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Characterization of transgenic zebrafish lines that express GFP in the retina, pineal gland, olfactory bulb, hatching gland, and optic tectum

Wei Fang^{1,*}, Sarah Bonaffini^{1,*}, Jian Zou^{1,*}, Xiaolei Wang¹, Cen Zhang¹, Taro Tsujimura^{3,4}, Shoji Kawamura³, and Xiangyun Wei^{1,2}

¹Department of Ophthalmology, University of Pittsburgh School of Medicine, 3501 Fifth Avenue, Pittsburgh, PA 15213

²Department of Developmental Biology, University of Pittsburgh School of Medicine

³Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8562, Japan

Abstract

Transgenic animals are powerful tools to study gene function in vivo. Here we characterize several transgenic zebrafish lines that express green fluorescent protein (GFP) under the control of the *LCR^{RH2}-RH2-1* or *LCR^{RH2}-RH2-2* green opsin regulatory elements. Using confocal immunomicroscopy, stereo-fluorescence microscopy, and Western blotting, we show that the *Tg(LCR^{RH2}-RH2-1:GFP)^{pt112}* and *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115}* transgenic zebrafish lines express GFP in the pineal gland and certain types of photoreceptors. In addition, some of these lines also express GFP in the hatching gland, optic tectum, or olfactory bulb. Some of the expression patterns differ significantly from previously published similar transgenic fish lines, making them useful tools for studying the development of the corresponding tissues and organs. In addition, the variations of GFP expression among different lines corroborate the notion that transgenic expression is often subjected to position effect, thus emphasizing the need for careful verification of expression patterns when transgenic animal models are utilized for research.

Keywords

Crumbs2b; olfactory system; transgenic zebrafish; green opsin promoter; photoreceptor; retina

Introduction

The developed vertebrate retina is composed of several layers of distinct types of cells (Dowling, 1970). Photoreceptors, the sensory neurons in the retina, are positioned in the outermost layer of the retina and are responsible for the conversion of light signals to chemical neurotransmitter signals. These signals are then transmitted to the ganglion cells through the interneurons of horizontal cells, bipolar cells and amacrine cells. Like other

Corresponding authors: Xiangyun Wei: weix@upmc.edu Tel: 412-383-5845 Fax: 412-647-5880.

⁴Present address: European Molecular Biology Laboratory, Heidelberg, Germany.

*The first three authors contributed equally to this work.

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epithelium-derived tissues, the multilayered mature retina is developed from a single sheet of undifferentiated polarized neuroepithelial cells.

During development, epithelial polarity genes play essential roles in maintaining retinal cytoarchitecture and integrity (Malicki, 2000). A growing body of evidence indicates that the functions of polarity proteins vary under different biological contexts. For example, in the undifferentiated neuroepithelium, Lin7c forms a complex with Nok to maintain tissue polarity and integrity, whereas in the developed retina, Lin7c does not colocalize with Nok (Wei et al., 2006; Yang et al., 2009). Because most polarity proteins in the mature retina are also expressed in undifferentiated retinal neuroepithelium as well as in various embryonic epithelia (Gosens et al., 2008; Knust and Bossinger, 2002), conventional forward genetic approaches, such as chemical mutagenesis, can result in embryonic lethality (Malicki et al., 2002), and thus are not suitable for studying the roles of polarity genes in developed retinal photoreceptors. Therefore, it is necessary to utilize other approaches that can manipulate gene function in a temporally-controlled manner. This need may be met through the utilization of two transgenic strategies: first, RNA interference (RNAi)-based approaches to mediate sequence-specific post-transcriptional degradation of RNA transcripts (Fire et al., 1998); second, antagonist-based approaches to interfere with the functions of endogenous proteins at the post-translational level. Both strategies may be used to manipulate photoreceptor genes by using photoreceptor specific promoters.

With this notion of temporally-controlled gene function manipulation in mind, we recently conducted a study with both types of transgenic approaches to investigate the functions of Crumbs (Crb) apicobasal polarity proteins in retinal photoreceptors (Zou et al., 2012). The Crb proteins are expressed broadly in various epithelia, including undifferentiated as well as mature retina (Gosens et al., 2008; Knust and Bossinger, 2002; Tepass et al., 1990); thus it is desirable to temporally and spatially manipulate Crb expression to study their functions in the retinal photoreceptors. Although in the Zou et al. study we were not able to suppress Crb2b through transgenic shRNA expression, we obtained a number of transgenic lines that express a green fluorescent protein (GFP) reporter in various expression patterns under the control of green opsin promoter elements.

Here, we present detailed characterization of GFP expression patterns in four of these zebrafish lines. All of these lines express GFP in certain types of photoreceptors and the pineal gland. Some of these lines also express GFP in the hatching gland, olfactory system, or optic tectum. Some expression patterns differ significantly from previously published lines, making these four fish lines useful tools for studying the development of certain tissues and organs.

Methods

Generation of transgenic fish

Attempting to suppress the *crb2b* gene, four *crb2b*-targeting shRNA transgenic constructs were used to generate stable transgenic fish. Each of these constructs contains a distinct *shRNA* gene and a *GFP* reporter gene in a tandem fashion. These two genes are individually driven by an *LCR^{RH2}-RH2-2* DNA regulatory element (Fig. 1A, B). This regulator element is composed of a 0.5 kb *RH2* locus control region (LCR) and the 2.8 kb promoter region upstream of the first codon of the zebrafish *RH2-2* green opsin gene (Tsujimura et al., 2007). Thus, the transgenes contain a 5' UTR region that is identical to that of the *RH2-2 opsin* gene (Tsujimura et al., 2007). Each shRNA gene contains a 21-nt anti-sense sequence that matches with a different region of the *crb2b* coding sequence, a loop sequence, a sense sequence that complements the upstream anti-sense sequence, and finally the 3' UTR of the *sv40* gene (Fig. 1A, B). A mismatching nucleotide was introduced into the sense strand in an

effort to prevent self-targeting of the transgenic transcripts and to increase the potency of RNAi (Addepalli et al., 2010; Hamada et al., 2002).

We also attempted to generate a transgenic fish line to express a GST-His fusion protein and a GFP reporter. Such a fish line may serve as a control for transgenic fish that express GST- or His-tagged antagonists. The construct uses the *LCR^{RH2}-RH2-1* DNA regulatory element to drive transgenic expression (Fig. 2A). This regulatory element is composed of the 0.5 kb LCR of the RH2 genes and a 1.5 kb promoter region that is upstream of the first codon of the zebrafish *RH2-1* green opsin gene (Tsujimura et al., 2007). Thus, the transgenes carry the identical 5' UTR of the green opsin *RH2-1* gene (81 nt). The 3' UTR of the *sv40* gene was used as the 3' UTR of the GST-His and *GFP* genes.

The above transgene cassettes were cloned into the I-SceI meganuclease-based system for transgenesis (Thermes et al., 2002) (vector provided by Dr. Michael Tsang). About 20 pg of each transgene construct and 0.01U of I-SceI enzyme was co-injected into each wildtype TU zebrafish embryo at the 1-4-cell stages. Founder fish were raised to adulthood and outcrossed with TU wildtype fish to obtain the F1 generation. The *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115}* transgenic F1 fish were identified by GFP expression and PCR genotyping with a pair of forward primer (Rh22-5UTR-F, TCTTCGCATACGTGCAACACCTCC), which binds to the 3' end of the *RH2-2* promoter, and a reverse primer (SV40-R1, CCTGAAACATAAAATGAATGCAATTG), which binds to the 3' end of *sv40* 3' UTR sequence. The *Tg(LCR^{RH2}-RH2-1:GFP)^{pt112}* transgenic fish were genotyped with a forward primer (RH21-5UTR-F, TGCTCATTCATATTTCCACATAGGC) and a reverse primer (GST-R1, CTTTTGGACAACCACCCAAC). As controls of genomic PCR and RT-PCR, primer pairs β -actin-F (CTTTGAGCAGGAGATGGGAA), and β -actin R (TGGAAGGAGCAAGAGAGGTG) were used. The efficiency of our I-SceI meganuclease-based transgenesis varies from experiment to experiment, ranging from 3% to 25%. The initial founder fish of these lines were generated in 2004 and nine generations of these stable lines have been maintained both as heterozygous and homozygous fish thus far. These fish develop normally as wildtype and they have the lifespan and behaviors indistinguishable from wildtype fish. Experimental animals were maintained in accordance with the guidelines of the University of Pittsburgh. The study was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh (Tge University of Pittsburgh animal protocol number is 0909487B-3; the assurance number is A3187-01). These fish lines will be available to other researchers upon request and we plan to deposit them at the ZFIN fish stock center.

Immunohistochemistry

Immunohistological analyses were performed using the procedure described previously (Wei et al., 2006). The double cones were labeled with mouse monoclonal zpr1 antibodies (<http://zfin.org/action/marker/view/ZDB-ATB-081002-43>). The rabbit anti-green opsin polyclonal antibodies (provided by Drs. David Hyde and Thomas Vihtelic) were used at a 1:200 dilution. The cell nuclei were labeled with DAPI. The rabbit polyclonal anti-Crb2b antibodies (Zou et al., 2012) were used at a 1:400 dilution.

RT-PCR analyses

The fish were anesthetized with 0.8mg/ml tricaine and killed on ice. The eyes were carefully removed with a sharp pair of forceps and tales were cut from the same fish. Total RNA of eyes was extracted using Trizol Reagent (Invitrogen). To eliminate potential contamination of genome DNA, the total RNA were treated by 0.2U/ μ L DNase I (Roche) at 37°C for 30min before reversal transcription. The first strand cDNA molecules, synthesized with an

oligo-dT primer using SuperScript® III First-Strand Synthesis System (Invitrogen), was used as templates for RT-PCR analyses.

Western blot analyses

The eyes of 9-month-old pt112 and wildtype fish were homogenized in a lysis buffer on ice. The lysates were added with 5X SDS-PAGE loading buffer and heated at 100°C for 10 min. Eye proteins were separated in a 12% SDS-PAGE gel; His-tag and GST-tag recombinant proteins were used as positive controls for Western blotting using monoclonal His(27E8) and GST(26H1) antibodies (Cell Signaling Technology, Inc.).

Results

Attempts of shRNA-based RNAi to suppress Crb2b expression

Crb2b is expressed at the inner segment junctional interfaces between green, red, and blue cones in zebrafish (Zou et al., 2012). During the course of the Zou et al. study, we attempted to use transgenic RNAi to suppress Crb2b expression. When the study was started in 2004, we had a limited selection of zebrafish cone promoters. We thus decided to transgenically express *crb2b*-tageting *shRNA* genes along with a GFP reporter under the control of the *LCR^{RH2}-RH2-2* DNA regulatory element. *LCR^{RH2}-RH2-2* or *LCR^{RH2}-RH2-1* elements show regional activation in the zebrafish retina (Tsujimura et al., 2007), which actually gave us two potential benefits: This allows us to compare any differences between the transgene-activated region and non-activated region within the same retina; in addition, should transgenic expression cause photoreceptor degeneration, regional activation might allow fish to maintain a certain level of vision and to survive to breeding age.

We obtained several fish lines that show positive expression of GFP. Although PCR genotyping confirmed the integration of the *shRNA* transgenes into the fish genome, RT-PCR analyses suggest that the *shRNA* transgenes were not expressed (Fig. 1C,D). Of course, we could not rule out the possibility that the transcript were processed into siRNAs immediately after transcription, thus escaping detection by RT-PCR analyses. It is unclear if the broad smear bands below the expected position in the RT-PCR gel represent intermediate siRNA processing products or non-specific amplification (Fig. 1D). Regardless, Crb2b expression was not suppressed by the *shRNA* transgenes either in founder fish or in stable lines (Fig. 1E and 1F). Thus, the *crb2b*-tageting *shRNA* genes were ineffective in silencing the *crb2b* gene in the photoreceptors, either due to the lack of expression of the *shRNA* genes or to ineffective silencing. Consequently, these transgenic fish lines develop normally as wildtype fish. To avoid confusion, we designated these fish lines *Tg(LCR^{RH2}-RH2-2:GFP)pt115-a, b, c, i, j, and k* for future reference, where a, b, c, i, j, and k indicate lines originating from different founder fish.

Attempt to generate a GST-His-expressing line

The specific effects of transgenic antagonists can be inferred by comparing them with the effects of biologically non-active proteins. Thus, we attempted to generate a transgenic line that expresses GST-His fusion protein along with a GFP reporter under the control of the opsin *RH2-1* promoter (Fig. 2A). Such a fish line can serve as a control for those fish that express GST- or His-tagged antagonists. Although we obtained a fish line that expresses GFP, immunohistochemistry and Western blotting analyses of the eye samples indicated that the retina did not express the GST-His fusion protein (Fig. 2B, 2D). But interestingly, genomic DNA PCR and RT-PCR analyses suggest that the transgene was present in the genome and was transcribed (Fig. 2C). The reasons that the transcript was not translated are unknown. These transgenic fish develop normally as wildtype fish. To avoid confusion, we designate this fish line *Tg(LCR^{RH2}-RH2-1:GFP)pt112* for future reference.

GFP expression patterns in *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115}* and *Tg(LCR^{RH2}-RH2-1:GFP)^{pt112}*

Although the primary transgenes were not expressed or functional in the *Tg(LCR^{RH2}-RH2-1:GFP)^{pt112}* and *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115}* fish lines, their varying GFP expression patterns make them excellent research tools. They differ significantly in ectopic GFP expression patterns in non-green cone photoreceptors as well as in certain non-eye tissues and organs (Table 1). These ectopic expression patterns differentiate these fish lines from one another. However, no difference in expression patterns was detected among the same types of transgenic fish at the same ages (number of fish = 5 for each line and for each age group). In addition, no difference was observed between founder fish, generation 4 and generation 7 at the histological level and among all generations at the stereomicroscopic fluorescent imaging level. The detailed GFP expression patterns of *Tg(LCR^{RH2}-RH2-1:GFP)^{pt112}*, *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-k}*, *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-c}*, and *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-j}* were analyzed in detail and presented as follows:

In *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-k}*, GFP is expressed in the retina, pineal gland, and hatching gland (Fig. 3). Apparent retinal GFP expression can be detected as early as 60 hpf (hours postfertilization) and persists throughout adulthood. Immunocytochemistry showed that GFP is specifically expressed in green cones (Fig. 3H-I). The coverage of GFP expression in the retina varies at different ages. GFP is expressed in the entire retina at 5 dpf and 1 mpf (month postfertilization) (Fig. 3B-E). By contrast, in adult retina, GFP is not expressed in the ventral, anterior peripheral, and posterior peripheral regions (Fig. 3F-G). Besides the retina, GFP is also weakly expressed in the hatching gland at 2 dpf (days postfertilization) (Fig. 3A; arrows, inset), and this expression disappears after 3 dpf.

In *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-c}*, GFP is expressed in the retina, pineal gland, hatching gland, and optic tectum (Fig. 4). The specific green cone GFP expression (Fig. 4M, N), the coverage of GFP expression in the entire retina at early stages (Fig. 4 G-J), and weak hatching gland GFP expression (data not shown) are similar to that of *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-k}*. Interestingly, unlike *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-k}*, in adult retina, GFP expression extends more in peripheral directions, particularly in the ventral direction (Fig. 4K-L). At 4 dpf, GFP is also expressed in about a dozen cells in a superficial region of the optic tectum (Fig. 4C-F). These cells appear to be neurons because they bear extensive nerve fiber-like processes (Fig. 4D-F; Supplementary Movie 1). The identity of these cells remains to be determined.

In *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-j}*, GFP is expressed in the retina, pineal gland, hatching gland, olfactory bulb, and various connective tissues (Fig. 5). In the adult retina, GFP is strongly expressed in green cones and weakly in rods, blue cones, and red cones (Fig. 5D, E). However, not all red cones and rods express GFP (Fig. 5D; asterisks). The GFP expression extends to all retinal regions including the ventral region at 5 dpf, 1 mpf, and 9 mpf (Fig. 5H-I; data for 5dpf not shown). GFP is also expressed in a distinct local region within the olfactory bulb; this region is adjacent to the olfactory epithelium (Fig. 5B, arrows). In the head and trunk regions, GFP is also extensively expressed in many connective tissues that are presumably derived from mesenchymes (Fig. 5C, arrows).

In *Tg(LCR^{RH2}-RH2-1:GFP)^{pt112}*, GFP is expressed in the retina and pineal gland. In the retina of 3-mpf (month post fertilization) fish, GFP is expressed strongly in both green and blue cones (Fig. 6A-C). Weaker GFP expression in the red cones can be visualized under stronger laser excitation conditions which saturate the GFP signals in the green and blue cones (Fig. 6D). At 7 mpf, while still much weaker than in green and blue cones, GFP in most red cones becomes apparent at milder laser excitation conditions (Fig. 6E), suggesting that the expression level of GFP in the red cones increases over time. Occasionally, some green, red, and blue cones do not express GFP (Fig. 6D, arrowheads; data not shown). These

GFP-negative cones appear to be distributed randomly, with relatively higher frequency in the peripheral retina. Unlike the *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-k}* line, GFP expression is present in all retinal regions in the photoreceptor layer at 5 dpf, 1 mpf, and 9 mpf (Fig. 6 F-I; data for 5dpf retina not shown). The distinct GFP expression patterns in *Tg(LCR^{RH2}-RH2-1:GFP)^{pt112}* make it a unique tool for simultaneously labeling the green, red and blue cones in the retina. By using zpr1 antibodies to visualize green/red double cones, the three types of long cones can be easily distinguished: blue cones should express strong GFP signals and are negative for zpr1; green cones should express strong GFP signals and are positive for zpr1; and red cones should be positive for zpr1 and weakly positive or negative for GFP (Fig. 6B-E).

Discussion

In this study, we utilized both the *LCR^{RH2}-RH2-1* and *LCR^{RH2}-RH2-2* regulatory elements of the green opsin genes to drive transgenic expression. We characterized the various GFP expression patterns in *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-k}*, *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-c}*, *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-j}*, and *Tg(LCR^{RH2}-RH2-1:GFP)^{pt112}*. Compared with the transgenic lines generated previously using a large P1-artificial chromosome DNA fragment that contains green opsin regulatory elements (Tsujimura et al., 2007), the distinct expression patterns of our fish lines establishes them as useful tools for visualizing certain cell types in the retina and other tissues.

Variations of GFP expression patterns among different transgenic lines

Opsins are the protein moiety of visual pigments. There are several types of opsins, each of which has a distinct light absorption spectrum (Applebury et al., 2000; Chinen et al., 2003; Yokoyama, 1997). Because each opsin is specifically expressed in a distinct type of photoreceptor, opsin promoters have been conveniently used to drive transgenic expression in photoreceptors (Chinen et al., 2003; Fadool, 2003; Hamaoka et al., 2002; Kennedy et al., 2007; Takechi et al., 2003; Takechi and Kawamura, 2005; Takechi et al., 2008; Tsujimura et al., 2007).

Although all *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115}* and *Tg(LCR^{RH2}-RH2-1:GFP)^{pt112}* lines express GFP in the green cones and pineal gland, the observation of ectopic GFP expression patterns in other tissues and cell types in *pt112*, *pt115-c*, and *pt115-j* is interesting. There are many possibilities to explain the phenomena of ectopic expression. The hatching gland expression of GFP in all *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115}* lines but not in *RH2-1*-driven *Tg(LCR^{RH2}-RH2-1:GFP)^{pt112}* suggests that unlike the *LCR^{RH2}-RH2-1* regulatory element, the *LCR^{RH2}-RH2-2* element may miss certain necessary elements that restrict its activation in the hatching gland (Table 1; data not shown). Alternatively, there is a hatching gland activating element in the *LCR^{RH2}-RH2-2* element that is normally suppressed in the endogenous chromatin environment. Another possibility is that the tandem configuration of two *LCR^{RH2}-RH2-2*-driven genes may compromise its specificity, which was observed previously (Tsujimura et al., 2007). Like the Tsujimura et al transgenic lines (Tsujimura et al., 2007), *RH2-1* and *RH2-2* regulatory elements are not activated in a significant large ventral retina in *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-k}*. However, in *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-c}* the ventral retinal region that does not express GFP is much smaller (Fig. 3G; Fig. 4L). Furthermore, in *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-j}* and *Tg(LCR^{RH2}-RH2-1:GFP)^{pt112}* fish GFP is expressed in the entire adult retina (Fig. 5H-I; Fig. 6H-I). Interestingly, at 5 dpf and 1 mpf, GFP expression is extended to the entire retina in all of these four lines, indicating a developmental regulation of the *RH2-1* and *RH2-2* activities (Figs. 3B-E; 4G-J; 5F-G; 6F-G).

The position effects may also contribute to ectopic GFP expression because the expression patterns vary from one transgenic line to another. For example, the olfactory bulb GFP expression in pt115-j and the optic tectum GFP expression in pt115-c are not present in pt115-k. In addition, GFP expression levels in the blue cones vary dramatically between pt111, *Tg(LCR^{RH2}-RH2-1:GFP)^{pt112}*, pt116, and *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-i}*, and *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-j}* (Table 1; data not shown). Similarly, Tsujimura also noticed transgenic expression of GFP in their *Tg(LCR-RH2-1:GFP)* fish (Tsujimura et al., 2007). Furthermore, these fish lines also vary in terms of the GFP expression in the ventral and peripheral retina regions. The lack of GFP expression in the peripheral retinal in pt115-k is very similar to the expression pattern of *Tg(RH2-1/GFP-PAC)* (Tsujimura et al., 2007). This suggests that the RH2-2up2.8kb element may regulate the regional specificity in retinal expression although this specificity can be easily influenced by other factors. These observations corroborate the necessity to strictly verify transgenic expression patterns with suitable reporters whenever transgenic approaches are used to manipulate gene functions. Despite the extensive ectopic expression in some of these lines, we have never found UV cones expressing GFP. This suggests the presence of certain mechanisms by which UV cones robustly suppress the activation of *LCR^{RH2}-RH2-1* and *LCR^{RH2}-RH2-2*.

Utility of *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115}* and *Tg(LCR^{RH2}-RH2-1:GFP)^{pt112}* as tools

Since the initial generation of the founder fish of these lines in 2006, nine generations of these lines have been maintained and the GFP expression patterns have maintained stable and strong in either homozygous or heterozygous of these lines, suggesting that the GFP expression is unlikely to alter in future generations.

The various GFP expression patterns in these lines make them useful tools to distinctively visualize and tag certain types of cells. Specifically, for *Tg(LCR^{RH2}-RH2-1:GFP)^{pt112}*, the strong GFP expression in both the green and blue cones in the entire retina differs significantly from the expression pattern reported by Tsujimura et al. (Tsujimura et al., 2007), in which GFP is only expressed in green cones in dorsal retina. As indicated in Figure 6, when combined with immunostaining with the *zpr1* antibody, the GFP expression in *Tg(LCR^{RH2}-RH2-1:GFP)^{pt112}* allows simultaneous distinguishing of green, red, and blue cones in all retinal regions. This staining pattern is more suitable for analyzing the mosaic distribution of cones in the zebrafish retina than using anti-opsin antibodies that stain the outer segments, which are not as neatly aligned as inner segments because the outer segments of different types of cones are position in different levels in the basal-apical axis. This utility has never been reported before. In addition, GFP expression in these lines allows the expansion of immunostaining to detect more proteins of interest in conjunction with visualization of specific cone types. Furthermore, GFP expression facilitates live imaging or FACS sorting analyses.

For *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-j}*, a smaller group of olfactory bulb cells express GFP. This pattern is different from that of the *Tg(dat:EGFP)* transgenic line, in which the entire olfactory bulb appears to express GFP (Xi et al., 2011). Thus this line can be used as a new tool to label and visualize a sub-region in the olfactory bulb.

For *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-c}*, the expression of GFP in the small group of surface cells in the optic tectum has not been reported before. It would be interesting to determine if these cells are the brain cells that facilitate the sensing of the light-dark zeitgeber. This fish line will potentially serve as a unique tool to study the involvement of optic tectum in circadian regulations in zebrafish.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

GFP expression in the green and blue cones in $Tg(LCR^{RH2}-RH2-1:GFP)^{pt112}$

GFP expression in the green cones in $Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-k}$

GFP expression in the optic tectum in $Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-c}$

GFP expression in the olfactory bulb in $Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-j}$

Figure 1.

Crb2b expression in photoreceptors is not suppressed in transgenic fish *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115}*. **A**. A schematic illustrates the structures and tandem arrangement of a *crb2b*-targeting *shRNA* gene and a *GFP* reporter gene in the transgenic constructs for generating the *Tg(RH2-2:GFP)^{pt115}* fish lines. The region amplified in the PCR genotyping assay (see panel C) is indicated with a double-headed arrow. **B**. The sequences of the sense and anti-sense regions of the *crb2b*-targeting *shRNA* genes are presented and the mismatching nucleotides are underlined. **C**, **D**. PCR analyses suggest that the *shRNA* transgenes are present in all stable pt115 lines (arrow) at 5 dpf (C) and 9 mpf (D). However, RT-PCR analyses did not suggest apparent *shRNA* transgene transcripts at the size of genomic DNA PCR product. Genomic DNA PCR and RT-PCR of β -actin gene were conducted as controls for the quality of PCR DNA template, especially for genomic DNA contamination of cDNA. **E**. The expression levels of *Crb2b* (red) in the transgenically expressing (arrowheads) and transgenically non-expressing retinal regions (arrows) were similar in adult *shRNA*-1 founder fish, suggesting no suppression of *Crb2b* by the *shRNA* transgenes. Transgenic cells were visualized by *GFP* reporter signals. Green cones are labeled with the letter G, and blue cones are labeled with letter B. The identification of cone types was based on the stereotypical spatial alignment of zebrafish cones in the order of UV-green-red-blue-red-green within the two dimensional photoreceptor mosaics (Robinson et al., 1993; Raymond et al., 1995; Zou et al., 2012) and green/red double cone (DC) staining with the *zpr1* antibody. This identification method was also applied to Figures 3-6. **F**. In homozygous adult transgenic *Tg(LCR^{RH2}-RH2-2:GFP)^{pt11-k5}* fish, the expression of *Crb2b* did not show apparent reduction because the expression levels at cone inner segment junctions next to transgenically-expressing cells (arrows) and non-transgenically-expressing cells (arrowheads) were identical.

Figure 2.

Transgenic zebrafish line $Tg(LCR^{RH2}-RH2-1:GFP)^{pt112}$, expresses GFP but not GST-His fusion in the retina. **A**. The schematic illustrates the structures and tandem arrangement of the *GST-His* and *GFP* reporter genes in the transgene construct that was used to generate the $Tg(LCR^{RH2}-RH2-1:GFP)^{pt112}$ line. The regions amplified by PCR genotyping reactions are indicated with double-headed arrows. **B**. In the adult $Tg(LCR^{RH2}-RH2-1:GFP)^{pt112}$ retina at 8 mpf (months postfertilization), the expression of the GST-His protein was undetectable, whereas GFP expression is very strong. **C**. RT-PCR and genomic DNA PCR analyses demonstrated that the *GST-His* gene was present in the $Tg(LCR^{RH2}-RH2-1:GFP)^{pt112}$ genome, and was apparently transcribed at both 5 dpf and 9 mpf. **D**. Western blotting analyses confirmed that GST-His fusion protein was not expressed in $Tg(LCR^{RH2}-RH2-1:GFP)^{pt112}$ fish at both 5 dpf and 9 mpf. The asterisks indicate the position where the fusion protein was supposed to be. Unrelated His-Tagged and GST-tagged recombinant proteins expressed in *E. coli* were used as controls for Western blotting conditions.

Figure 3.

The *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-k}* line expresses GFP in the green cones and hatching gland. **A.** Stereo-fluorescent microscopy shows weak GFP signals in the hatching gland at 2 dpf (arrows). The inset shows a magnified image of the GFP-positive hatching gland cells. The green fluorescent signals in the yolk were non-specific auto-fluorescence. **B-E.** GFP expression is present in the entire photoreceptor layer in the retina at 5 dpf and 1 mpf. A-P and D-V arrows are for anterior-posterior and dorsal-ventral direction, respectively. **F-G.** At 9 mpf, the GFP expression is absent in anterior and posterior peripheral retinal regions as well as a significantly large ventral retinal area. **H-I.** Vertical (H) and transverse (I) imaging of photoreceptors show GFP (green) is specifically expressed in green cones (arrow G), but not in red cones (arrow R) or other types of photoreceptors (8 mpf). The green/red double cones were visualized with *zpr1* antibodies. The cell nuclei were labeled with DAPI.

Figure 4.

The *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-c}* line expresses GFP in the green cones, hatching gland, and optic tectum. **A-B.** Bright field (A) and stereo-fluorescence (B) imaging shows GFP signals in the retina at 4 dpf. The top fish is a wildtype larval control, and the bottom is a *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-c}* transgenic larva. **C.** There are about one dozen GFP positive cells on each side of the brain in the optic tectum region at 4 dpf (arrows). **D.** A confocal image illustrates the extensive cell processes of the GFP-positive cells in the optic tectum, suggesting that they are neurons (arrowheads for the cell bodies). **E-F.** Confocal imaging of the cross sections of the 4-dpf brain revealed that the GFP positive cells in the optic tectum are in the superficial regions. Insets show a magnified superficial region of the optic tectum (OT) and the arrowheads indicate the cell bodies of GFP positive cells. The nuclei were labeled with TO-PRO. Actin was labeled with phalloidin in blue. **G-J.** GFP expression is present in the photoreceptor layer of the entire retina at 5 dpf and 1 mpf. A-P and D-V arrows indicate anterior-posterior and dorsal-ventral directions, respectively. **K-L.** At 9 mpf, the entire retina maintains strong GFP expression, except for a small ventral retinal region (arrowhead, L). **M-N.** Only green cones in 8-mpf adult retina express GFP. The double cones (red) were visualized by the *zpr1* antibodies. M shows a vertical section and N shows a transverse section.

Figure 5.

The *Tg(LCR^{RH2}-RH2-2:GFP)^{pt115-j}* line expresses GFP in several types of photoreceptors, the hatching gland, pineal gland, and olfactory bulb. **A.** Bright field and fluorescence images show GFP signals (green) in the hatching gland, pineal gland, and a small region in the forebrain (arrowhead) at 3 dpf. **B.** Imaging on the frontal plane revealed that the GFP-positive cells in the forebrain at 48 hpf (panel A, arrowhead) correspond to a small region in the olfactory bulb (arrows), immediately next to the olfactory epithelia (OE). The anterior is on top. **C.** A more ventral-positioned imaging on the frontal plane at 48 hpf (compared to panel B) revealed extensive GFP expression in the mesenchyme-derived tissues in the head and trunk (arrows). **D-E.** Vertical (D) and transverse (E) confocal imaging of photoreceptors in 8-mpf adult retina revealed GFP signals in the green, red, and blue cones as well as some rods (arrowheads). However, certain rods do not express GFP (asterisks). Double cones were labeled with *zpr1* antibody in red; arrow G, green cones; arrow B, blue cones; arrow R, red cones. **F-G.** GFP is expressed in the photoreceptor layer in the entire retina at 1 mpf. **H-I.** At 9 mpf, strong GFP expression is maintained in the photoreceptor layer in the entire retina. A-P and D-V arrows indicate anterior-posterior and dorsal-ventral directions, respectively.

Figure 6.

The *Tg(LCR^{RH2}-RH2-1:GFP)^{pt112}* line expresses GFP strongly in green and blue cones and weakly in red cones in the retina. **A, B.** At a low non-saturating laser excitation condition, similarly strong GFP expression levels were detected in the green and blue cones in 3-mpf retina. The panels in B are magnified local regions from the panels in A. **C.** Transverse imaging of the photoreceptor layer shows the mosaic organization of the GFP-expressing green and blue cones at 3 mpf. **D.** At a strong saturating laser excitation condition, weak GFP expression was detected in some but not all red cones (arrowheads) in the 3-mpf retina. **E.** At 7 mpf, weak GFP expression was detectable in most red cones even under non-saturating laser excitation conditions. **F-I.** GFP expression is present in the photoreceptor layer in the entire retina at 1 mpf and 9 mpf. The nuclei were labeled with TO-PRO nuclear dye or DAPI (blue, A-C). UV, green, red, and blue cones are indicated with lettered-arrows U, G, R, and B, respectively.

GFP expression patterns among different transgenic fish lines

Table 1

Table 1 summarizes the distinct patterns and intensity of *LCR^{RH2-RH2-1}*-driven and *LCR^{RH2-RH2-2}*-driven GFP expression in various types of photoreceptors, the hatching gland, optic tectum, and olfactory bulb in various transgenic lines. Stronger GFP expression levels are indicated with more positive signs. Non-detectable GFP expression is indicated with negative signs. Simultaneous positive and negative signs indicate that some cells express GFP while others of the same types do not. Transgenic lines pt111 and pt116 were generated to express GFP as well as Crb2b-lf and Pard3 in green cones, respectively (unpublished data).

regulatory elements	Transgenic lines	Green	Red	Blue	UV	Rod	Hatching gland	Or	OB	VR	PG	Figure presentation
<i>LCR^{RH2-RH2-2}</i>	pt115-k	+++	-	-	-	-	+	-	-	-	+	Fig. 3
<i>LCR^{RH2-RH2-2}</i>	pt115-c	+++	-	-	-	-	+	+	-	-	+	Fig. 4
<i>LCR^{RH2-RH2-2}</i>	pt115-j	+++	-	+	-	+	+++	-	+	+	+	Fig. 5
<i>LCR^{RH2-RH2-2}</i>	pt115-i	++	+	+	-	++	+++	-	-	N/A	+	Data not show
<i>LCR^{RH2-RH2-2}</i>	pt115-b	+++	-	-	-	+	+++	-	-	N/A	+	Data not show
<i>LCR^{RH2-RH2-2}</i>	pt115-a	+++	-	-	-	-	++	-	-	N/A	+	Data not show
<i>LCR^{RH2-RH2-1}</i>	pt112	+++	+	+++	-	-	-	-	-	+	+	Fig. 6
<i>LCR^{RH2-RH2-1}</i>	pt111	+++	-	+	-	-	-	-	-	N/A	+	Unpublished data
<i>LCR^{RH2-RH2-1}</i>	pt116	+++	+/-	+/-	-	-	-	-	-	N/A	+	Unpublished data

Green, green cones; Red, red cones; Blue, blue cones; UV, UV cones; OT, the optic tectum; OB, olfactory bulb; VR, ventral retina; PG, pineal gland