PROCEEDINGS B

rspb.royalsocietypublishing.org

Research

Cite this article: Ogawa Y, Shiraki T, Kojima D, Fukada Y. 2015 Homeobox transcription factor Six7 governs expression of green opsin genes in zebrafish. *Proc. R. Soc. B* **282**: 20150659.
http://dx.doi.org/10.1098/rspb.2015.0659

Received: 24 March 2015
Accepted: 16 June 2015

Subject Areas:
genetics, developmental biology, evolution

Keywords:
photoreceptor development, cone opsin, green opsin, *six7*, zebrafish (*Danio rerio*)

Author for correspondence:
Yoshitaka Fukada
e-mail: sfukada@mail.ecc.u-tokyo.ac.jp

†These authors contributed equally to this study.

Electronic supplementary material is available at http://dx.doi.org/10.1098/rspb.2015.0659 or via http://rspb.royalsocietypublishing.org.

THE ROYAL SOCIETY PUBLISHING

Homeobox transcription factor Six7 governs expression of green opsin genes in zebrafish

Yohey Ogawa†, Tomoya Shiraki†, Daisuke Kojima and Yoshitaka Fukada

Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan

YO, 0000-0001-9031-4393; TS, 0000-0003-0447-007X; DK, 0000-0003-2658-780X

Colour discrimination in vertebrates requires cone photoreceptor cells in the retina, and high-acuity colour vision is endowed by a set of four cone subtypes expressing UV-, blue-, green- and red-sensitive opsins. Previous studies identified transcription factors governing cone photoreceptor development in mice, although loss of blue and green opsin genes in the evolution of mammals make it difficult to understand how high-acuity colour vision was organized during evolution and development. Zebrafish (*Danio rerio*) represents a valuable vertebrate model for studying colour vision as it retains all the four ancestral vertebrate cone subtypes. Here, by RT-qPCR and *in situ* hybridization analysis, we found that *sine oculis* homeobox homolog 7 (*six7*), a transcription factor widely conserved in ray-finned fish, is expressed predominantly in the cone photoreceptors in zebrafish at both the larval and the adult stages. TAL effector nuclease-based *six7* knock-out revealed its roles in expression of green, red and blue cone opsin genes. Most prominently, the *six7* deficiency caused a loss of expression of all the green opsins at both the larval and adult stages. *six7* is indispensable for the development and/or maintenance of the green cones.

1. Introduction

In vertebrates, vision is triggered by absorption of photons by visual opsins localized in outer segments of retinal photoreceptor cells. The photoreceptor cells are classified into rod and cone cells, which are distinct from each other in morphology and photoresponse [1–3]. Rods are responsible for twilight vision, while cones function under the daylight condition. Combination of multiple cone subtypes (i.e. UV-, blue-, green- and red-sensitive cones) confers many vertebrates with high-acuity colour vision [4,5], which is based on mutually exclusive expression of one subtype of the opsin in single cells. One of the key vertebrate species that helps us understand visual system evolution is the Southern Hemisphere lamprey, *Geotria australis*: it is a jawless vertebrate whose ancestor diverged from the lineage leading to jawed vertebrates probably before 500 Ma [6], and it has four subtypes of the cone opsins [7], namely UV (short wavelength sensitive 1: SWS1), blue (short wavelength sensitive 2: SWS2), green (medium wavelength sensitive: RH2) and red (long wavelength sensitive: M/LWS). The ancestral species of the jawed vertebrate is thus thought to have acquired four spectrally distinct cone subtypes by two rounds of chromosome duplications and appears to have had the tetrachromatic colour vision system [8].

The developmental process of visual photoreceptors has been well studied in mice, and important transcription factors governing the development of the photoreceptors have been identified [9,10]. In particular, cone-rod homeobox (*Crx*) is a master transcriptional regulator for differentiation of retinal progenitor cells into photoreceptor cells [11–13]. *Crx* determines the cell fate of the photoreceptor progenitor cells in combination with other transcription factors such as thyroid hormone receptor beta (*Thrb*), which regulates development of the cone photoreceptors expressing *M/LWS* opsin gene [14]. On the other hand, transcriptional regulatory mechanisms of *SWS2* and *RH2* opsin genes remain elusive because

mammals lost *SWS2* and *RH2* opsin genes during evolution. Hence, the vertebrate species having all four cone subtypes provide an excellent platform to study detailed mechanisms of the development of the four cone photoreceptors.

Zebrafish (*Danio rerio*) is a diurnal teleost having four cone subtypes, and it is a valuable animal model to study genetic mechanisms of vertebrate retinal development and diseases [15]. Recent studies isolated the promoter and/or enhancer regions sufficient for exclusive expression of the zebrafish cone opsin genes in a specific subtype of cones, such as UV-(*sws1*) [16], blue-(*sws2*) [17], green-(*rh2-1*, *rh2-2*, *rh2-3* and *rh2-4*) [18] or red-sensitive (*lws1* and *lws2*) cones [19]. Two transcription factors, T-box 2b (*tbx2b*) and *thrb*, were identified to be crucial for expression of a particular subtype of cone opsins in zebrafish: *tbx2b* is required for gene expression of *sws1* [20], while *thrb* is involved in the expression of *lws1* and *lws2* [21]. The role of *thrb* in M/LWS opsin gene expression is conserved between mouse and zebrafish, suggesting that these two vertebrate species share a mechanism underlying exclusive expression of the opsin genes in cone cells. Despite these advances, studies so far have been unable to identify any transcription factors responsible for expression of SWS2 and RH2 in vertebrates.

To understand regulation of *sws2* and *rh2* opsin genes, we searched for cone-specific transcription factors in zebrafish and found that *sine oculis* homeobox homolog 7 (*six7*) is predominantly expressed in cone photoreceptors. The Six family is a group of evolutionarily conserved transcription factors found in diverse organisms ranging from worms to humans [22]. Previous studies reported that *six7* is expressed during gastrulation and early segmentation [23], and that combined inactivation of two Six3/6/7 group members, *six7* and *six3b*, results in loss of the eyes [24]. Despite the involvement of *six7* in early eye development, it still remains unclear whether and how *six7* is engaged in cone photoreceptor development. We investigated roles of *six7* in cone development and in cone opsin expression by generating *six7* knock-out (KO) zebrafish with TAL effector nucleases (TALENs). The *six7* KO zebrafish lost expression of the green opsins at both larval and adult stages, suggesting that *six7* is indispensable for the development of the green cones.

2. Material and methods

Full methods and any associated references are available in the electronic supplementary material, methods.

(a) Zebrafish

Zebrafish were treated in accordance with the guidelines of the University of Tokyo. The wild-type (WT) zebrafish strain Ekk-Will were raised in a 14 L : 10 D cycle, and fed twice per day with living baby brine shrimps. Embryos were raised at 28.5°C in egg water (artificial seawater diluted 1.5 : 1000 in water).

(b) Construction of TAL effector nuclease targeting-vector

We designed TAL effector repeats recognizing exon 1 of zebrafish *six7* gene with the Golden Gate method [25]. These repeats were cloned into our original vectors (pTAL7DD and pTAL7RR), which were modified from pTAL3 vector (Addgene plasmid 31034), based on pCS2TAL3DD and pCS2TAL3RR [26]. Information about construction of pTAL7DD and pTAL7RR vectors is in the electronic supplementary material, methods.

(c) Immunohistochemistry and *in situ* hybridization

Immunohistochemistry and *in situ* hybridization were performed as described previously [27–30] with some modifications. Detailed information about the methods is in the electronic supplementary material, methods.

(d) Isolation of rods and cones

EGFP-positive rods and cones were isolated by fluorescence activating cell sorter (FACS) from the adult retinas of the two lines of transgenic zebrafish, *Tg*(*rho:egfp*)^{ja2} [31] and *Tg*(*gnat2:egfp*)^{ja23}, respectively. These lines express green fluorescent proteins in rods and all the cone subtypes, respectively. *Tg*(*gnat2:egfp*)^{ja23} was generated according to the method described in the previous study [32]. Detailed procedures for isolation of photoreceptors are described in the electronic supplementary material, methods.

(e) RNA extraction and RT-qPCR

Total RNA was extracted and purified using RNeasy Micro Kit (74004, Qiagen) from the eyes of adult or larval zebrafish. The ocular RNA (450 ng for the adult eye or 90 ng for the larval eye) was reverse-transcribed into cDNA using the oligo $(dT)_{15}$ primer with GoScript Reverse Transcriptase (A5003, Promega). The cDNA was subjected to quantitative PCR using GoTaq qPCR Master Mix (A6001, Promega) with the StepOnePlus Real-time PCR system (Applied Biosystems), following the manufacturer's protocol. The gene-specific primers used for RT-qPCR are listed in the electronic supplementary material, table S3. In all the figures, the mRNA levels were normalized to beta-actin 2 (*actb2*) mRNA levels.

3. Results

(a) Cone-specific expression of *six7*

In microarray analysis, we compared gene expression profiles between rod and cone cells purified from the adult zebrafish retina (yet to be published). Among approximately 500 genes that were found to be abundantly expressed in cones (more than 10 times higher than in rods), we focused on *sine oculis* homeobox homolog 7 (*six7*), whose signal intensity in cones was the highest among the cone-enriched transcription factors in our microarray analysis. Six7 belongs to Six family, and Six7 is found in ray-finned fish but not in mammals (figure 1). The phylogenetic tree showed that, among Six3/6/7 group, Six7 subfamily was separated before the divergence between Six3 and Six6 subfamilies, both of which are widely conserved in vertebrate species (figure 1).

We investigated the expression pattern of *six7* in zebrafish. RT-qPCR assay confirmed that *six7* was far more abundantly expressed in cones than in rods (more than 10 times; figure 2*a*). Among the adult tissues, *six7* was predominantly expressed in the retina (figure 2*b*). *In situ* hybridization analysis of the retina indicated that *six7* was expressed specifically in the photoreceptor layer (figure 2*d*). In particular, the *six7*-positive cells were mainly located in the layer of the red and green cones (figure 2*e*), suggesting that *six7* regulates transcription in the green and red cones.

We then analysed expression profiles of *six7* during the retinal development. At 4 days post fertilization (4 dpf), *six7* expression was detected in the anterior regions of the

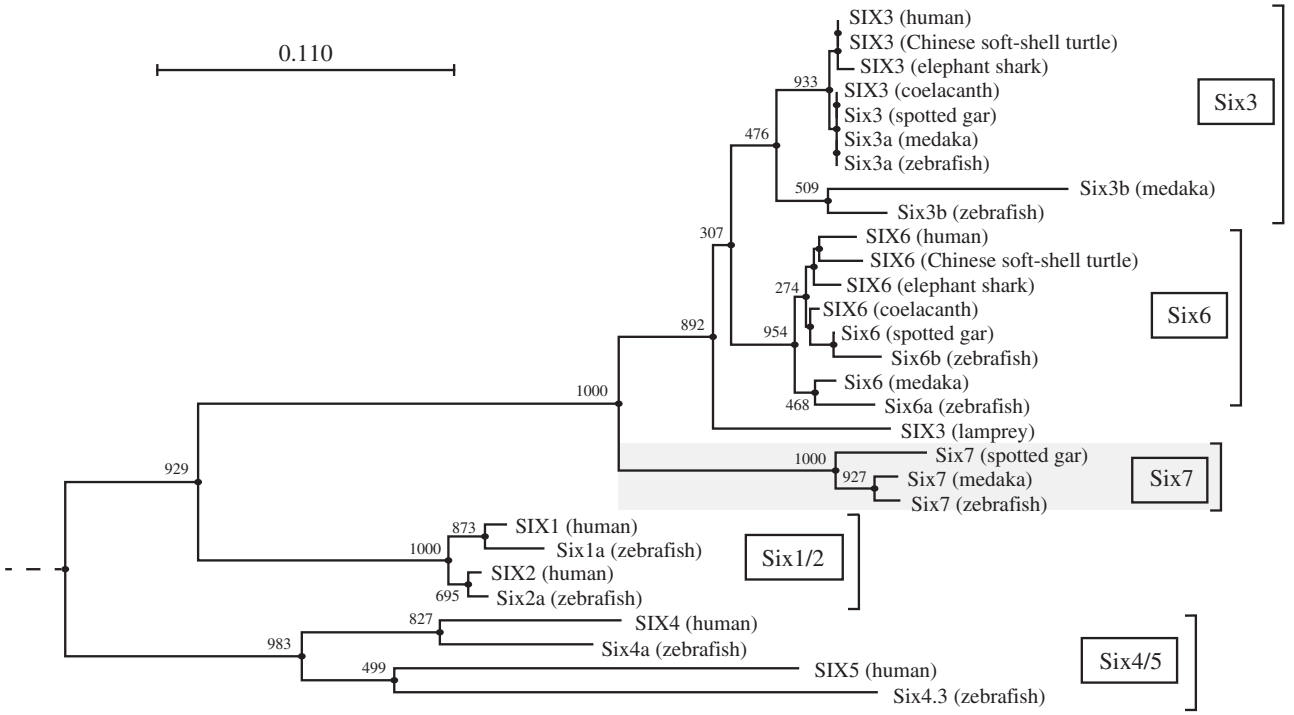

Figure 1. Phylogenetic tree of the members in Six family. A neighbour-joining (NJ) tree was constructed from amino acid sequences of SIX domain and homeodomain with 1000 bootstrapping replications. Numerical values indicating bootstrap support are shown at the base of each node. Nematode CEH-34 was employed as an outgroup. Scale bar, 0.11 substitutions per site. A phylogenetic tree including additional members of Six1/2 and Six4/5 groups is presented in the electronic supplementary material, figure S6*a*.

larvae (including the eyes), but not in the posterior region (figure 2*c*). Eye-specific expression of *six7* was confirmed by whole-mount *in situ* hybridization (figure 2*f*). The *six7* expression in the eye began at 1.5 dpf, when the retinal development just starts, suggesting the *six7* expression in the retinal progenitor cells. *In situ* hybridization using 3.5 dpf zebrafish ocular sections revealed that *six7*-positive cells were localized in the photoreceptor cell layer, in which the positive cells were distributed uniformly across the whole retina (figure 2*g*). Considering that rods are tightly clustered together in the ventral retina at this developmental stage [33] (see also figure 3*c*), the expression pattern of *six7* was similar to that of the cone-specific phototransduction component genes. Although we cannot rule out the possibility that *six7* is also expressed in rods at the larval stages, these results imply that *six7* is predominantly expressed in cones at 3.5 dpf and suggest that *six7* plays a role in the cone development.

(b) Decrease of green and blue cones in the larva of *six7* knock-out zebrafish

To examine the role of *six7* in photoreceptor development, we generated KO zebrafish with TALENs. The KO zebrafish had a mutation in the *six7* coding region: the ja51 mutation caused a frame shift of the amino acid sequence of Six7 by 8 bp loss, thereby introducing an immature stop codon (figure 3*a*,*b*). The KO zebrafish apparently had the normal body shape throughout the developmental stages (data not shown).

We examined expression levels of phototransduction component genes in the eyes of the WT zebrafish and $six7^{ja51/ja51}$ KO zebrafish larvae by RT-qPCR. Notably, mRNA levels of the middle wavelength-sensitive opsins, green opsins (*rh2-1* and *rh2-2*) and blue opsin (*sws2*) were all reduced in 3.5 dpf $six7^{ja51/ja51}$ larvae (figure 3*d*). Like other teleosts, zebrafish has multiple copies of green opsin genes, *rh2-1*, *rh2-2*, *rh2-3* and *rh2-4*, among which *rh2-3* and *rh2-4* are expressed at extremely low levels during larval stage in WT [34] and also in $six7^{ja51/ja51}$ (data not shown).

In situ hybridization analysis (figure 3*f*) revealed that green opsin expression was hardly detected in the eye of $six7^{ja51/ja51}$ larvae, while blue opsin expression was reduced approximately by half in $six7^{ja51/ja51}$ larvae, suggesting decreases in the numbers of middle wavelength-sensitive cones (i.e. green and blue cones). Consistently, $six7^{ja51/ja51}$ larvae exhibited reduced mRNA levels of arrestin 3a (*arr3a*) and arrestin 3b (*arr3b*) (figure 3*d*), which are expressed in the double cones (green and red cones) and the single cones (UV and blue cones), respectively [35]. This observation further supports the reduction of the number of blue and green cones in $six7^{ja51/ja51}$ larvae at 3.5 dpf.

The reduction in larval mRNA levels of blue and green opsins was also observed in another *six7* mutant line, $six7^{ja52/ja52}$(electronic supplementary material, figure S1*b*). Using this line, we generated $six7^{ja52/ja52}$;$six3b^{ja53/ja53}$ double KO larvae and found that the double mutant had extremely small eyes (electronic supplementary material, figure S1*e*): the loss of the eyes recapitulated the morpholino-induced phenotypes of *six7*/*six3b*-deficient larvae reported in the previous study [24], supporting that the *six7* deficiency is caused by the ja52 mutation. The expression profiles of opsin genes in $six7^{ja52/ja52}$ larvae were quite similar to those in $six7^{ja51/ja51}$ larvae, suggesting that the phenotype of *six7* mutants did not arise from any off-target effect of TALENs but from the *six7* deficiency. In the following experiments, we used $six7^{ja51/ja51}$ zebrafish, which we refer to as *six7* KO.

(c) Increase of rod cells in the larva of *six7* knock-out zebrafish

In contrast to the remarkable reduction in the number of blue and green cones, the mRNA levels of rod-specific

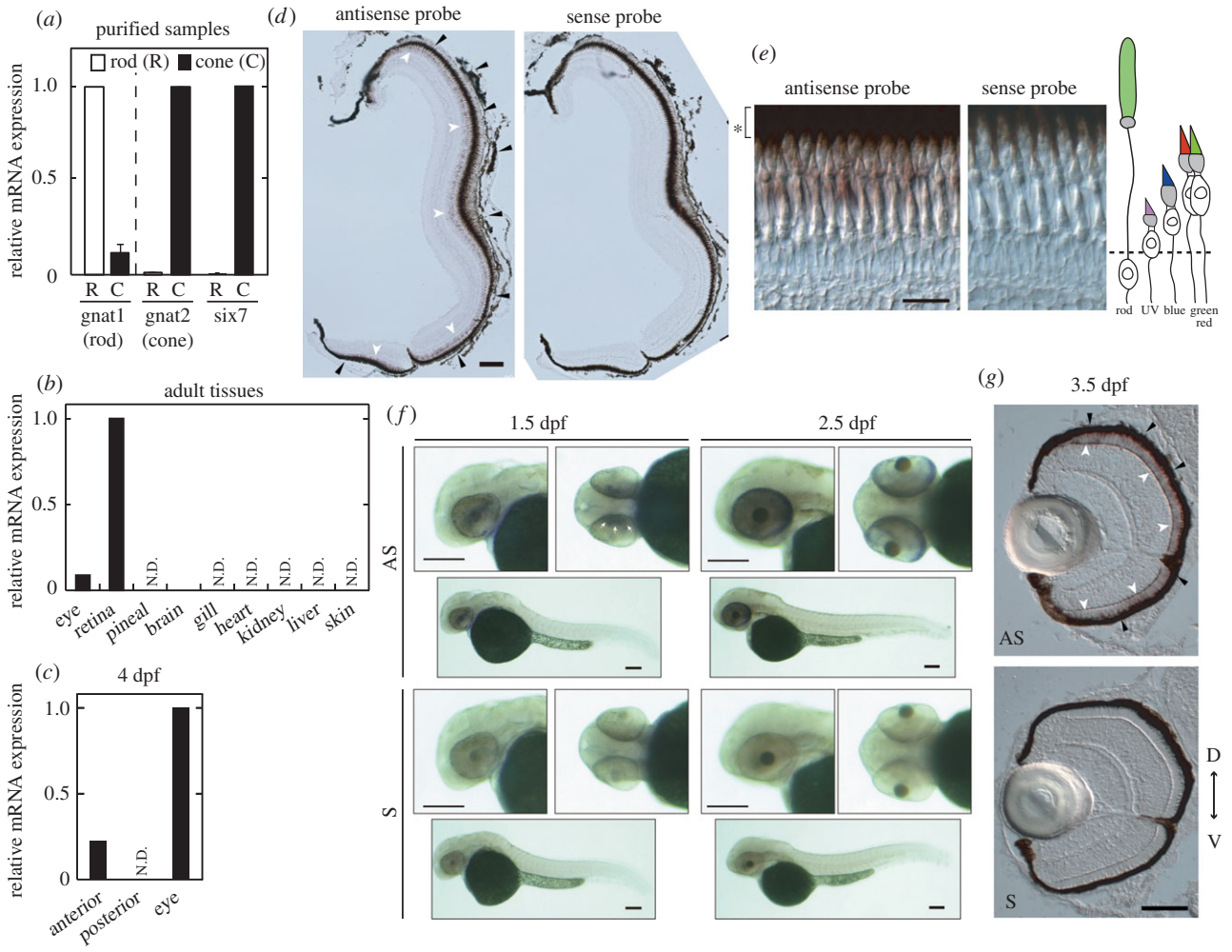

Figure 2. Expression patterns of *six7* at the larval and adult stages. (*a–c*) Relative expression levels of *six7* were quantified by RT-qPCR using the following samples: (*a*) rods and cones isolated from *Tg*(*rho:egfp*) and *Tg*(*gnat2:egfp*) zebrafish, respectively, at the adult stage ($n = 2$), (*b*) adult tissues and (*c*) larval anterior segments, posterior segments and eyes at 4 days post fertilization (4 dpf). N.D., not detected. (*d*) *In situ* hybridization using cryosections of the light-adapted adult zebrafish retina at 7 months post-fertilization (mpf). The *six7* expression was detected in the photoreceptor layer (white arrowheads), which is adjacent to the retinal pigmented epithelium (RPE, black arrowheads). Scale bar, 100 µm. (*e*) Magnified images of (*d*). The five subtypes of photoreceptor cells are located in distinct layers of the light-adapted retina (as illustrated in the schematic drawing). RPE is indicated by the asterisk. Scale bar, 20 µm. (*f*) Whole-mount *in situ* hybridization using larval zebrafish. The *six7* expression was observed at both 1.5 dpf (arrowheads) and 2.5 dpf. Scale bars, 100 µm. (*g*) *In situ* hybridization using cryosections of the 3.5-dpf larval zebrafish. D, dorsal side; V, ventral side. Scale bar, 50 µm. AS, antisense probe; S, sense probe. (Online version in colour.)

phototransduction component genes, rhodopsin (*rho*) and rod transducin α-subunit (*gnat1*), were upregulated in *six7* KO larvae as compared with WT (figure 3*d*). The number of Gnat1-positive cells was increased in KO larvae when assessed by immunohistochemistry with anti-Gnat1 antibody (figure 3*c*). It is also evident that *six7* KO exhibited increased mRNA levels of rod-specific transcription factors, neural retina leucine zipper (*nrl*) and nuclear receptor subfamily 2, group E, member 3 (*nr2e3*) (figure 3*e*). These two transcription factors are both essential for rod development in mice [36,37]. Together, these data support the notion that KO larvae had many more rods than WT larvae at 3.5 dpf.

(d) Decrease of green cones in adult *six7* knock-out zebrafish

To examine whether the number of photoreceptors expressing green opsins are reduced throughout developmental stages, we analysed expression profiles of opsin genes in adult zebrafish. The number of green cones expressing *rh2-1*, *rh2-2*, *rh2-3* or *rh2-4* was severely reduced in *six7* KO at the adult stage (figure 4*a*,*b*), indicating that transcription of all the green opsin genes was suppressed throughout developmental stages in KO. The reduction of the number of green cone cells in KO at both adult and larval stages suggests that *six7* is indispensable for development of green cones.

(e) Developmental stage-dependent phenotypes of blue cones and rods in *six7* knock-out zebrafish

The mRNA levels of rod-specific genes, *rho* and *gnat1*, were upregulated in *six7* KO larvae (figure 3*d*), but at the adult stage, they were not significantly different between WT and KO (figure 4*a*). Similarly, the suppressed expression of blue opsin gene (*sws2*) in *six7* KO was observed only at the larval stage (figure 3*d*) but not at the adult stage (figure 4*a*). The number of blue cones was reduced in the adult KO central retina, where early differentiated photoreceptors were located (figure 4*b*; electronic supplementary material, figure S2), suggesting that *six7* is necessary for the development of blue cones only during the larval stage.

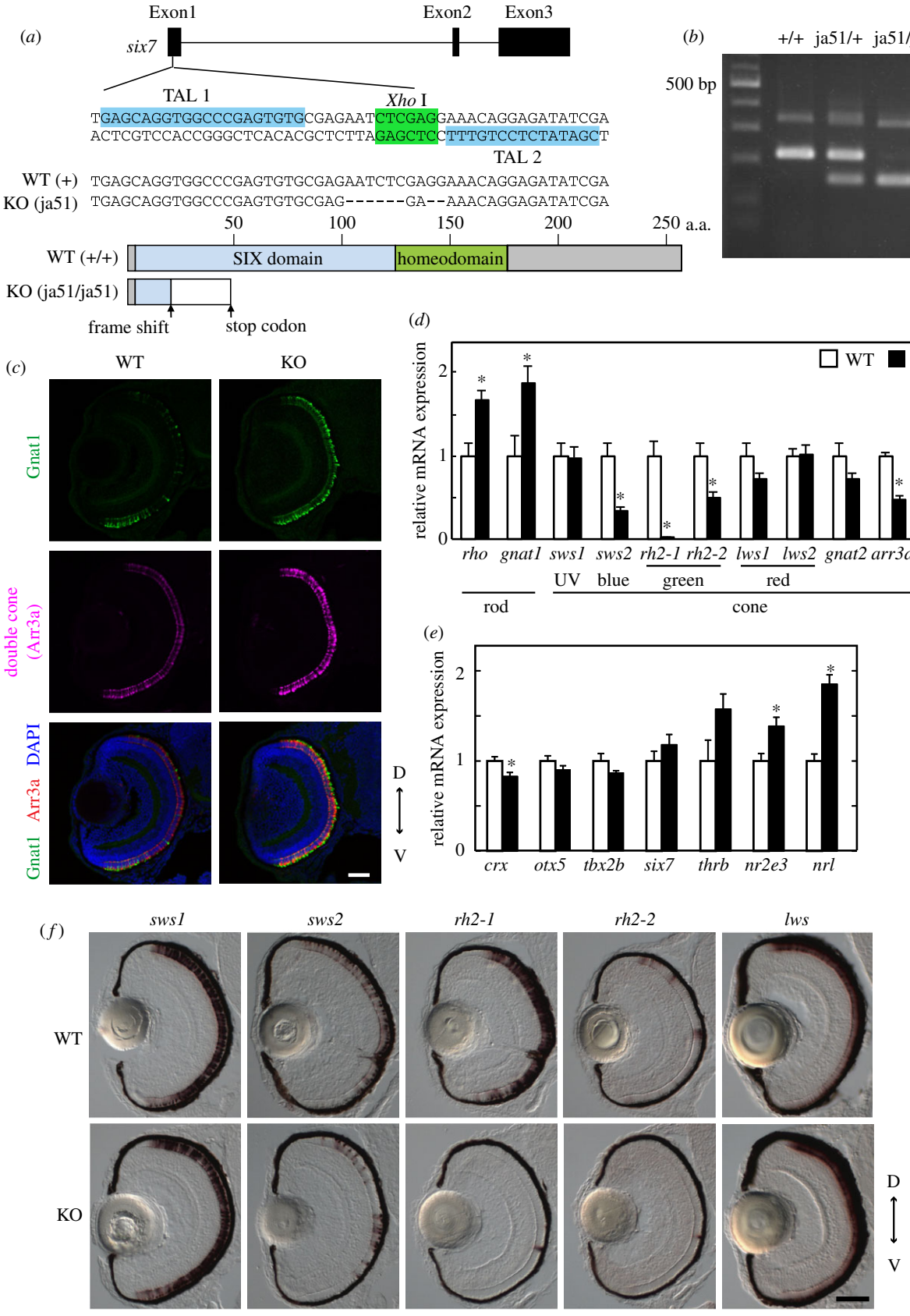

Figure 3. Analysis of the WT zebrafish and the *six7* KO zebrafish at the larval stage. (*a*) Top: exon–intron organization and partial nucleotide sequences of zebrafish *six7*. The binding sites of the left and right TALENs are highlighted in blue. The recognition site of the endonuclease *Xho*I is highlighted in green. Middle: the nucleotide sequence of the KO (ja51 mutant) fish is compared with the WT sequence. Deletions are indicated by dashes. Bottom: schematic of Six7 and its mutant protein. (*b*) Genotyping of the *six7* KO zebrafish. See details in the electronic supplementary material, methods. (*c*) Immunofluorescence of ocular sections of the WT and KO larvae at 3.5 dpf. The ocular sections were immunostained with the anti-Gnat1 antibody for rods (green) and with the zpr1 antibody for double cones (i.e. red and green cones; magenta). DAPI staining identified all the cell nuclei (blue). Scale bar, 40 µm. (*d*,*e*) Expression profiles of the phototransduction component genes (*d*) and the transcription factors contributing to the photoreceptor development in mouse and/or zebrafish (*e*) in the WT and *six7* KO larval eyes at 3.5 dpf. The mRNA levels were quantified by RT-qPCR. The data were represented by mean + s.e.m. ($n = 5$). Statistical significance between WT and *six7* KO data is shown by the asterisks (*$p < 0.05$ by Student's *t*-test). (*f*) Expression profiles of the cone opsin genes by *in situ* hybridization using the larval eyes of the WT and KO at 3.5 dpf. The *lws* probe was designed to recognize both of the *lws1* and *lws2* opsin genes. D, dorsal side; V, ventral side. Scale bar, 50 µm. (Online version in colour.)

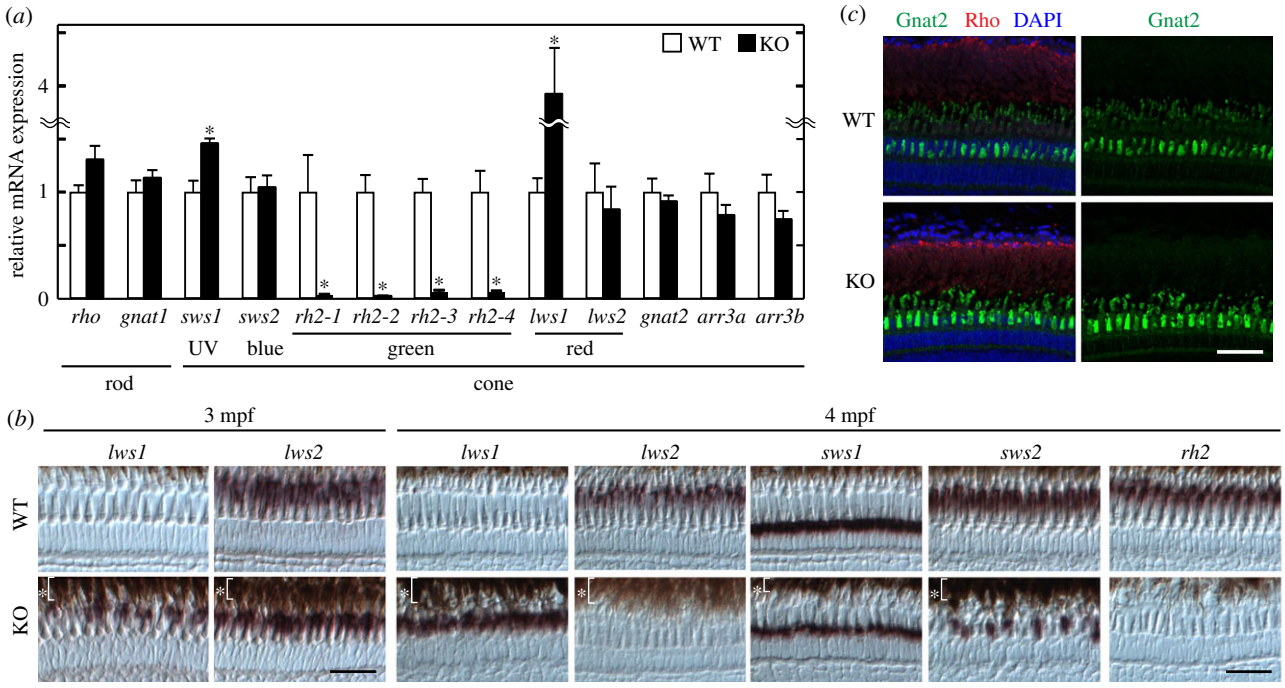

Figure 4. Analysis of the WT zebrafish and the *six7* KO zebrafish at the adult stage. (*a*) mRNA expression levels of phototransduction component genes in the WT and *six7* KO eyes at 3 mpf. The RT-qPCR data were represented by mean + s.e.m. ($n = 4$). Statistical significance between WT and *six7* KO data is shown by the asterisks (*$p < 0.05$ by Student's *t*-test). (*b*) Expression profiles of cone opsin genes in the central retina of the adult WT and KO by *in situ* hybridization. All subsets of green cones were stained with a mixture of four different RNA probes, each of which specifically recognizes the corresponding subtype of the green opsin (*rh2*) gene. RPE is indicated by the asterisks. Demagnified images are shown in the electronic supplementary material, figure S2. Scale bars, 30 μm. (*c*) Fluorescent images in the central retina of the adult WT and KO at 3 mpf. The cryosections of the adult zebrafish retina were immunostained with the anti-Gnat2 antibody for cones (green) and with the anti-rhodopsin antibody for rods (red). DAPI staining identified all the cell nuclei (blue). Scale bar, 40 μm. (Online version in colour.)

(f) Ectopic expression of red opsin gene *lws1* in the central retina of adult *six7* knock-out zebrafish

Zebrafish have two duplicated red opsin genes, *lws1* and *lws2*, and their expression is spatially regulated in the retina [19,34]. In the eyes of adult *six7* KO, the *lws1* mRNA level was markedly upregulated (figure 4*a*), in contrast to its normal expression in *six7* KO larvae (figure 3*d*). Spatial expression pattern of *lws1* in the adult KO retina was also distinct from that in the adult WT retina. In WT, the *lws1* expression was confined to the peripheral retina, while the *lws2* expression was more localized in the central retina (figure 4*b*; electronic supplementary material, figures S2 and S4*b*). In *six7* KO, the *lws1*-expressing cells were observed across the whole retina (figure 4*b*; electronic supplementary material, figures S2 and S4*b*). At this adult stage (3 mpf), the number of *lws2*-expressing cells in the central retina was mostly unaffected by the loss of *six7* (figure 4*b*), as is consistent with the RT-qPCR analysis (figure 4*a*), but surprisingly, at the later stage (4 mpf), *lws2* showed a severely reduced expression in the central retina of KO (figure 4*b*; electronic supplementary material, figure S2). Considering that the loss of *six7* does not affect the development of red cone, at the larval stage (figure 3) and that *six7* KO maintains the laminar structure of the retina (figure 4), we suppose that *six7* controls only the expression pattern of the duplicated red opsin genes in the red cones. We further validated the number of the red cones in *six7* KO by whole-mount immunohistochemistry with 1D4 antibody as a red cone marker [38] and with anti-Gnat2 antibody to stain all the cone subtypes. The loss of *six7* caused reduction in the number of the Gnat2-positive cells in the double cone layer, while apparent increase or decrease in the red cones was not observed in *six7* KO retina at 6 mpf (electronic supplementary material, figure S4*c*). Collectively, the deficiency of *six7* is likely to cause gradual switching of the expression of red opsins from *lws2* to *lws1* in the central retina without affecting the total number of the red cones. These results suggest that *six7* also plays an important role in spatio-temporally regulated expression of red opsin subtypes.

(g) Expression levels of transcription factors responsible for photoreceptor development in *six7* knock-out zebrafish

Two transcription factors (*tbx2b* and *thrb*) are known to contribute to development of UV and red cone cells in zebrafish, respectively [20,21]. The mRNA levels of these transcription factors were mostly unaffected by the loss of *six7* at both the larval and adult stages (figure 3*e*; electronic supplementary material, figure S3). The unaltered expression of these transcription factors indicates that the developmental process of the green cones might be independent of that of the UV and red cones. Importantly, no significant change was observed between WT and KO in the mRNA levels of *crx* and orthodenticle homolog 5 (*otx5*) (figure 3*e*; electronic supplementary material, figure S3), the master transcriptional regulators of photoreceptor cells in zebrafish [39,40]. It should be noted that the loss of *six7* did not affect the laminar structure of the larval retina (figure 3*c*,*f*), nor the positions and the morphologies of each photoreceptor cells in the adult retina (figure 4*c*; electronic supplementary material, figure S2). Together, our results indicate that *six7* governs the expression of green opsin genes without affecting gene expression of the key transcription factors.

4. Discussion

We investigated the role of *six7*, which is predominantly expressed in cone photoreceptors throughout the development of zebrafish (figure 2). We generated *six7* KO zebrafish and found that *six7* was essential for the expression of all subsets of the green opsin genes (figures 3 and 4), which have been duplicated and diverged in the teleost lineage. The loss of *six7* caused expression switching between the two red opsin genes, from *lws2* to *lws1*, in the central retina at the adult stage (figure 4). These results demonstrate that *six7* is important for the effective detection of the middle- and long-wavelength light by controlling the expression of the green and red opsins in zebrafish. In *six3b* KO larvae, on the other hand, no significant alteration of mRNA levels of the opsin genes was observed (electronic supplementary material, figure S5), suggesting that *six7* plays specialized roles in regulating the expression of these opsin genes, in contrast to the redundant role of *six3b* and *six7* in eye formation [24] (electronic supplementary material, figure S1).

We observed an increase in the number of rods and reduced number of blue cones in *six7* KO at the larval stage (figure 3). Recently, Saade *et al.* [41] also reported an increased number of rods due to the loss of *six7*. In their report, a forward genetic screen of the genes involved in rod development led to isolation of a mutant called *lots-of-rods jr* (*ljr*), which was characterized by an increased number of rods at the larval stage. They mapped the *ljr* mutation on chromosome 7, in which *six7* is located. The morpholino-mediated knockdown of *six7* phenocopied the *ljr* mutation [41]. In this study, by using the *six7* KO zebrafish, we analysed the role of *six7* in the photoreceptor development at the adult stage. The *six7* KO caused the increase in number of rods as well as the suppression of the mRNA level of the blue opsin only at the larval stage (figure 3*d*), while the number of the green cones was reduced by *six7* KO at both the larval and adult stages (figures 3*d* and 4*a*). By contrast, the ectopic expression of the red opsin *lws1* in the central retina of *six7* KO was observed only at the adult stage (figure 4*a*). These phenotypes observed at either the larval or the adult stage suggest that the differentiation programmes of the retinal progenitor cells change as zebrafish grow, and that *six7* contributes to stage-dependent differentiation in photoreceptor development.

So far, *six7* has been found widely in the ray-finned fish genomes but not in birds nor mammals. The emergence of the Six7 subfamily at the early evolutionary stage (figure 1; electronic supplementary material, figure S6*a*) suggests that *six7* is lost in some vertebrate species. We noticed a gene termed *SIX7* in the Ensembl Genome database of reptilian species, green anole and Chinese soft-shell turtle, and our subsequent tBLASTn searches identified *SIX7*-like sequences in the NCBI genome database of green sea turtle and Indian python. These reptilian SIX7 sequences are quite diverged from those of ray-finned fish Six7, as is evident from too many amino acid substitutions and gaps in the sequence alignment (electronic supplementary material, figure S6*b*). Additional genome information will be required to make clear whether these *SIX7* genes are true orthologues of ray-finned fish *six7*.

Although it is likely that *six7* directs expression of *rh2* (the green opsin) genes in ray-finned fish, *SIX7* is lost in birds and elephant shark, both of which retain *RH2* genes. In these species, expression of the *RH2* gene could be regulated by a *six7*-independent mechanism. To gain insight into evolutionary aspects of the regulatory mechanisms, we compared the gene synteny around the *RH2* gene locus among various vertebrate species (electronic supplementary material, figure S7). Intriguingly, the gene synteny can be classified into two types: (i) the non-teleost type and (ii) the teleost type (electronic supplementary material, figure S7). The non-teleost type was found in the species ranging from elephant shark to tetrapods, suggesting that this synteny represents an ancient type. In the teleost lineage, the genes around *RH2* are likely to have been rearranged into the teleost type, in which *six7* could have acquired the important role in the expression of *rh2* genes.

Previously, Tsujimura *et al.* [18] identified a 0.5 kbp enhancer region (termed locus control region; LCR) located 15 kbp upstream of the tandem array of *rh2* genes, as a regulator essential for expression of all the duplicated *rh2* genes in zebrafish [18]. Interestingly, LCR contains the binding sites of both Six7 (TAATGTC) [42] and Crx (TAATC) [11]. The loss of expression of all subsets of *rh2* genes in the *six7* KO zebrafish (figure 4*a*,*b*) suggests that *six7* may activate expression of the *rh2* genes through the LCR. Teleosts possess a variable number of duplicated *rh2* genes [43] (electronic supplementary material, figure S8), whose spectral sensitivities are diverged from each other [44]. The *six7* regulation of the duplicated *rh2* expression may enable teleosts to adapt to diverse aquatic environments.

Ethics. All research described here adhered to local guidelines and all appropriate ethical approval and licences were obtained.

Data accessibility. The datasets supporting this article have been uploaded as part of the electronic supplementary material.

Authors' contributions. T.S. and Y.F. conceived and designed the research. Y.O., supported by T.S., carried out the molecular laboratory work, participated in data analysis and carried out sequence alignments. T.S. performed flow cytometry. D.K. and Y.F. supervised the project. Y.O., T.S., D.K. and Y.F. wrote the manuscript. All authors approved the manuscript for publication.

Competing interests. The authors declare no conflict of interest.

Funding. This work was supported by Grants-in-Aid for scientific research from MEXT, Japan (to D.K. and Y.F.), by Grant for Basic Science Research Projects from the Sumitomo Foundation (to D.K.) and by Research Foundation for Opto-Science and Technology (to D.K.).

Acknowledgements. We thank the members of Fukada lab for valuable discussion and also thank S. Kuraku (RIKEN) for valuable comments. We thank members of FACS core laboratory, the University of Tokyo, for support of cell sorting. We also thank D. F. Voytas (University of Minnesota) and B. N. Kennedy (UCD) for supplying plasmids, and ZIRC for providing zpr1 antibody.

References

1. Ebrey T, Koutalos Y. 2001 Vertebrate photoreceptors. *Prog. Retin. Eye Res.* **20**, 49–94. (doi:10.1016/S1350-9462(00)00014-8)
2. Kawamura S, Tachibanaki S. 2008 Rod and cone photoreceptors: molecular basis of the difference in their physiology. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **150**, 369–377. (doi:10.1016/j.cbpa.2008.04.600)
3. Lamb TD. 2013 Evolution of phototransduction, vertebrate photoreceptors and retina. *Prog. Retin. Eye Res.* **36**, 52–119. (doi:10.1016/j.preteyeres.2013.06.001)
4. Okano T, Fukada Y, Yoshizawa T. 1995 Molecular basis for tetrachromatic color vision. *Comp. Biochem. Physiol. B. Biochem. Mol.*

Biol. **112**, 405–414. (doi:10.1016/0305-0491(95)00085-2)

5. Goldsmith TH. 2013 Evolutionary tinkering with visual photoreception. *Vis. Neurosci*. **30**, 21–37. (doi:10.1017/S095252381200003X)
6. Shu D-G *et al.* 2003 Head and backbone of the Early Cambrian vertebrate Haikouichthys. *Nature* **421**, 526–529. (doi:10.1038/nature01264)
7. Collin SP, Knight MA, Davies WL, Potter IC, Hunt DM, Trezise AEO. 2003 Ancient colour vision: multiple opsin genes in the ancestral vertebrates. *Curr. Biol*. **13**, R864–R865. (doi:10.1016/j.cub.2003.10.044)
8. Lagman D, Ocampo Daza D, Widmark J, Abalo XM, Sundström G, Larhammar D. 2013 The vertebrate ancestral repertoire of visual opsins, transducin alpha subunits and oxytocin/vasopressin receptors was established by duplication of their shared genomic region in the two rounds of early vertebrate genome duplications. *BMC Evol. Biol*. **13**, 238. (doi:10.1186/1471-2148-13-238)
9. Swaroop A, Kim D, Forrest D. 2010 Transcriptional regulation of photoreceptor development and homeostasis in the mammalian retina. *Nat. Rev. Neurosci*. **11**, 563–576. (doi:10.1038/nrn2880)
10. Adler R, Raymond PA. 2008 Have we achieved a unified model of photoreceptor cell fate specification in vertebrates? *Brain Res*. **1192**, 134–150. (doi:10.1016/j.brainres.2007.03.044)
11. Furukawa T, Morrow EM, Cepko CL. 1997 Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. *Cell* **91**, 531–541. (doi:10.1016/S0092-8674(00)80439-0)
12. Chen S, Wang QL, Nie Z, Sun H, Lennon G, Copeland NG, Gilbert DJ, Jenkins NA, Zack DJ. 1997 Crx, a novel Otx-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes. *Neuron* **19**, 1017–1030. (doi:10.1016/S0896-6273(00)80394-3)
13. Freund CL *et al.* 1997 Cone-rod dystrophy due to mutations in a novel photoreceptor-specific homeobox gene (CRX) essential for maintenance of the photoreceptor. *Cell* **91**, 543–553. (doi:10.1016/S0092-8674(00)80440-7)
14. Ng L, Hurley JB, Dierks B, Srinivas M, Saltó C, Vennström B, Reh TA, Forrest D. 2001 A thyroid hormone receptor that is required for the development of green cone photoreceptors. *Nat. Genet*. **27**, 94–98. (doi:10.1038/83829)
15. Fadool JM, Dowling JE. 2008 Zebrafish: a model system for the study of eye genetics. *Prog. Retin. Eye Res*. **27**, 89–110. (doi:10.1016/j.preteyeres.2007.08.002)
16. Luo W, Williams J, Smallwood PM, Touchman JW, Roman LM, Nathans J. 2004 Proximal and distal sequences control UV cone pigment gene expression in transgenic zebrafish. *J. Biol. Chem*. **279**, 19 286–19 293. (doi:10.1074/jbc.M400161200)
17. Takechi M, Seno S, Kawamura S. 2008 Identification of cis-acting elements repressing blue opsin expression in zebrafish UV cones and pineal cells. *J. Biol. Chem*. **283**, 31 625–31 632. (doi:10.1074/jbc.M806226200)
18. Tsujimura T, Chinen A, Kawamura S. 2007 Identification of a locus control region for quadruplicated green-sensitive opsin genes in zebrafish. *Proc. Natl Acad. Sci. USA* **104**, 12 813–12 818. (doi:10.1073/pnas.0704061104)
19. Tsujimura T, Hosoya T, Kawamura S. 2010 A single enhancer regulating the differential expression of duplicated red-sensitive opsin genes in zebrafish. *PLoS Genet*. **6**, e1001245. (doi:10.1371/journal.pgen.1001245)
20. Alvarez-Delfin K *et al.* 2009 Tbx2b is required for ultraviolet photoreceptor cell specification during zebrafish retinal development. *Proc. Natl Acad. Sci. USA* **106**, 2023–2028. (doi:10.1073/pnas.0809439106)
21. Suzuki SC, Bleckert A, Williams PR, Takechi M, Kawamura S, Wong ROL. 2013 Cone photoreceptor types in zebrafish are generated by symmetric terminal divisions of dedicated precursors. *Proc. Natl Acad. Sci. USA* **110**, 15 109–15 114. (doi:10.1073/pnas.1303551110)
22. Kawakami K, Sato S, Ozaki H, Ikeda K. 2000 Six family genes—structure and function as transcription factors and their roles in development. *Bioessays* **22**, 616–626. (doi:10.1002/1521-1878(200007)22:7<616::AID-BIES4>3.0.CO;2-R)
23. Seo HC, Drivenes O, Ellingsen S, Fjose A. 1998 Transient expression of a novel Six3-related zebrafish gene during gastrulation and eye formation. *Gene* **216**, 39–46. (doi:10.1016/S0378-1119(98)00328-X)
24. Inbal A, Kim S-H, Shin J, Solnica-Krezel L. 2007 Six3 represses nodal activity to establish early brain asymmetry in zebrafish. *Neuron* **55**, 407–415. (doi:10.1016/j.neuron.2007.06.037)
25. Cermak T *et al.* 2011 Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Res*. **39**, e82. (doi:10.1093/nar/gkr218)
26. Dahlem TJ, Hoshijima K, Jurynec MJ, Gunther D, Starker CG, Locke AS, Weis AM, Voytas DF, Grunwald DJ. 2012 Simple methods for generating and detecting locus-specific mutations induced with TALENs in the zebrafish genome. *PLoS Genet*. **8**, e1002861. (doi:10.1371/journal.pgen.1002861)
27. Kojima D, Mano H, Fukada Y. 2000 Vertebrate ancient-long opsin: a green-sensitive photoreceptive molecule present in zebrafish deep brain and retinal horizontal cells. *J. Neurosci*. **20**, 2845–2851.
28. Kojima D, Torii M, Fukada Y, Dowling JE. 2008 Differential expression of duplicated VAL-opsin genes in the developing zebrafish. *J. Neurochem*. **104**, 1364–1371. (doi:10.1111/j.1471-4159.2007.05093.x)
29. Barthel LK, Raymond PA. 2000 *In situ* hybridization studies of retinal neurons. *Methods Enzymol*. **316**, 579–590.
30. Mano H, Kojima D, Fukada Y. 1999 Exo-rhodopsin: a novel rhodopsin expressed in the zebrafish pineal gland. *Brain Res. Mol. Brain Res*. **73**, 110–118. (doi:10.1016/S0169-328X(99)00242-9)
31. Asaoka Y, Mano H, Kojima D, Fukada Y. 2002 Pineal expression-promoting element (PIPE), a cis-acting element, directs pineal-specific gene expression in zebrafish. *Proc. Natl Acad. Sci. USA* **99**, 15 456–15 461. (doi:10.1073/pnas.232444199)
32. Kennedy BN, Alvarez Y, Brockerhoff SE, Stearns GW, Sapetto-Rebow B, Taylor MR, Hurley JB. 2007 Identification of a zebrafish cone photoreceptor-specific promoter and genetic rescue of achromatopsia in the nof mutant. *Invest. Ophthalmol. Vis. Sci*. **48**, 522–529. (doi:10.1167/iovs.06-0975)
33. Raymond PA, Barthel LK, Curran GA. 1995 Developmental patterning of rod and cone photoreceptors in embryonic zebrafish. *J. Comp. Neurol*. **359**, 537–550. (doi:10.1002/cne.903590403)
34. Takechi M, Kawamura S. 2005 Temporal and spatial changes in the expression pattern of multiple red and green subtype opsin genes during zebrafish development. *J. Exp. Biol*. **208**, 1337–1345. (doi:10.1242/jeb.01532)
35. Renninger SL, Gesemann M, Neuhauss SCF. 2011 Cone arrestin confers cone vision of high temporal resolution in zebrafish larvae. *Eur. J. Neurosci*. **33**, 658–667. (doi:10.1111/j.1460-9568.2010.07574.x)
36. Mears AJ, Kondo M, Swain PK, Takada Y, Bush RA, Saunders TL, Sieving PA, Swaroop A. 2001 Nrl is required for rod photoreceptor development. *Nat. Genet*. **29**, 447–452. (doi:10.1038/ng774)
37. Chen J, Rattner A, Nathans J. 2005 The rod photoreceptor-specific nuclear receptor Nr2e3 represses transcription of multiple cone-specific genes. *J. Neurosci*. **25**, 118–129. (doi:10.1523/JNEUROSCI.3571-04.2005)
38. Yin J, Brocher J, Linder B, Hirmer A, Sundaramurthi H, Fischer U, Winkler C. 2012 The 1D4 antibody labels outer segments of long double cone but not rod photoreceptors in zebrafish. *Investig. Ophthalmol. Vis. Sci*. **53**, 4943–4951. (doi:10.1167/iovs.12-9511)
39. Gamse JT, Shen Y-C, Thisse C, Thisse B, Raymond PA, Halpern ME, Liang JO. 2002 Otx5 regulates genes that show circadian expression in the zebrafish pineal complex. *Nat. Genet*. **30**, 117–121. (doi:10.1038/ng793)
40. Shen Y, Raymond PA. 2004 Zebrafish cone-rod (crx) homeobox gene promotes retinogenesis. *Dev. Biol*. **269**, 237–251. (doi:10.1016/j.ydbio.2004.01.037)
41. Saade CJ, Alvarez-Delfin K, Fadool JM. 2013 Rod photoreceptors protect from cone degeneration-induced retinal remodeling and restore visual responses in zebrafish. *J. Neurosci*. **33**, 1804–1814. (doi:10.1523/JNEUROSCI.2910-12.2013)
42. Suh CS, Ellingsen S, Austbø L, Zhao X-F, Seo H-C, Fjose A. 2010 Autoregulatory binding sites in the zebrafish six3a promoter region define a new recognition sequence for Six3 proteins. *FEBS J*. **277**, 1761–1775. (doi:10.1111/j.1742-4658.2010.07599.x)
43. Rennison DJ, Owens GL, Taylor JS. 2012 Opsin gene duplication and divergence in ray-finned fish. *Mol. Phylogenet. Evol*. **62**, 986–1008. (doi:10.1016/j.ympev.2011.11.030)
44. Yokoyama S. 2008 Evolution of dim-light and color vision pigments. *Annu. Rev. Genomics Hum. Genet*. **9**, 259–282. (doi:10.1146/annurev.genom.9.081307.164228)