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# Identification of a retina-specific *Otx2* enhancer element active in immature developing photoreceptors

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ABSTRACT

The homeodomain protein, Otx2, is a critical regulator of vertebrate photoreceptor genesis. However, the genetic elements that define the expression of *Otx2* during photoreceptor development are unknown. Therefore, we sought to identify an *Otx2* enhancer element that functions in photoreceptor development in order to better understand this specification event. Using the technique of electroporation, we tested a number of evolutionarily conserved elements (ECRs) for expression in the developing retina, and identified ECR2 as having robust activity in the retina. We have characterized this element using a number of assays, including Cre-fate mapping experiments. We found that ECR2 recapitulates expression/function of *Otx2* primarily in newly postmitotic photoreceptor cells (PRs), as well as in a subset of retinal progenitor cells (RPCs). ECR2 was also found to be expressed in a subset of horizontal cells (HCs), in keeping with the role of Otx2 in HC development. Furthermore, we determined that the ECR2 element is not active in other Otx2-positive cells such as retinal bipolar cells (BPs), retinal pigmented epithelium (RPE), or the tectum, suggesting that the transcriptional networks controlling *Otx2* expression in these cells are unique from those of developing PRs and HCs. These results reveal a distinct molecular state in dividing retinal cells and their newly postmitotic progeny, and provide genetic access to an early and critical transcriptional node involved in the genesis of vertebrate PRs.

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## Introduction

The vertebrate retina has been used as a model for studies of the development of the nervous system, including studies on the determination of cell fates. The six classes of retinal neurons and the single retinal glial type arise from multipotent RPCs in a stereotypical order across phylogeny (Livesey and Cepko, 2001). The classes of neurons can be further subdivided into many anatomically and physiologically distinct types of cells, such that more than 50 types of retinal neurons are now recognized (Masland and Raviola, 2000). Many transcription factors have been found to affect the genesis, differentiation and/or survival of one or more retinal cell types. To learn how these factors work together in networks to produce the large variety of retinal cell types will require more sophisticated analyses than simple gain and loss of function experiments. One such type of analysis is the dissection of the cis-regulatory elements of key transcription factors that operate at nodes in networks. The network that controls the production of PRs is a critical one for light sensing tissues, as PRs are the defining cell type for light detection and vision in a wide range of organisms across phylogeny. Elucidation of this network will not only likely reveal the developmental mechanism for production of this cell type, but also may shed some light on the evolution of PRs.

The homeobox transcription factor, *Otx2*, in vertebrates, and the invertebrate homologue, *Otd*, have been established as critical regulators of PR development. In *Drosophila*, *Otd* hypomorphic alleles lead to poor PR development (Vandendries et al., 1996). In mice, conditional removal of *Otx2* in the neural retina leads to loss of PRs, BPs, and HCs. These losses are accompanied by an increase in the number of amacrine cells (ACs) (Koike et al., 2007; Nishida et al., 2003; Sato et al., 2007). Conversely, postnatal viral misexpression of *Otx2* in the rat retina leads to an increase in the number of PRs and the concomitant loss of all other postnatal cell types (Nishida et al., 2003). Furthermore, introduction of *Otx2* into non-neural cells can induce PR gene expression (Akagi et al., 2004; Inoue et al., 2010). Due to the nature of retinal development, with RPCs producing multiple cell classes in overlapping temporal and spatial windows, the actual coincidence of *Otx2* expression with the formation of these cell types during development has not been established. It is likely that there are multiple regulators of *Otx2* expression, operating within different RPC subpopulations and their descendents, offering a window into the transcriptional networks that underlie the generation of multiple cell types within the retina.

The transcriptional control of *Otx2* has been examined previously in several developmental contexts. Enhancer elements for *Otx2* have previously been identified for the anterior neuroectoderm, visceral endoderm, cephalic mesoderm and early eye field (Kimura et al.,

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1997; 2000; Kurokawa et al., 2004a; 2004b; Visel et al., 2007). Within the eye, enhancer elements have not been defined beyond the qualitative activity of a very early eye element defined by the Encode project (referred to below as the hs1150 enhancer) (Visel et al., 2007). Expression of Otx2 in the neural retina has been observed in RPCs, retinal ganglion cells (RGCs), adult PRs, BPs, and a subset of Mueller glial cells (MGs) (Baas et al., 2000; Bovolenta et al., 1997; Brzezinski et al., 2010; Fossat et al., 2007; Trimarchi et al., 2008a), though no Otx2 enhancer elements that function in these cells have been described. Therefore, it is currently unknown if *Otx2* expression in the various retinal populations is under the control of a single or multiple transcriptional networks.

As *Otx2* is one of the factors that is expressed in a subset of RPCs, and appears to control specific and multiple cell fates, this study sought to identify enhancers that control its expression. We report here the identification of the Otx2ECR2 element, which directs reporter expression in a subset of the Otx2-dependent cell types of the retina. Otx2-positive RPCs, PR precursor cells and HCs all utilize the ECR2 element, while another Otx2-dependent cell-type, the BP cell, does not. Furthermore this study shows that electroporation can be used to identify meaningful enhancers that track with RPC states in the developing retina, as well as the transitional state from RPC to more mature cell types.

## Materials and methods

### *DNA electroporations: general methodology*

The reporter plasmid used was the Stop Tata Enhanced Green Fluorescent Protein Ires Placental Alkaline Phosphatase version 3 (Stagia3) reporter plasmid consisting of a multiple cloning site for insertion of ECRs, a minimal promoter (the TATA box from the thymidine kinase promoter of the herpes simplex virus), and an Enhanced Green Fluorescent Protein, internal ribosomal entry site, and Placental Alkaline Phosphatase (EGFPiresPLAP) reporter cassette (Supplemental Fig. 1A) (Billings et al., 2010). For electroporation experiments in which retinas were to be processed for immunofluorescent detection of the EGFP reporter, a CAG-AU1 coelectroporation plasmid was used to mark electroporated cells (Materials and methods, Supplemental Fig. 1B). This plasmid has a broadly active promoter, CAG (Niwa et al., 1991), which drives the expression of an mRNA encoding an AU1 epitope-tagged Gapdh, which can be detected with immunofluorescence using a monoclonal antibody directed against the AU1 epitope (see below under DNA plasmids). For electroporation experiments in which the PLAP reporter of the Stagia3 plasmid was employed, a CAG-mCherry co-electroporation plasmid was used, which allowed the fluorescence of mCherry to be used as an indication of electroporation efficiency (Supplemental Fig. 1C). For *ex vivo* electroporation (Supplemental Fig. 1D), retinas were placed into an electroporation chamber filled with a mixture of plasmids and voltage pulses were applied to drive the plasmids into retinal cells found in roughly the central 50% of the retina (Step 1, Supplemental Fig. 1D). Retinas were then cultured *ex vivo* on floating filters for 1 to 8 days (Sparrow et al., 1990) (Step 2, 2a Supplemental Fig. 1D). For example, after 2 days *ex vivo*, Otx2-positive cells were scattered throughout the extent of the retina, with the retina tissue still too immature to exhibit distinct or homogeneous layers (Step 2a, Supplemental Fig. 1D). At this stage, some of the Otx2-positive cells would be expected to be RPCs, as assessed by tritiated thymidine labeling preformed previously (Trimarchi et al., 2008b). After 8 days in *ex vivo* culture, the retina had assumed the basic morphological characteristics of the retina with a distinct outer nuclear layer (ONL) where PRs reside, an inner nuclear layer (INL) containing HCs, BPs, ACs, and MGs, and a RGC layer (GCL) containing RGCs and ACs. Otx2 protein was highly expressed in BPs and a small number of MGs in the INL and was relatively weakly expressed in the PRs found in the ONL (Step 2b, Supplemental Fig. 1D). Electroporation of an empty Stagia3 plasmid that had no active enhancer elements showed very little AP activity following histochemical detection compared to one that contains an active enhancer element (Step 3, Supplemental Fig. 1). Immunofluorescent processing of electroporated retinas also allows for the detection of the electroporated population of cells based on AU1 staining and the subsets of cells expressing the EGFP reporter and endogenous proteins such as Otx2 (Step 4, Supplemental Fig. 1).

### *DNA electroporation: specific methodology*

For mouse *ex vivo* electroporation experiments, P0 mouse retinas were dissected in 50% DMEM/50% F12 and then electroporated with typically 200 ng/μl of each plasmid. Electroporations were performed with a BTX Electro Square Porator ECM830 electroporator and a homemade chamber using 5 pulses of 25 V with a pulse length of 50 ms and 950 ms interpulse interval. Chicken *ex vivo* electroporations were performed on embryonic day 5 (E5) retinas with 160 ng/μl of reporter constructs and 100 ng/μl of Cre plasmids, using the same electroporation parameters described for the mouse. Retinas were cultured on 0.2 μm, 13 mm Nuclepore Track-Etch Membrane Whatman filters as reported previously (Kim et al., 2008). After *ex vivo* culture, retinas were taken off the filters and fixed with 4% paraformaldehyde. For electroporated retinas that were to be alkaline phosphatase (AP)-stained, CAG-mCherry was co-electroporated to verify electroporation. A thin border of RPE was left around the ciliary margin as removal of it caused too much damage to the retina. Lens tissue was removed completely after electroporation and before culture. Chicken *in ovo* electroporations were performed on E3 chicks. DNA solution (2.64 μg/μl plasmid Otx2ECR2SF2, 2.19 μg/μl CAG-AU1, 2 μg/μl Fast Green dye) was introduced with a pulled glass needle into the subretinal space of the right eye. A sharp tungsten negative electrode was used to pierce the head region just caudal to the eye and a gold plated electrode was used as a positive electrode anterior to the eye. 10 V pulses were applied for 50 ms for a total of 3 times, 950 ms apart.

### *DNA plasmids*

Due to an inability to completely separate the emission spectra of EGFP and genetically encoded red fluorescent molecules in our paradigm (unpublished observations), an epitope-based co-electroporation plasmid (CAG-AU1) was designed. This allowed the use of a Cy3 fluorescently conjugated secondary antibody that was completely spectrally-segregated from the signal derived by EGFP. The AU1 sequence is a 6 amino acid sequence that can be detected with a specific monoclonal antibody and has been validated to give a good signal-to-noise ratio in the retina (Lim et al., 1990; Shevtsova et al., 2006). To generate a CAG-AU1 plasmid, EGFP was removed from the Stagia3 reporter plasmid and replaced with the mouse Gapdh coding sequence (based on the expressed sequence tag AK164415) that was amplified from adult mouse cDNA. Two copies of the AU1 sequence were placed at the N-terminus of Gapdh (which served only as a carrier protein for the AU1 tags) and separated by glycine residues to give the following protein (MG**DTYRYI**G**DTYRYI**ASvkvgv…) with AU1 peptides shown in bold and Gapdh from the second amino acid shown in lower case. The CAG promoter from CAG-EGFP (Matsuda and Cepko, 2004) was cloned into the Sal1/EcoR1 sites of this AU1Gapdh modified version of Stagia3. No deleterious side effects have been observed by introduction of the CAG-AU1 plasmid into the retina. To generate a Cre responsive AU1 plasmid (CALNL-AU1), the CALNL-EGFP plasmid (Matsuda and Cepko, 2007) was digested with Age1 and BsrG1 to remove the EGFP coding sequence and AU1Gapdh from CAG-AU1 was excised with Age1 and BsrG1 and ligated to the CALNL fragment. A Cre recombinase version of Stagia3 was made by excising EGFP with Age1 and BsrG1, filling in the ends with Klenow polymerase and cloning in a Cre EcoR1/Not1 fragment from CAG-Cre (Matsuda and Cepko, 2007) with ends filled

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