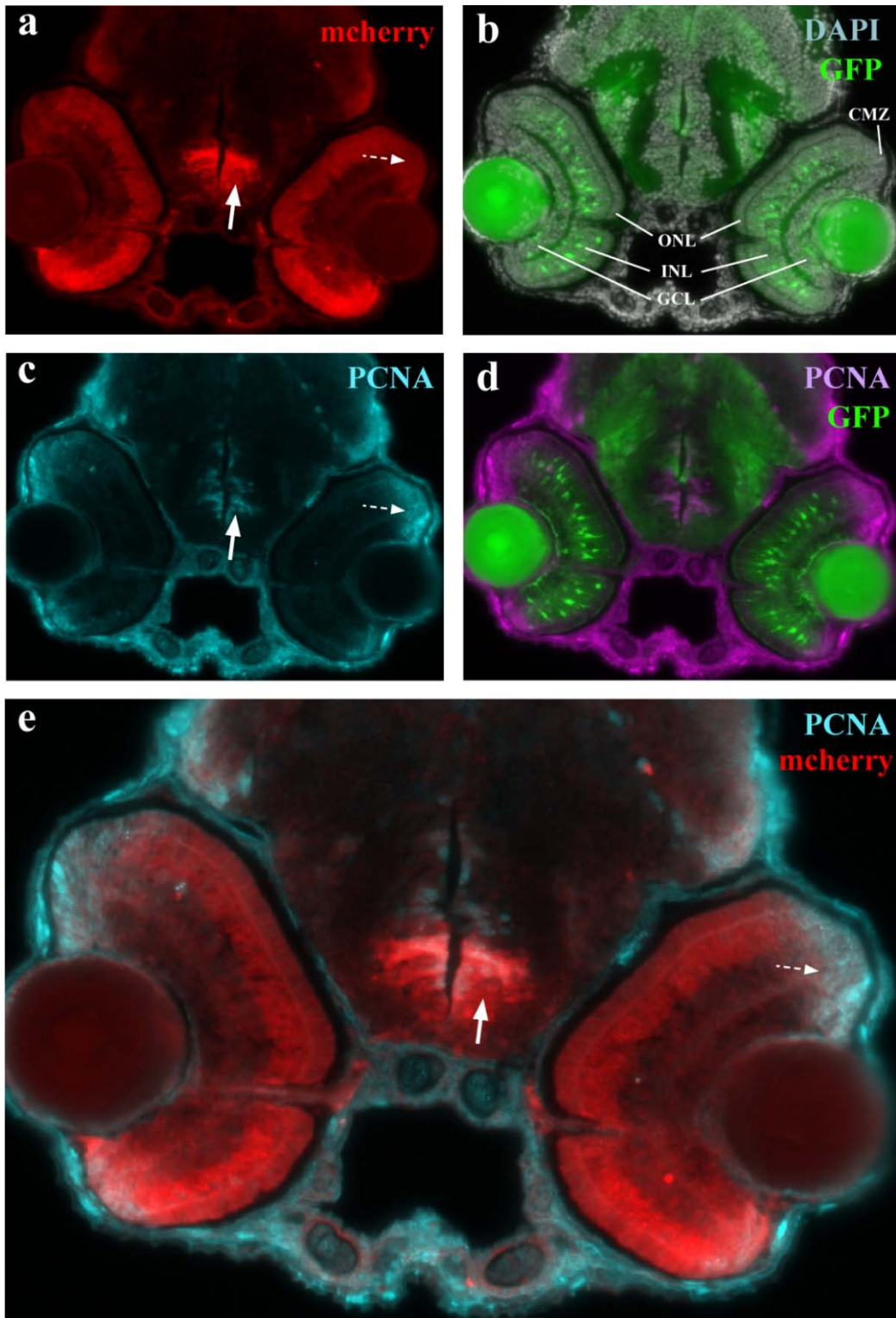


Figure 11: *gfap:EGFP-ccnb1:mcherry* embryos 3dpf cranial sections, ICC. Panel a: mCherry, b: DAPI, GFP merge, c:PCNA, d: PCNA, GFP merge, e: PCNA, mcherry merge. Arrows point to proliferating cells in the diencephalon, dashed arrows point to proliferating cells in the CMZ.