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Distinct cis-acting regions control *six6* expression during eye field and optic cup stages of eye formation

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ABSTRACT

The eye field transcription factor, Six6, is essential for both the early (specification and proliferative growth) phase of eye formation, as well as for normal retinal progenitor cell differentiation. While genomic regions driving *six6* optic cup expression have been described, the sequences controlling eye field and optic vesicle expression are unknown. Two evolutionary conserved regions 5′ and a third 3′ to the *six6* coding region were identified, and together they faithfully replicate the endogenous *X. laevis six6* expression pattern. Transgenic lines were generated and used to determine the onset and expression patterns controlled by the regulatory regions. The conserved 3′ region was necessary and sufficient for eye field and optic vesicle expression. In contrast, the two conserved enhancer regions located 5′ of the coding sequence were required together for normal optic cup and mature retinal expression. Gain-of-function experiments indicate endogenous *six6* and GFP expression in F_1 transgenic embryos are similarly regulated in response to candidate trans-acting factors. Importantly, CRISPR/CAS9-mediated deletion of the 3′ eye field/optic vesicle enhancer in *X. laevis*, resulted in a reduction in optic vesicle size. These results identify the cis-acting regions, demonstrate the modular nature of the elements controlling early versus late retinal expression, and identify potential regulators of *six6* expression during the early stages of eye formation.

1. Introduction

Early vertebrate retina formation can be broadly separated into eye field, optic vesicle and optic cup stages. Eye field stage begins shortly after gastrulation when an anterior region of the neural plate (the eye field) is specified then determined to eventually form the retina (reviewed in Sinn and Wittbrodt (2013) and Zuber (2010)). During neurulation, the flat sheet of neural plate cells curl up to form the neural tube, while the eye field cells simultaneously evaginate on both sides of the forming tube as out-pockets to generate the optic vesicles. Optic cup stages begin once the optic vesicles make contact with the overlying surface ectoderm, at which point the vesicles invaginate to form a cup, into which the lens develops (reviewed in Martinez-Morales and Wittbrodt (2009)). The eye field and optic vesicles consist of proliferating retinal progenitor cells (RPCs). The very first retinal neurons are born at late optic vesicle stage, however, the vast majority of RPCs exit the cell cycle and differentiate into the seven classes of mature retinal cell types during optic cup stages.

The eye field becomes specified and determined under the control of an evolutionarily conserved set of eye field transcription factors (EFTFs) that pattern the anterior neural plate and maintain retinal progenitors in a proliferative state during eye field and optic vesicle formation and growth. Unexpectedly, the same eye field transcription factors that maintain retinal progenitors in a proliferative state, are also required for the differentiation of specific retinal cell types at optic cup stages, and the transcription of these genes is often maintained even in differentiated retinal cells of the functionally, mature retina. An outstanding example is the EFTF, Six6/Optx2 (SIX homeobox 6/Optic Six gene 2), which was originally described in zebrafish and chicken, and found to be expressed throughout retinal development, first in the eye field, but also in differentiated cells of the mature retina (Toy et al., 1998; Seo et al., 1998). Similar expression patterns were subsequently reported for human, mouse, *X. laevis* and medaka fish *six6* (Aijaz et al., 2005; Jean et al., 1999; López-Ríos et al., 1999, 2003; Toy and Sundin, 1999; Zuber et al., 1999). Early work in model organisms demonstrated a role for Six6 in RPC specification and proliferation (Bernier

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et al., 2000; Toy et al., 1998; Li et al., 2002; López-Ríos et al., 2003; Zuber et al., 1999). Consistent with this role, mutations in *SIX6* have been linked to anophthalmia and microphthalmia in patients (Aldahmesh et al., 2013; Cheng et al., 2015; Elliott et al., 1993; Gallardo et al., 1999, 2004; Lemyre et al., 1998; Nolen et al., 2006; Bennett et al., 1991; Yariz et al., 2015). However, linkage studies also suggest SIX6 plays a part in retinal ganglion cell maintenance as some mutations in the *SIX6* gene, and surrounding genomic regions, are linked to primary open angle glaucoma (reviewed in Abu-Amero et al., 2015). The generation of retinal horizontal, amacrine and photoreceptor cells are also regulated by Six6 at optic cup stages (Conte et al., 2010; Wang and Harris, 2005). These distinct, stage dependent roles of Six6 during eye formation, may be regulated by independent enhancer elements that control early and late *six6* expression. Consistent with this hypothesis, enhancers that regulate optic cup and mature retinal expression have been identified, but these elements were not reported to control eye field and optic vesicle *six6* expression (Conte et al., 2010; Lee et al., 2012).

Here we identify and characterize three evolutionarily conserved regions that replicate the expression pattern of *six6* in *Xenopus laevis*. Regions 1 and 2 (R1 and R2) are 5′ (proximal) to the *six6* coding region and transgenes containing *X. tropicalis* R1 and R2 sequences can drive expression in the optic cup and mature retina of *X. laevis*. These regions coincide in position and include previously identified mouse and medaka fish *six6* enhancers (Conte et al., 2010; Lee et al., 2012; Tétreault et al., 2009). Importantly, these regions were not sufficient for either eye field or optic vesicle *six6* expression. Region 3, by contrast, is located 3′, is more distant (distal) to the *six6* coding region, and is sufficient to drive eye field and optic vesicle expression. We identify consensus binding sites in R3 for Pax6, Onecut1 and FoxD1. We show Pax6 activates, while Onecut1 and FoxD1 repress the transcription of both endogenous *six6* and GFP in transgenic animals. Finally, we used CRISPR/Cas9 genome editing to delete R3 from the *X. laevis* genome and observed a reduction in optic vesicle size, consistent with a role for Region 3 in controlling the expression of Six6 during the early proliferative growth phase of eye development.

2. Methods

2.1. Animals and transgenic generation

Both the SceI meganuclease and restriction enzyme mediated integration (REMI) methods were used to generate transgenic *Xenopus laevis* (Haeri and Knox, 2012; Ogino et al., 2006; Pan et al., 2006). Tadpoles were genotyped as previously described (Zuber et al., 2012), using primers 5′ (GATGGATTGCACGCAGGTTC) and 3′ (CGATAGAAGGCGATGCGCTGC). To generate tadpoles for analysis, transgenic females were induced to lay eggs and in vitro fertilized with wild-type sperm, while transgenic males were crossed with wild-type females by natural mating as previously described (Viczian and Zuber, 2010; Zuber et al., 2012). All procedures were in accordance with IACUC approved protocols. Experiments using CRISPR/Cas9 were performed using J-strain male and female *Xenopus laevis* obtained from the National *Xenopus* Resource (NXR, Woods Hole, MA).

2.2. Constructs for transgenesis

To generate *Xtr six6* R2R1→GFP: *Xenopus tropicalis* genomic DNA was PCR amplified with Taq polymerase (Fisher Scientific, #FB600025), according to the manufacturer's protocol using primers 5‘XtrSix6-MMP and 3‘XtrSix6-EP (Table S1). The R2R1 PCR product was TA-subcloned into the pGEMT-easy vector (Promega) to generate pGEMT-easy.R2R1. pGEMT-easy+R2R1 was digested with EcoR1 and the R2R1 containing fragment was subcloned into the EcoR1 site of the pEGFP-plasmid (Viczian et al., 2004) to generate pEGFP-R2R1→GFP. The *Hin*dIII/*Not*I fragment of pEGFP-.R2R1→GFP containing R2R1→GFP was subcloned into the *Hin*dIII/*Not*I site of I-SceI-pBSII-SK +(Ogino et al., 2006) to generate *Xtr six6* R2R1→GFP.

Xtr six6 R2R1→GFP was digested with *Ppu*MI/*Xho*I or *Bgl*II and religated to generate *Xtr six6* NCR1→GFP and *Xtr six6* R1→GFP, respectively.

To generate *Xtr six6* R3R2R1→GFP: *X. tropicalis* genomic DNA was PCR amplified with primers 5XtrSix6-2661 and 3XtrSix6-1268 (Table S1), digested with *Ppu*MI and subcloned into the *Ppu*MI site of *Xtr six6* R2R1→GFP to generate *Xtr six6* NCR2R1→GFP. *X. tropicalis* genomic DNA was then PCR amplified with primers 5′XtrSix6 R3 and 3′XtrSix6 R3 (Table S1) and TA-cloned into pGEMT-easy to generate pGEMT-easy+R3. R3 was removed from pGEMT-easy+R3 by digestion with *Sac*II/*Nsi*I and subcloned into the *Nsi*I/*Xho*I site (after blunt ending) of *Xtr six6* NCR2R1→GFP to generate *Xtr six6* R3R2R1→GFP.

To generate deletion Construct 1 (C1): the *Pvu*II/*Dra*I fragment of pGEMT-easy+R3 containing R3 was subcloned into the *Nsi*I/*Xho*I site (after blunt ending) of *Xtr six6* NCR2R1→GFP. To generate deletion Construct 2 (C2): *X. tropicalis* genomic DNA was PCR amplified with primers 5′XtrSix6 R3-2 and 3′XtrSix6 R3-2 (Table S1) and TA-subcloned into pGEMT-easy to generate pGEMT-easy+R3-2. pGEMT-easy+R3-2 was digested with *Nsi*I and the R3-2 containing fragment was cloned into the *Nsi*I site of *Xtr six6* R1→GFP. To generate deletion Construct 3 (C3): the *Pvu*II/*Dra*I fragment of pGEMT-easy+R3 containing R3 was subcloned into the *Nsi*I/*Bam*HI site (after blunt ending) of *Xtr six6* NCR2R1→GFP.

2.3. In situ hybridization and immunohistochemistry

Previously published in situ hybridization methods were used on whole embryos with a 2–3 day coloration step (Viczian et al., 2003). *Six6* in situ probe was generated using *X. laevis six6-L* (NM_001088464) and including both coding and 3'UTR sequences; antisense eGFP probe was generated using the entire eGFP cDNA. Samples were immunostained as previously described (Viczian et al., 2003) except slides were washed with 1X phosphate buffered saline (PBS) for 2 min, 100% methanol for 10 min prior to the published protocol. Antibodies were used at the following concentration: 1:3000 dilution of 4D2 anti-opsin and 1:500 dilution of anti-GFP (Molecular Probe cat#A-11122) antibodies.

2.4. Bioinformatics

Identification of conserved regions was done using ECR Browser (http://ecrbrowser.dcode.org) and MultiPIPMaker, as previously described (Ogino et al., 2012). Briefly, NCBI:Gene was used to identify genomic location for each *six6* gene; 10 kb upstream and downstream was downloaded. SeqBuilder software (DNAStar software; Lasergene) was used to import the sequence and generate FASTA files; mask files were generated using RepeatMasker.org. All sequence analysis was performed using the Lasergene Software Package (DNASTAR, Madison, WI). Possible transcription factor binding sites were identified using the Genomatrix MatInspector module (Quandt et al., 1995) and Transfac software (Biobase, Qiagen).

2.5. RNA injections and quantitation of GFP expression

Capped RNA was prepared using the SP6 mMessage machine kit (Life Technologies, Inc.). RNA was transcribed from pCS2R plasmids containing mCherry, Pax6 or Smad1-DVD, as previously described (Wong et al., 2015). To obtain Onecut1, FoxD1 and FoxM1 RNA, we cloned each from either stage 15 cDNA or plasmid DNA (Source Bioscience) using Herculase II Fusion DNA polymerase (Agilent Technologies Inc., Santa Clara, CA) and sequence specific primers indicated in Table S3. RNA was injected into 4-cell stage embryos at concentrations indicated in the figure. Tracer β-gal or mCherry RNA was injected at 200 pg and 250 pg, respectively. Embryos were

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