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**REGULATION OF FOXN4 DURING RETINA DEVELOPMENT**

**by**

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Professor Li Cai

And approved by

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## **ABSTRACT OF THE DISSERTATION**

### **REGULATION OF FOXN4 DURING RETINA DEVELOPMENT**

**By MOHAMMED MONIRUL ISLAM**

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Transcription factor forkhead box N4(Foxn4) is a key regulator involved in a variety of biologic processes in development and metabolism. In particular, Foxn4 plays an essential role in the genesis of amacrine and horizontal neurons from neural progenitors in the retina. Although the functions of Foxn4 have been well established, the transcriptional regulation of *Foxn4* expression during progenitor cell differentiation remains unclear. The goal of this dissertation is to identify regulatory mechanisms that define the expression of *Foxn4* during retinogenesis. Four evolutionarily conserved regions (CR1-CR4) from non-coding sequences of *Foxn4* gene were computationally predicted as *cis*-elements. Their gene regulatory potential was individually tested in developing chick and mouse embryonic retina using electroporation transfection technique with a reporter assay system. In this dissertation, I describe that CR4.2 (a 129 bp DNA fragment of CR4, located approximately 50kb upstream of *Foxn4* transcription start site) functions as a novel *cis*-regulator that directs retinal cell type specific gene expression. CR4.2 is preferentially active in the Foxn4 expressing cells, primarily in the

differentiating and differentiated horizontal and amacrine cells as shown by reporter assays. Specific *trans*-acting factors, e.g., Meis1, were found to interact with CR4.2 by electrophoretic mobility shift assays (EMSA). Mutation and/or deletion of the Meis1 binding motif through site-directed mutagenesis diminishes the ability of CR4.2 to drive reporter GFP expression. Furthermore, the role of Meis1 in regulating Foxn4 expression during progenitor cell differentiation was determined using a RNAi-based gene silencing assay. Knockdown of Meis1 by short hairpin RNA (shRNA) specific to *Meis1* genes abolishes gene regulatory activity of CR4.2, and further diminishes the endogenous level of Foxn4 expression. In addition, cells with Meis1 knockdown failed to differentiate into horizontal cells. Taken together, I demonstrate that Meis1 transcription factor regulate the expression of *Foxn4* expression and horizontal cell lineage development in the vertebrate retina via their interactions with CR4.2. These findings provide new insights into molecular mechanisms that govern gene regulation in retinal progenitors and vertebrate retinal cell development.

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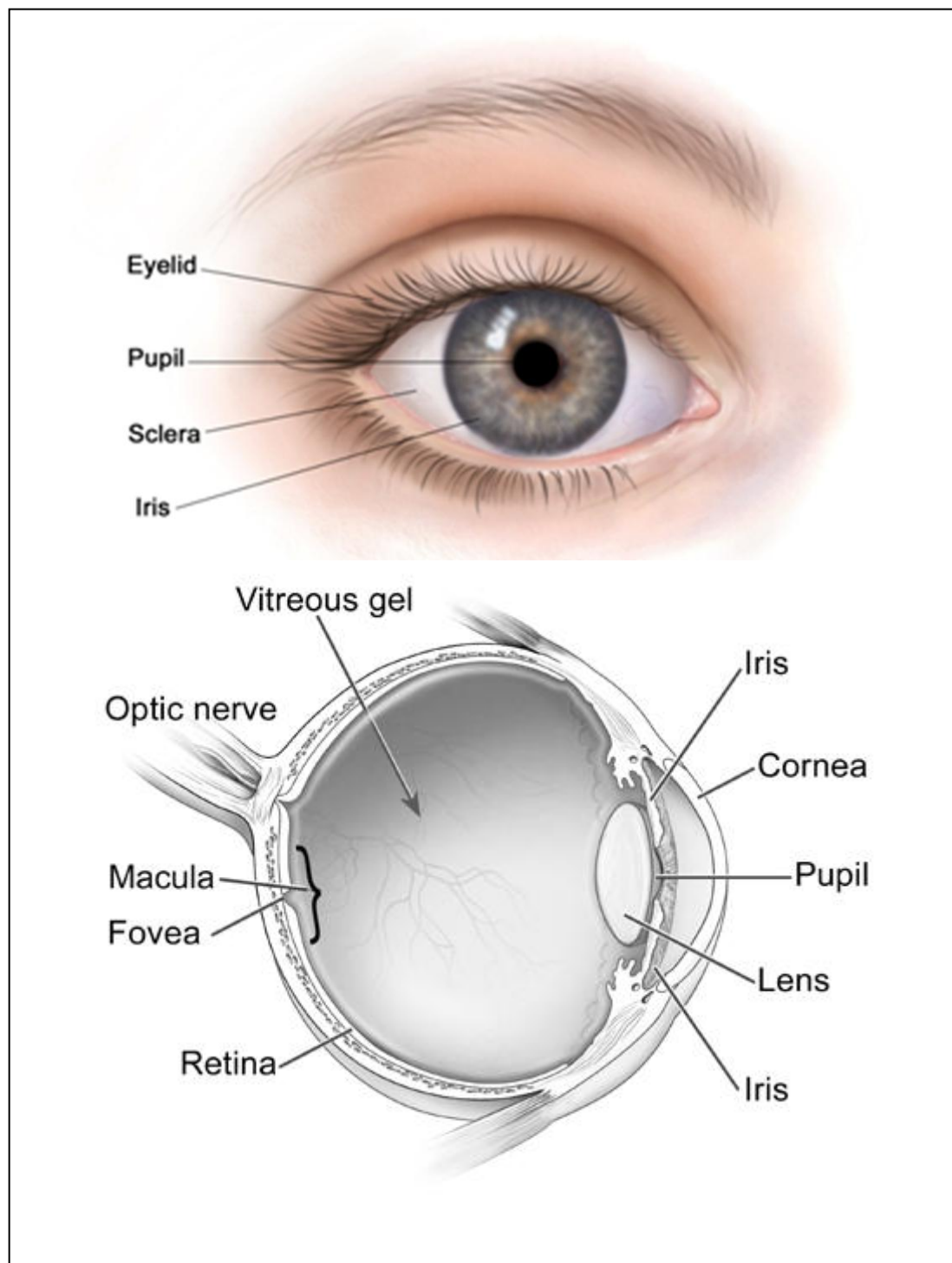
## **CHAPTER 1**

### **INTRODUCTION**

According to latest estimates from World Health Organization (WHO), 285 million people are visually impaired worldwide; 39 million are blind and 246 have low vision. However, 80% of all visual impairment can be prevented, treated or cured. A leading cause of visual impairment is age-related retinal degeneration that leads to huge social and economical impact.

### **1.1 Anatomy of the Eye**

The primary function of the eye is to capture light and transduce that data into neurological impulses that can be processed into images in the brain. The eye is a slightly asymmetrical small globe measuring 25 millimeters along the anterior-posterior axis in the average person, with very little variance among individuals (Coleman, 1969) . The globe is covered with a tough, white tissue called the sclera that protects and physically supports the delicate components of the eye. The surface of the eye not covered by white scleral tissue is called the cornea. The transparent cornea refracts light as it enters the eye. The cornea covers the iris, lens and pupil. The colored part of the eye visible directly underneath the cornea is the iris. The iris contains a sphincter muscle that controls the level of light entering the eye by contracting or relaxing. The pupil is the opening in the center of the iris through which light enters the eye.





**Figure 1. The human eye viewed in front and in cross-section.**

A thick, transparent vitreous gel maintains the shape of the eye. Light passes through the cornea to enter the eye. The papillary sphincter of the iris contracts or relaxes to allow the optimal amount of light to pass through the lens. The opening in the center of the iris is called the pupil. The curvature of the lens is adjusted to focus an image on the portion of the retina called the fovea. The fovea is used for tasks demanding high visual acuity. The fovea sits at the center of the macula, the region of the retina specialized for perception of color and detail. Light stimuli are converted to changes in membrane potential in the neurosensory retina, enabling information received by the eye to be sent to the brain via the optic nerve. This image is provided courtesy of the National Eye Institute, National Institutes of Health.

The pupil appears larger or smaller dependent on the action of the papillary sphincter.

The biconvex, transparent lens lies behind the iris and aids in focusing light on the retina.

The retina covers the posterior surface of the interior of the globe. The space between the lens and the retina is filled with a clear, gel-like substance called the vitreous humor

(Figure 1).

## **1.2 Vertebrate Retina**

The retina is the neurosensory surface lining the back of the eye. It is used to perceive light. The retina is considered part of the central nervous system, based upon its embryologic origin in the forebrain. Hence, the vertebrate retina has become an excellent model system for studying the development of the nervous system including the cell differentiation process. A complex arrangement of diverse cell types gives the retina its unique functionality. Although, more than 50 types of retinal neurons have been identified (Masland, 2001) the vertebrate retina is mainly composed of six major types of neurons and one major type of glial cell (Fig. 2).

The mature vertebrate retina consists of three distinct cellular layers and two synaptic layers (Livesey and Cepko, 2001; Masland, 2001; Masland and Raviola, 2000). The photoreceptor cells (rods and cones) are the deepest or outer nuclear layer (ONL) of the retina, farthest away from incoming light. Short projection neurons (bipolar cells) and local circuit neurons (horizontal and amacrine cells) are located in the inner nuclear layer (INL), while long projection neurons (ganglion cells) are located in the ganglion cell layer (GCL) (Kaneko, 1979). During early stages of retinal development, the outer neuroblastic layer (ONBL) consists almost entirely of mitotic progenitor cells, while newborn neurons (mostly consisting of amacrine and ganglion cells) reside in the inner

neuroblastic layer (INBL). The position of mitotic progenitors within the ONBL varies depending upon their progress through the cell cycle, with S phase cells being found on the vitreal side of the ONBL near the border with the INBL and M phase cells being found on the scleral side of the ONBL abutting the retinal pigment epithelium (Young, 1985a, b).

The photoreceptor outer segments interdigitate with the apical microvilli of the retinal pigment epithelium (RPE). The retinal pigment epithelium is a monolayer of polarized cells that supports the neural retina. It absorbs light by virtue of its pigment, facilitates the exchange of nutrients and waste between the retina and the blood, and participates in the regeneration of Vitamin A moieties for visual photo transduction (Strauss, 2005). The retinal pigment epithelium forms the blood-brain barrier between the neural retina and the choroid. The choroid is a rich vascular network that supplies the retina with nutrients. The segmental distribution of the choroid is complementary to but distinct from that of the central retinal artery, necessitating both arterial sources for adequate oxygenation of the retina.

### **1.3 Determination of Retinal Cell Fate**

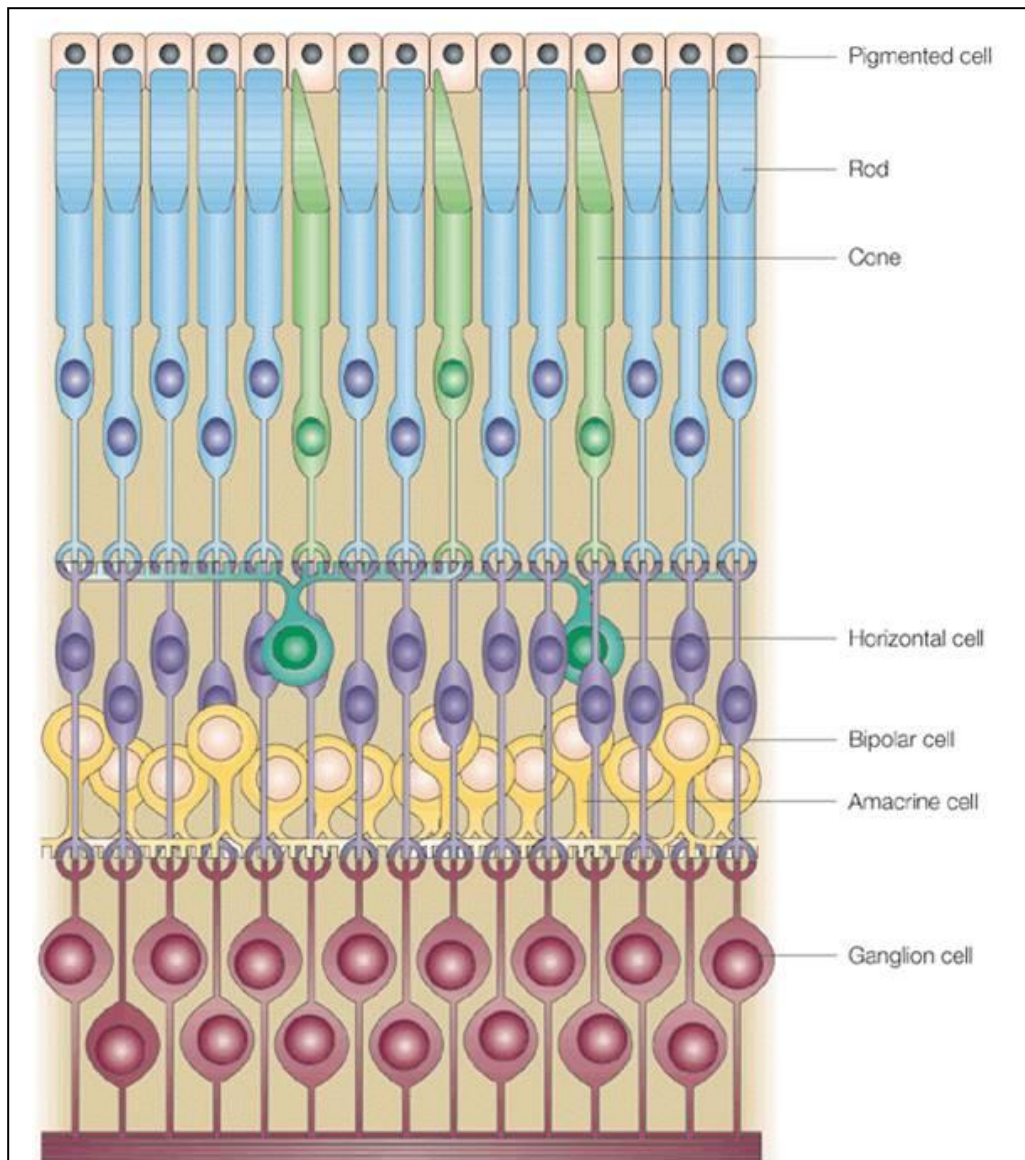
The seven cell types are derived from a common pool of multipotent retinal progenitor cells (RPC) that differentiate in a conserved chronological order (Livesey and Cepko, 2001). Retinal ganglion cells, cones, horizontal (HC) and amacrine (AC) cells are produced first, whereas rods, Müller glial cells and bipolar cells are generated last. Retinal development is performed in an orderly combination of four major tasks: proliferation, migration, differentiation, and formation of synaptic connections. The RPC

differentiation pathway choice is determined by cell-intrinsic, i.e., transcription factors, and cell-extrinsic factors. First, secreted signaling molecules and cell-cell interactions must accurately transmit a combination of spatial and temporal cues (extrinsic). Second the developing cell must correctly interpret these cues and appropriately respond (intrinsic). This results in the generation of each of the cells types of the retina in the proper temporal order, relative number, and laminar location within the retina.

Previous studies have revealed that the development of the vertebrate retina is a conserved process of cell genesis with the following order of cell birth: ganglion cells, horizontal cells, cone photoreceptors, amacrine cells, bipolar cells, rod photoreceptors, and Müller glia. Birthdating studies have shown that retinal cell types are generated in two major waves of overlapping intervals, with ganglion cells, cone photoreceptors, amacrine cells, and horizontal cells generated prior to birth, and bipolar neurons and Müller glia generated after birth in mice. Rod photoreceptor cells (rods), the most abundant cell type in the retina, are born both pre- and postnatally, with a peak of genesis coincident with the day of birth in the mouse (Young, 1985a, b).

Retinal cell fate specification is regulated by multiple transcription factors. Multiple studies shown that the Math5–Brn3b pathway regulates retinal ganglion cell genesis (Liu et al., 2001; Yang et al., 2003). It has been shown that bipolar cell genesis is regulated by two different classes of transcription factors, the bHLH genes Mash1 and Math3, and the homeobox gene Chx10 (Hatakeyama et al., 2001) et al., 2001). Multiple factors, NeuroD, Math3, Ptf1, Pax6, Six3 and Foxn4 (Liu et al., 2001) have been shown to regulate the genesis of amacrine cell (Fujitani et al., 2006; Hatakeyama et al., 2001; Inoue et al., 2002; Li et al., 2004). Horizontal cell genesis is regulated by Foxn4, Ptf1a and Prox1. If

any of these three genes are mutated, horizontal cell genesis is compromised (Dyer et al., 2003; Fujitani et al., 2006; Li et al., 2004). The homeobox gene Crx and Otx2 are key molecules regulating photoreceptor cell development. Otx2 transactivates Crx and is required for photoreceptor cell fate determination, because deletion of Otx2 results in the conversion from photoreceptor cells to amacrine- like cells (Chen et al., 1997; Furukawa et al., 1997; Nishida et al., 2003). Likewise, Crx-mutant retinas display defects in photoreceptor cell genesis (Furukawa et al., 1999). Even though above mentioned factors are shown to be involved in the determination of retinal cell fate, how these genes are coordinated and regulated in retinal cell genesis remains elusive.



**Figure 2. The laminar organization of vertebrate retina.**

Although, more than 50 types of retinal neurons have been identified, the vertebrate retina is mainly composed of six major types of neurons and one major type of glial cells. Figure source: (Livesey and Cepko, 2001).

## **CHAPTER 2**

### **RATIONALE**

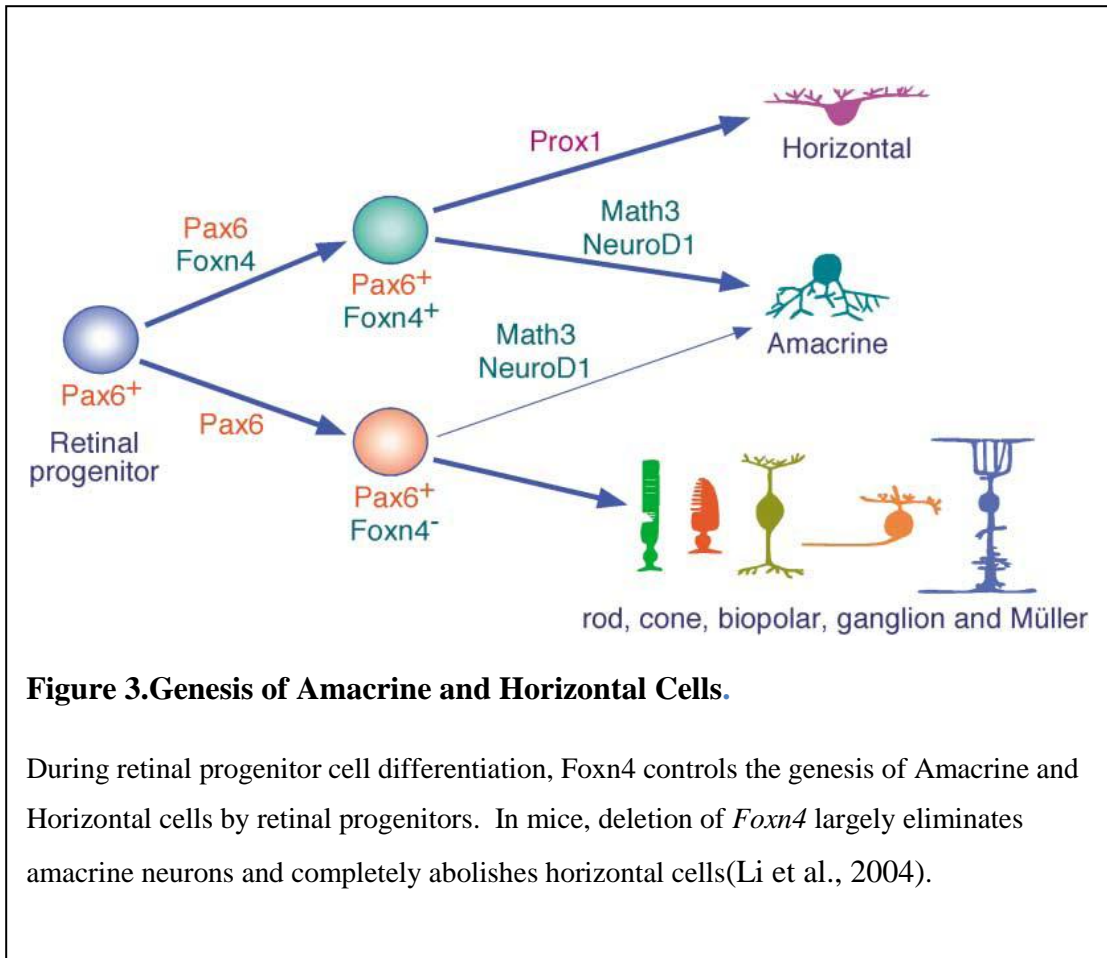
Many transcription factors have been found to regulate the genesis and differentiation of one or more retinal cell types. An excellent way to gain the understanding how these factors work together in networks is the dissection of the cis-regulatory elements of key transcription factors. Many advances have been made in the area of in ovo/vivo electroporation methods to study key developmental genes and their regulatory elements using overexpression, knockdown, or promoter/enhancer specific reporter gene assays.

## **2.1 Role of Foxn4 during Retinal Cell Differentiation**

Transcription factor Foxn4 belongs to the subfamily N of forkhead transcription factors, which includes Foxn1 to Foxn6. In mice, chicken and lower vertebrates like fish and tadpole (*Xenopus laevis*) the gene is expressed in brain tissue, spinal cord, olfactory organs, lung and the retina (Boije et al., 2008; Danilova et al., 2004; Gouge et al., 2001; Kelly et al., 2007; Li and Xiang, 2011). In addition, Foxn4 is expressed in the atrium ventricle canal (Chi et al., 2008) and in the thymus (Danilova et al., 2004; Schorpp et al., 2002) of adult zebrafish. The Foxn4 expression was analyzed in the retina and the atrium ventricle canal in a *Foxn4* knock-out mouse and an ENU mutant zebrafish, respectively. Because of early lethality the phenotype of both animals was only analyzed during the embryonic and larval stages (Chi et al., 2008; Li et al., 2004). In mice Foxn4 influences alveologenesis during lung development (Li and Xiang, 2011). In the retina Foxn4 is necessary and sufficient for the commitment to the amacrine cell fate and is non-redundantly required for the generation of horizontal cells. Both are interneurons that modulate and integrate visual signals in the retina and are born early from multipotent progenitors (Fig-3) (Li et al., 2004). Prox1, Foxn4, and Ptf1a are transcription factors that



are necessary for horizontal cell-fate determination of retinal progenitor cells (Dyer et al., 2003; Fujitani et al., 2006; Li et al., 2004; Nakhai et al., 2007). Foxn4 is upstream of Math3, NeuroD1, and Prox1 since expression of these genes are reduced in Foxn4-KO mice retinas, and accordingly, overexpression of Foxn4 induced these genes (Fujitani et al., 2006; Li et al., 2004; Nakhai et al., 2007).



**Figure 3. Genesis of Amacrine and Horizontal Cells.**

During retinal progenitor cell differentiation, Foxn4 controls the genesis of Amacrine and Horizontal cells by retinal progenitors. In mice, deletion of *Foxn4* largely eliminates amacrine neurons and completely abolishes horizontal cells (Li et al., 2004).

## 2.2 Non-Coding Sequences and Gene Regulation

Annotation efforts and gene prediction methods have begun the process of identifying protein coding genes, however robust high-throughput methods for detecting functional non-protein coding elements remains elusive (Frazer et al., 2003). Only about 2 percent of the human or mouse genomes consist of DNA sequences that are protein-coding regions (Makalowski, 2001). The remaining vast majority of the genome consists of non-coding sequences (Nobrega et al., 2003; Vavouri et al., 2007; Woolfe et al., 2005). It has been shown that gene regulatory elements (GREs) reside in the non-coding sequences. GREs have been broadly placed into two major functional groups: promoters and enhancers. Promoters are sequences that direct the precise locations of transcription start sites. Promoters are therefore usually located close to the 5' start of the gene. Enhancers are sequences that bind gene regulatory proteins and influence the transcription activity of a gene. Enhancers can be located upstream, downstream, or even internal to the target gene. Enhancers, therefore, act as switches to turn gene expression on or off and as modulators to increase or decrease expression. Traditionally, non-coding sequences have not received as much attention from investigators as protein coding sequences and GREs are generally poorly defined, mostly as only sequence motifs. Research is now increasingly focusing on non-coding sequences and specifically the search for non-coding sequences with regulatory function. Identifying functional non-coding sequences and understanding their mechanism of operation will shed new insights into the understanding of the regulatory functions of transcription, DNA replication, chromosome pairing, and chromosome condensation (Kellis et al., 2003; Makalowski, 2001).

### **2.3 Identification of Gene Regulatory Elements through Computational Prediction**

Highly conserved noncoding sequences are extensively associated with temporal, spatial, and quantitative regulation of gene expression, development and disease (Davidson and Erwin, 2006; Kleinjan and van Heyningen, 2005). There are two commonly used methods for identification of functional GREs. The first uses gene expression analysis and the second uses comparative genomics. DNA microarray gene expression profiling is capable of evaluating thousands of genes across various experimental conditions.

Bioinformatics approaches are used to cluster subsets of genes that show similar patterns of expression. Once genes with similar patterns of expression are identified, they are searched within their upstream non-coding sequences to identify over-represented or conserved sequence motifs (Brazma et al., 1998; Bucher, 1999; Fujibuchi et al., 2001; Roth et al., 1998). The second method utilizes sequence alignment algorithms to identify conserved sequences from diverse species in non-coding regions located within and around genes with the same function, known as homologous genes. Genes from different species with the same function should also share some of the same regulatory mechanisms and elements. Therefore, non-coding regions that are highly conserved amongst distantly related species should have regulatory function that can be identified using alignment analysis. Functional regions (which consist of protein coding regions along with regulatory regions) should experience selective pressure against change and therefore have a higher level of sequence conservation across a wide range of species than non-functional regions. Ideally, selective pressures allow for nonfunctional sequences to diverge due to evolutionary drift while leaving functional regions with high similarity (Bejerano et al., 2004; Brudno et al., 2003; Frazer et al., 2003; Frazer et al.,

2004; Mayor et al., 2000; Schwartz et al., 2003a; Schwartz et al., 2003b; Schwartz et al., 2000; Stojanovic et al., 1999). Comparative genomic sequence analysis has been used successfully to identify evolutionarily conserved regulatory elements that drive cell-specific gene expression (Aparicio et al., 1995; de la Calle-Mustienes et al., 2005; Fisher et al., 2006; Levy et al., 2001; Marshall et al., 1994; Nobrega et al., 2003; Pennacchio et al., 2007; Prabhakar et al., 2006; Xie et al., 2005). Transcriptional regulatory regions in genes from humans, mouse, Fugu fish, *Caenorhabditis elegans*, *Drosophila*, and yeast have been identified through identifying conserved non-coding sequences (Bergman and Kreitman, 2001; Cliften et al., 2001; Kellis et al., 2004; Kellis et al., 2003; Loots et al., 2000; Thacker et al., 1999; Woolfe et al., 2005). The power of comparative genomics analysis is enhanced significantly when genomic sequences are available from a number of related species that have diverged sufficiently.

## **2.4 Functional Verification of Predicted Gene Regulatory Elements**

Gain-of-function assay using transgenic mice is a well-established method for studying development (Lewandoski, 2001). However, the use of transgenic mice is very time-consuming. Electroporation can be used with mice, rats, or chicks and multiple plasmids can be electroporated at once. Thus, *in vivo/ovo* electroporation offers a rapid alternative to the use of transgenic mice for gain-of-function assays. While the expression of transfected genes is not permanent it is long enough to study the development of the animal model (Matsuda and Cepko, 2004, 2007). This method has been used to study the function of various genes (Matsuda and Cepko, 2007), the cell specificity of particular

promoters (Matsuda and Cepko, 2004), and the study of enhancers (Timmer et al., 2001). In ovo/ex vivo electroporation will be used in this as a key method for studying normal development and for the experimental verification of CRs for enhancer function.

## **2.5 Regulation of *Foxn4* Expression**

Although the functions of *Foxn4* during tissue development have been well studied, little is known about the molecular mechanisms that regulate the spatial and temporal expression of *Foxn4*. Genome comparisons using the human, mouse, chicken, and other vertebrate sequences reveal remarkable conservation of the *Foxn4* gene. To identify novel regulatory elements involved in the transcriptional regulation of the *Foxn4* gene in the retina, we assessed 4 highly conserved noncoding sequences using a reporter assay system with the aid of *in-ovo* electroporation technique (Doh et al., 2010). 2 conserved regions were found to have the ability to drive gene expression in the retina. A highly conserved sub-region with 129 bp long noncoding sequence (*Foxn4*CR4.2) was shown to drive gene expression preferentially in horizontal and amacrine cells. These results demonstrate the presence of a novel enhancer for *Foxn4* located between the *Myo1h* and *Acab* genes. Furthermore, *Meis1* transcription factor was determined to interact with *Foxn4*CR4.2 using the electrophoretic mobility shift assay (EMSA) and site-directed mutagenesis assay. Knocking down the expression of *Meis1* using transfection of short hairpin RNA (shRNA) specific to *Meis1* gene into the retina diminish the transcription activity of this novel enhancer. Also, absence of *Meis1* severely affects the expression of

*Foxn4* in the retinal cells. Together, my thesis provides a new insight into the regulatory mechanism of *Foxn4* expression during retinal progenitor cell differentiation.

## CHAPTER 3

### MATERIALS AND METHODS

#### **3.1 *Sequence Alignments***

The sequences and annotations of the mouse *Foxn4* gene along with its homologs from the human, rat, cow, chicken and other vertebrate genomes were retrieved using Non Coding sequence Retrieval System (NCSRS) (Doh et al., 2007). This web-based sequence retrieval system was developed by our lab which can quickly and easily extracts noncoding sequences associated with a specific user defined gene set from a single and/or multiple genomes. The NCSRS efficiently delivers non-coding sequences for specified genes or gene sets using a user-friendly interface from a single site. This system



eliminates the need to manually sift through genome sequences and look for annotation information from multiple sources. This helped eliminate human errors as well as increase throughput for those investigating gene regulatory elements.

These sequences were then aligned using multi-LAGAN to identify sequences of at least 75% identity over a 100 bp span. Annotations were submitted to multi-LAGAN (Brudno et al., 2003) and mVISTA (Frazer et al., 2004) along with their respective sequences to ensure that highly conserved regions of interest were non-coding regions. The percent identity and the length of the conserved sequence were used to calculate a score for each conserved region ( $\text{score} = \text{percent identity} + (\text{length}/60)$ ). A limit of 2 kb was implemented in order to isolate individual enhancers for study. Based on this scoring system the percent identity was more heavily weighted to ensure that shorter very highly conserved sequences are not ranked below longer sequences with lower levels of conservation (Fig. 6).

Motif-based sequence analysis tools, MEME (Multiple Em for Motif Elicitation) suite was used to find highly conserved motifs within conserved regions (Bailey et al., 2009). CR4 sequence along with its homologs from the human, rat, cow, chicken and other vertebrate genomes were retrieved using NCBI genome database and submitted to MEME (<http://meme.sdsc.edu/meme/cgi-bin/meme.cgi>).

### **3.2 DNA plasmid**

Computational predicted conserved regions were amplified using *Taq* PCR Kit (New England Biolabs, MA) following the routine *Taq* PCR reaction protocol. Primers used were summarized in Table 2. Mouse genomic DNA was extracted from an adult mouse

tail and used as the PCR template for all primers. A random extension sequence and the SpeI recognition sequence (ACTAGT) was added to the 5' end of the forward primer and the random extension sequence and FseI recognition sequence (GGCCGGCC) to the 5' end of the reverse primer. These modifications produced PCR products with ends that could be digested with their respective restriction enzymes to produce sequence specific “sticky ends”, allowing for sequence specific ligation. The sticky end inserts were gel purified after digestion and ligated into the  $\beta$ GP-GFP backbone linearized with FseI and SpeI to produce enhancer test constructs (Fig. 1b).

### **3.3 *Chicken embryos***

Fertilized pathogen-free (SPF) white leghorn chicken (*Gallus domesticus*) eggs were obtained from Sunrise Farms (Catskill, NY). These eggs were incubated at 37.5°C and 60% humidity (GQF manufacturing, Savannah, GA) for 96-100 hours (~ 4 days) to obtain embryos that are at the developmental stage HH22. Stages of the chick embryo were determined according to Hamburger and Hamilton (Hamburger, 1992; Hamburger and Hamilton, 1992). All of the animal experiments were approved by the Institutional Animal Care and Facilities Committee at Rutgers University.

### **3.4 *Retinal cell cultures and ex-vivo electroporation***

Retinal explant cultures were prepared as described previously (Tabata et al., 2004). Briefly, retinas derived from mouse embryos were placed on a Millicell chamber filter insert (Millipore). Filters were placed into a six-well plate containing 1 ml of explant

media and cultured. Monolayer culture was set up as described earlier (Koso et al., 2006; Matsuda and Cepko, 2004). Electroporation was done using Electroporator BTX ECM 830 (Harvard Apparatus), Round Platinum 2mm Petridish Electrode, CUY700-P2E (Protech) and Round Platinum 2mm Cover Electrode CUY700-P2L (Protech) as described before.

### ***3.5 In ovo electroporation***

Targeted retinal injection and *in ovo* electroporation was performed according to the protocol developed in our lab (Doh et al., 2010) with few modification. I have advanced the technique to significantly increase the success rate and recently, published the video protocol of this technique in the Journal of Visual Experiment (JoVE). Therefore, it is described in detail at the end of this chapter. Briefly, the pulled glass micropipette needles were attached to a 0.1 ml Hamilton Gastight 1710 syringe (Reno, NV) mounted on a WPI M3301-M3 micromanipulator (Sarasota, FL). The needle was loaded with a mixture of 2  $\mu$ l of reporter plasmid DNA solution with a concentration ranging 3-6  $\mu$ g/ $\mu$ l and 0.2  $\mu$ l of fast green (0.025%). Plasmid constructs were directly delivered into the embryonic chick subretinal space (Fig. 1c) and electroporated with 5 square pulses of 15V for 50 ms with 950 ms intervals using a pulse generator ECM 830 (BTX Harvard Apparatus).

### ***3.6 Tissue processing and sectioning***

Chick embryos were harvested at various times after electroporation, and placed in cold 1x PBS (Phosphate buffered saline, Fischer Scientific). Retinas were dissected at E6, E7 or E8. Tissues from chick retina were harvested at different development stages (E6, E7, and E8) and fixed in 4% paraformaldehyde (in 1x PBS) for up to 4 hours and washed in PBS 3 times for 5 minutes at 4°C and then infiltrated overnight in 30% sucrose (in 1x PBS). For embryos injected with reporter plasmid constructs, successful transfection (Fig. 2) was verified by examining the retinas under a fluorescent dissection microscope, Leica MZ16FA (Leica Microsystems, Germany) before embedding and sectioning. Tissues were embedded in OCT (Electron Microscopy Sciences, Hatfield, PA) and stored at -80°C until ready for sectioning. Retina tissues sections at 10-15 µm were cut using a cryostat (Thermo 0620E), mounted on Superfrost slides (Fisher Scientific) and air-dried. Immunohistochemistry was performed immediately afterwards.

### ***3.7 Immunofluorescence***

Immunostaining were performed using Shandon Slide Rack (Thermo Scientific, MA). Sections were incubated in blocking solution (0.05% Triton X-100, 10% goat serum or donkey serum, 3% BSA in 1x PBS) for 1 hour at room temperature followed by overnight primary antibody application. Primary antibodies and dilutions used were as follows: goat or rabbit anti-GFP (1:500, Abcam), mouse anti-Foxn4 (1:1000, Aviva), mouse anti-Lim1+2 (1:20, 4F2 supernatant, DSHB), mouse anti-Brn3a (1:200, Millipore), mouse anti-NeuN (1:1000, Millipore), mouse anti-Visinin (1:20, 7G4 supernatant, DSHB) and goat anti-Meis1/2 (1:250, Santa Cruz). Slides were then washed with PBS

and secondary antibodies carrying fluorescence from the appropriate host were applied (1:300 dilution; Jackson Immuno Research, West Grove, PA). The slides were washed with PBS and cover slipped.

### **3.8 *Imaging***

Microscopy and imaging analysis were performed using an upright fluorescence microscope (Zeiss Axio Imager A1) with a monochrome digital camera Axiocam MRM (Zeiss, Germany). Images of GFP-expressing cells and secondary antibody Cy3 labeled cells were taken separately using FITC and DsRed filters, respectively. Images of GFP-expressing cells and secondary antibody Cy3 labeled cells were taken separately using 488nm and 543nm wavelengths, respectively. Images of Cy3 and GFP channels were then overlaid using Adobe Photoshop CS to create pseudo-colored double-labeled images.

### **3.9 *Data quantification***

All experiments where percentages of cells were calculated represent the averages calculated from at least three independently electroporated retinas. Typically, an entire field (160  $\mu\text{m}$   $\times$  360  $\mu\text{m}$ ) of a section of a retina was scored for the relevant markers and electroporated plasmid reporters. In cases where the particular cells to be scored were less abundant, more than one field of a given retina was examined. The minimum number of a particular cell type that was scored ranged from 20 to 140 cells per retina, depending on the abundance within the sample, and each percentage shown in the figures was the

combined average for three separate retinas. Each graph shows the indicated percentage of cells that were scored for a particular feature, with the types of cells being analyzed for that feature as the denominator (described in the figure legends). Error bars in figures represent the standard error of the mean. In cases where results were tested for statistical significance, a student's t-test was applied with a cutoff of  $P < 0.05$ .

### **3.10 *Electrophoretic mobility shift assay (EMSA)***

Matinspector (Genomatix) was used to predict potential sequence specific binding sites for protein factors. Double stranded DNA probes ranging 30-35bp were designed to span Foxn4CR4.2. Probes were synthesized by IDT (Piscataway, New Jersey) as single stranded oligonucleotides. Single stranded oligonucleotides were biotinylated using Biotin 3' End DNA Labeling Kit (Thermo Fisher Scientific Inc, IL) and annealed at room temperature an hour immediately prior to binding. Unlabeled single stranded probes were annealed and used as double stranded competition probes. The molar ratio of 40:1 was used for competition probe to labeled probes. Nuclear extracts were prepared from chick retina at E6, E7 and E8. The EMSA binding reaction and competition reaction were performed according to the LightShiftChemiluminescent EMSA Kit (Thermo Fisher Scientific Inc, IL) protocol. The reaction mixture was loaded onto a 10% non-denaturing polyacrylamide gel containing 0.5x TBE (40 mM Tris, 40 mM borate, 1 mM EDTA). Mini (8 x 8 x 0.1 cm) gels were run at 100 V for 3 h at 4°C and transferred to membrane.

### **3.11 *shRNA targeting Meis proteins***

For RNA interference transfection experiment, knockdown of Meis1 and Meis2 expression was performed by transfecting embryonic chick retina with shRNA specific to *Meis1* and *Meis2* genes or a non-targeting control shRNA (OriGene Technologies, Inc., Rockville, MD). Each shRNA clone was constructed within the pRFP-C-RS vector. The Meis-targeting sequence was

5' - AGGTGATGGCTTGGACAACAGTGTAGCT -3' (Meis1 and Meis2),

### **3.12 *In Ovo* Electroporation in Embryonic Chick Retina**

This section is a video protocol that is accepted for publication in the Journal of Visual Experiments (JoVE) (2011, in press). The technique described here was originally developed in the Cai laboratory by Dr. Sung Tae Doh, a former graduate student in this lab. I have modified and improved the technique significantly to increase the success rate of electroporation and the survival of the embryos.

#### **3.12.1 Short Abstract:**

The overall goal of this video was to show how to perform targeted retinal injection and *in ovo* electroporation of DNA/RNA constructs into the embryonic chick retina at the Hamburger and Hamilton stage 22-23, which is about embryonic day 4 (E4). This technique is very useful to study gene regulation and morphological change in developing chick retina.

#### **3.12.2 Long Abstract:**

Chicken embryonic retina is an excellent tool to study retinal development in higher vertebrates. Because of large size and external development, it is comparatively very easy to manipulate embryonic chick retina using recombinant DNA/RNA technology.

Electroporation of DNA/RNA construct into the embryonic retina has a great advantage to study gene regulation in retinal stem/progenitor cells during retinal development.

Different type of assays such as reporter gene assay, gene over-expression, gene knock down (shRNA) etc. can be performed using electroporation technique. This video demonstrates targeted retinal injection and *in ovo* electroporation into the embryonic chick retina at the Hamburger and Hamilton stage 22-23, which is about embryonic day 4 (E4).



Here we showed how a plasmid DNA that expresses green fluorescent protein (GFP) as a marker was directly delivered into the embryonic chick subretinal space and followed by electric pulses to facilitate DNA uptake by retinal stem/progenitor cells using a rapid and convenient *in ovo* electroporation technique. The new method of retinal injection and electroporation at E4 allows the visualization of all retinal cell types, including the late-born neurons (Doh et al., 2010), which has been difficult with a conventional method with injection and electroporation at E1.5 (Blank et al., 2007).

### **3.12.3 Protocol**

#### **3.12.3a Egg Handling and Needle Preparation**

1. Eggs can be stored in a wine cooler at about 13°C for up to 1 week. If the temperature is too high, embryos will start to develop abnormally, while lower temperature causes high mortality. Once the eggs are ready to incubate take out the eggs from the wine cooler and set the eggs vertically with the larger end up. Keep the eggs in the room temperature for at least 2 hours before putting them in the 37.5 °C incubator.
2. Incubate the eggs for 96-100 hours which is about embryonic day 4 to obtain embryos that are at the Hamburger and Hamilton stage 22(Hamburger, 1992; Hamburger and Hamilton, 1992).
3. Prepare micropipette needles from pulled glass capillary tubes and break the tip under a dissecting microscope with a tweezers to get a tip opening about 0.1 µm in diameter and a 20 mm taper. Needles with larger tips have difficulty piercing

the vitelline membrane while smaller tips have difficulty loading and delivering the DNA solution.

4. Attach the needle to a 0.1 ml Hamilton Gastight syringe mounted on a micromanipulator. Use a small piece of Masterplex silicone tube for attaching the needle to the syringe. Because of that you do not need any mineral oil to seal this attachment.
5. Mix 2  $\mu\text{l}$  of reporter plasmid DNA solution with a concentration ranging 3-6  $\mu\text{g}/\mu\text{l}$  and 0.2  $\mu\text{l}$  of fast green (0.025%) on a piece of parafilm. Fast green dye will help to visualize the injection. Slowly, load the needle with the mixture.
6. To free the vitelline membrane from the inner membrane rotate the egg gently about  $180^\circ$  and wait for few minutes then rotate it back to original position and set it for Electroporation.
7. Wipe the forceps and egg shell with 70 % ethanol to avoid infection to the embryo.
8. Make a small hole on the egg immediately above the air cell with a pair of size AA forceps. Be careful not to crack the egg shell. Remove small pieces one at a time to make a small window. Carefully remove the inner membrane using the forceps without touching the vitelline membrane.

### **3.12.3b Injection and Electroporation**

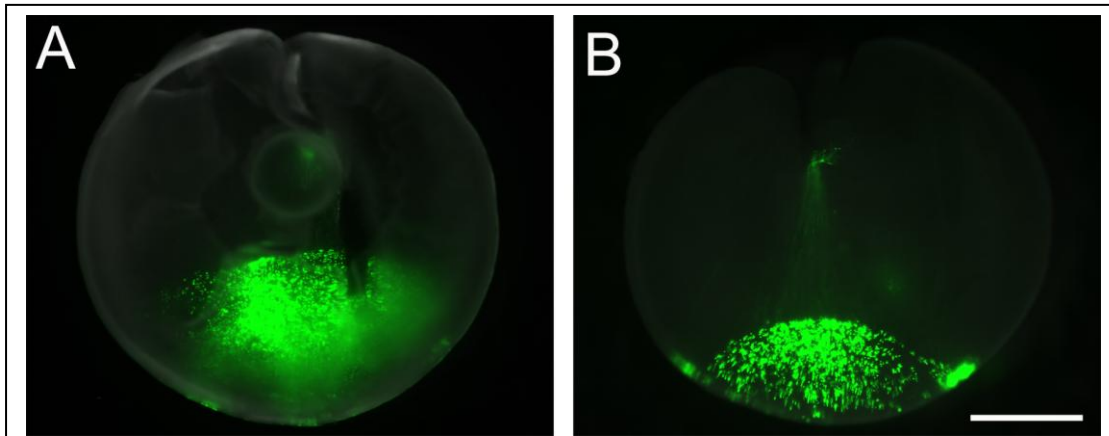
1. To prevent damage to the brain or the heart, position the needle contra lateral to the main bundle of blood vessels entering the eye and pointing towards the beak.

2. Pierce through the vitelline membrane, sclera, retina and vitreous humor by a sudden mild push of the needle. If the needle pierces through the other end of the eye it should be alright unless it damages any major blood vein.
3. Slowly pull back the needle at the edge of the opening and place it almost tangent to the outer wall of the eyeball.
4. Insert the needle into the sub retinal space between sclera and retina.
5. Inject the DNA until you can visualize the green solution filling the side of the eyeball and pushing the retina inwards by creating a bulge.
6. If your needle placing is not correct then you will see the DNA solution is spreading inside the vitreous humor filling up the middle of the eye ball. Also, if you damage the retina too much then you will see the DNA solution is coming out of the eyeball.
7. Slowly remove the needle and immediately place the electrodes in parallel inside the egg after soaking in PBS. Push down the electrodes to submerge into the amniotic fluid in a way so that the injected eye is located between the electrodes. Avoid touching any major blood vein or heart with the electrodes while placing them. Negative electrode should be at the injection side so that DNA can be transported from sub retinal space into the retina towards positive electrode. Electroporate the retina with 5 pulses of 15V for 50 ms with 950 ms intervals.
8. Carefully remove the electrodes and seal the window of the egg with pieces of clear scotch tape.

9. Label and date the injected egg before putting it back to the incubator. Typically, it takes about 3 to 5 minutes to complete the whole electroporation process.
10. GFP expression can be seen as early as 8 hours after electroporation. However, you may wait until the embryo reaches the desired stage before harvesting.

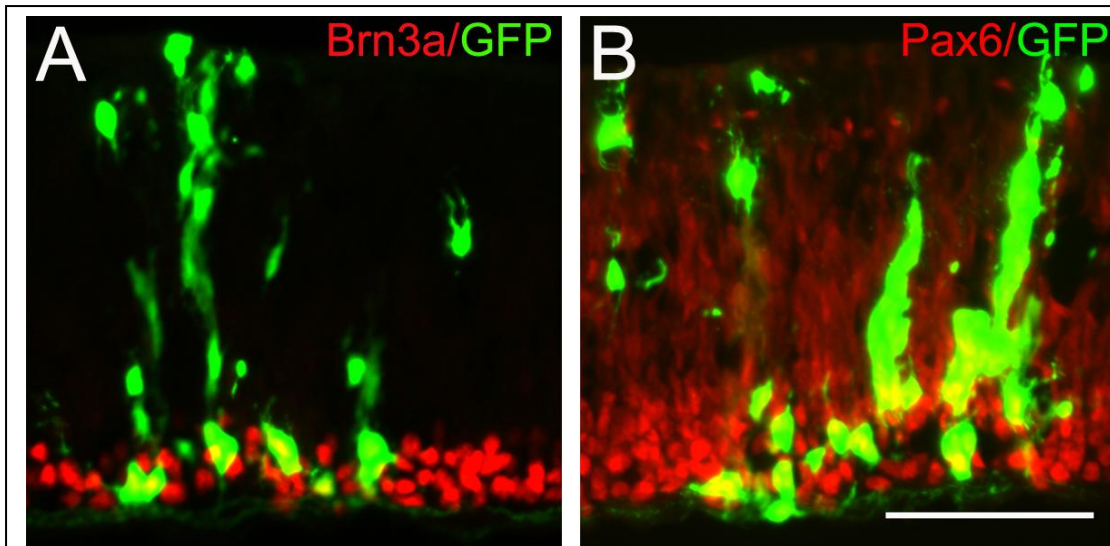
#### **3.12.4 Representative Results**

In our study we use various plasmid constructs to study the regulation of gene expression that involved retinal cell development. In this video protocol pCAG-GFP (transfection control) was used to follow a successful injection and electroporation. However, any plasmid construct with reporter gene (GFP, RFP etc.) can be used. Even though GFP expression can be seen as early as 8 hours after electroporation, we typically start harvesting the egg on day 6 (E6) and onwards. Electroporated retinas were dissected out of the embryo and analyzed under fluorescent dissection microscope before embedding and sectioning. Typically, reporter gene expression can be seen at least in a quarter of the retina after a successful electroporation (Fig 4). The transfected retinal tissues were further analyzed through sectioning for clear visualization of cell morphologies. Immunohistochemistry using cell type specific markers (Brn3a, Pax6 etc.) allowed characterizing cell specific GFP expression (Fig 5).



**Figure 4. Successful electroporation of reporter plasmid results positive GFP expression.**

Chicken embryonic retinas were injected and electroporated at embryonic day 4 and harvested at embryonic day 6. At least 25% of the retina was successfully transfected (A= anterior view, B= Posterior view). Scale Bar = 1mm.



**Figure 5. Characterization of GFP expressing retinal cells using immunohistochemistry method.**

GFP expressing retinal tissues at E7 stage were fixed and sectioned. These sections were then stained with various cell specific markers. Brn3a was used to determine ganglion cells (A) while Pax6 was used to determine horizontal, amacrine and ganglion cells (B). Scale bar = 50  $\mu$ m.

### 3.12.5 Discussion

After we published this technique in our previous paper (Doh et al., 2010), several scientist in this field contacted us for further assistance on this technique. So we decided to produce this video for visualization. In addition, we have advanced our technique since then which is described in this video. Targeted retinal injection and *in ovo* electroporation in embryonic retina at E4 stage can specifically target retinal progenitor cells resulting in the ability to visualize all six major retina cell types at the single cell level. *In ovo* electroporation at HH10 (~E1.5) targeting the optic vesicle is able to transfect cells that develop to form the eye. However, these cells have a very high turnover at this time and this method is not specific for retina cells. It may be that the high cell turnover rate prevents sustained stable expression. By E4, the embryo is developed enough that the major structures of the eye are all formed but young enough that the majority of cells in the retina are still retinal stem cells.

This method can also be applied to gain/loss of function studies where a gene of interest can be targeted to study normal development and/or disease of the retina. To perform this technique successfully, following critical factors worth to be discussed here:

The most critical factor is to place the needle precisely in the subretinal space. Firstly, one needs to be careful to align the needle contra lateral to the main bundle of blood vessels entering the eye and pointing towards the beak. This position is almost parallel to the heart and reduces the chance to damage the brain and the heart. When piercing through vitelline membrane, sclera, retina and vitreous humor, needle should not travel far and pierce through any blood vein. If the needle is sharp enough then this can be done very easily with few practices. Next step is to slowly pulling back the needle and

position it at the edge of the opening of the retina. There is always a chance to pull it out completely. If that should happen, then one can try again to put the needle tip back at the opening. Placing the needle in the subretinal space (between sclera and retina) requires practice and patience. In the beginning, it looks very difficult; however with some practices it becomes easy. While injecting the DNA solution inside the subretinal space, it is very important to observe the bulge formation. Afterwards, the green solution starts filling outline of the eye. This indicates a successful injection. If the solution diffuses away or starts filling into the middle of the eye, that indicates an incorrect injection and one should not waste time to continue this electroporation.

Second factor is the making of an optimal needle via pulling capillary micropipette and breaking the pulled needle tip. Needles with large tips have difficulty to pierce through the vitelline membrane and increase chance to damage the retina. Usually the sharper the needle tips the better for this purpose. However, needle with very small tips have difficulty loading and delivering the DNA and have increased chance to break down inside the eyeball. For this reason we make needle with a tip opening at about  $0.1\ \mu\text{m}$  in diameter and a 20 mm taper (Doh et al., 2010). Also, while loading the DNA mixture on a piece of parafilm, it is very important not to take the last bit as it increases the chance to get air inside the needle.

Finally, placing the electrodes is very critical to achieve successful electroporation. The electrodes should be placed in parallel so that the developing eye is situated between the electrodes. Extra caution should be taken not to touch any major blood vessels or the



heart with the electrodes. Damaging any of these may result death to the embryo even after a successful injection. Furthermore, the two eyes are differently oriented. So, it has to be kept in mind to change the electrode orientation (positive vs. negative) so that negative electrode is always at the injection side to allow the DNA diffuse into the retinal cells under the electric current.

### **3.12.6 Acknowledgments**

We would like to thank Mr. MaazEnver for the use of his HD camera (Cannon VIXIA HFS100). This work was supported in part by grants from NIH (EY018738), New Jersey Commission on Spinal Cord Research (08-3074-SCR-E-0 and 10-3091-SCR-E-0), and Busch Biomedical Research Award.

### **3.12.7 Disclosures**

All of the animal experiments were approved by the Institutional Animal Care and Facilities Committee at Rutgers University.

**Table 1: List of specific reagents and equipment used for Inovo electroporation**

<b>Name of the reagent</b>	<b>Company</b>	<b>Catalogue number</b>	<b>Comments</b>

Fertilized pathogen-free (SPF) white leghorn chicken eggs	Sunrise Farms (Catskill, NY)		
Chicken egg incubator	GQF manufacturing, Savannah, GA		Set to 60% humidity and 37.5°C.
Glass capillary tubes	World Precision Instrument Inc.	TW150F-4	
0.1 ml syringe	Hamilton Co. Reno, Nevada	Gastight 1710	Alternatively 1ml syringe can be used as well
Masterflex Silicone (peroxide) Tubing	Cole-Parmer	HV-96400-13	Cut a small piece (1 cm) for attaching the glass needle to the syringe
Tweezers	Dumont	AA	
Micromanipulator	World Precision Instrument Inc.	M3301-M3	
Plasmid DNA: CAG-GFP			borrowed from Dr. Connie Cepko
Fast Green FCF	Sigma-Aldrich	F7252	Dilute it to 0.025 % with PBS
Pulse generator	Harvard Apparatus, MA	BTX ECM 830	Square wave generator
Electrodes	Harvard Apparatus, MA	BTX model 514	Our electrodes were spaced 3-5 mm apart
Monochrome Digital Camera	Zeiss, Germany	Axiocam MRM	
Fluorescent Dissection Microscope	Leica Microsystems, Germany	Leica MZ16FA	
Upright Fluorescence Microscope	Zeiss, Germany	Zeiss Axio Imager A1	



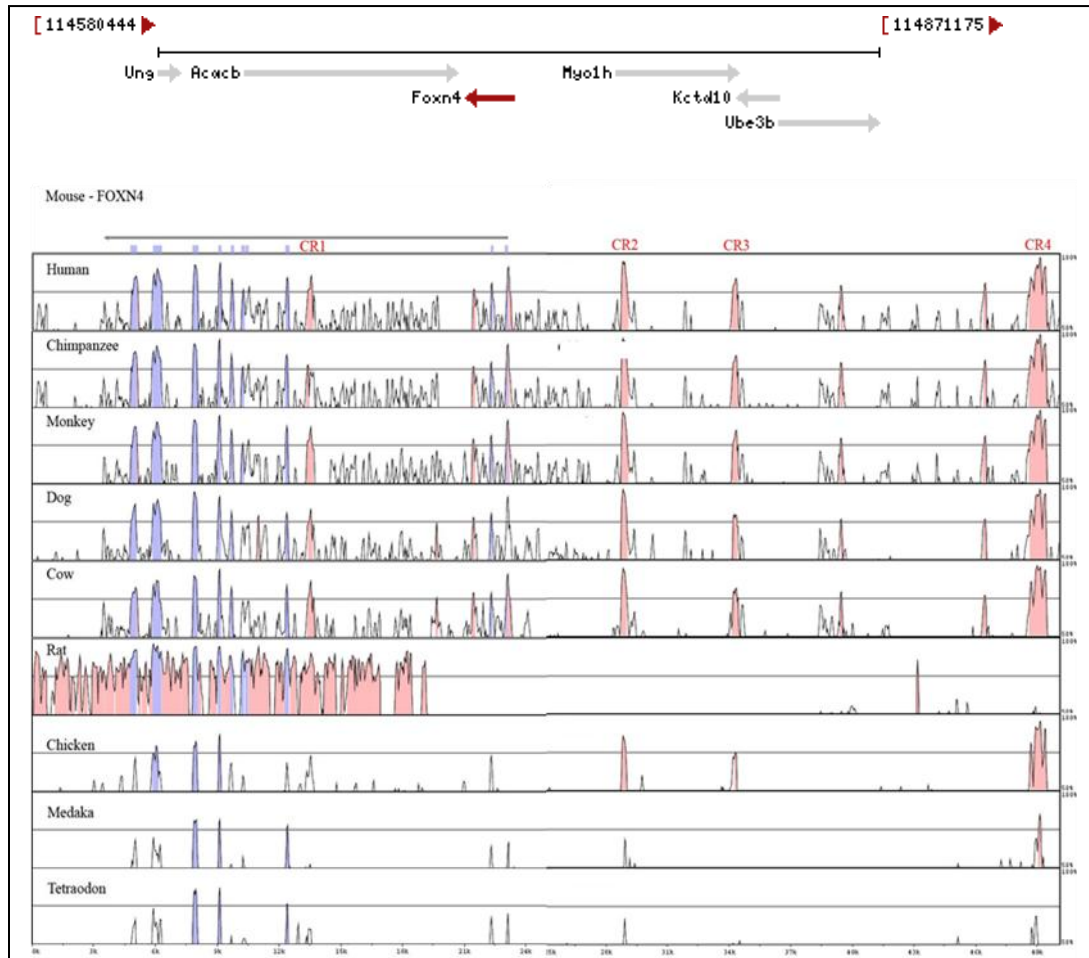
## **CHAPTER 4**

### **RESULTS**

#### **4.1 Identification of *cis*-regulatory elements at the *Foxn4* locus**

During retinal progenitor cell differentiation, *Foxn4* controls the genesis of amacrine and horizontal cells by retinal progenitors (Li et al., 2004). To gain insight into the regulation of *Foxn4* gene, we have performed comparative DNA sequence analysis to identify

evolutionarily conserved non-coding genomic sequences that may serve as *cis*- regulatory elements. The intergenic sequences spanning the 5' and 3' regions of *Foxn4* from various species, including human, mouse, chicken, and other vertebrate species were retrieved using our sequence retrieval system NCSRS (Doh et al., 2007), and aligned using multi-LAGAN and mVISTA (Brudno et al., 2003; Frazer et al., 2004) programs. The coding region of *Foxn4* gene is about 19 kb and it is in between two non-coding regions: 83 kb sequences to upstream adjacent gene *Myo1h* and 4 kb sequences to downstream adjacent gene *Acab*. The resulting sequence alignments revealed four highly conserved regions, and thus, predicted as Foxn4 enhancer candidates (Foxn4CR1-4, Fig.6). One of the regions resides within the intronic region of the gene (Foxn4CR1) while the other three reside upstream of the Foxn4 gene. All four regions were amplified and isolated from the mouse genome using PCR. These regions were then inserted into the  $\beta$ GP-GFP construct upstream of  $\beta$ GP to make four separate constructs (Fig. 7) which were used for electroporation of E4 chick and E15 mouse retinas.



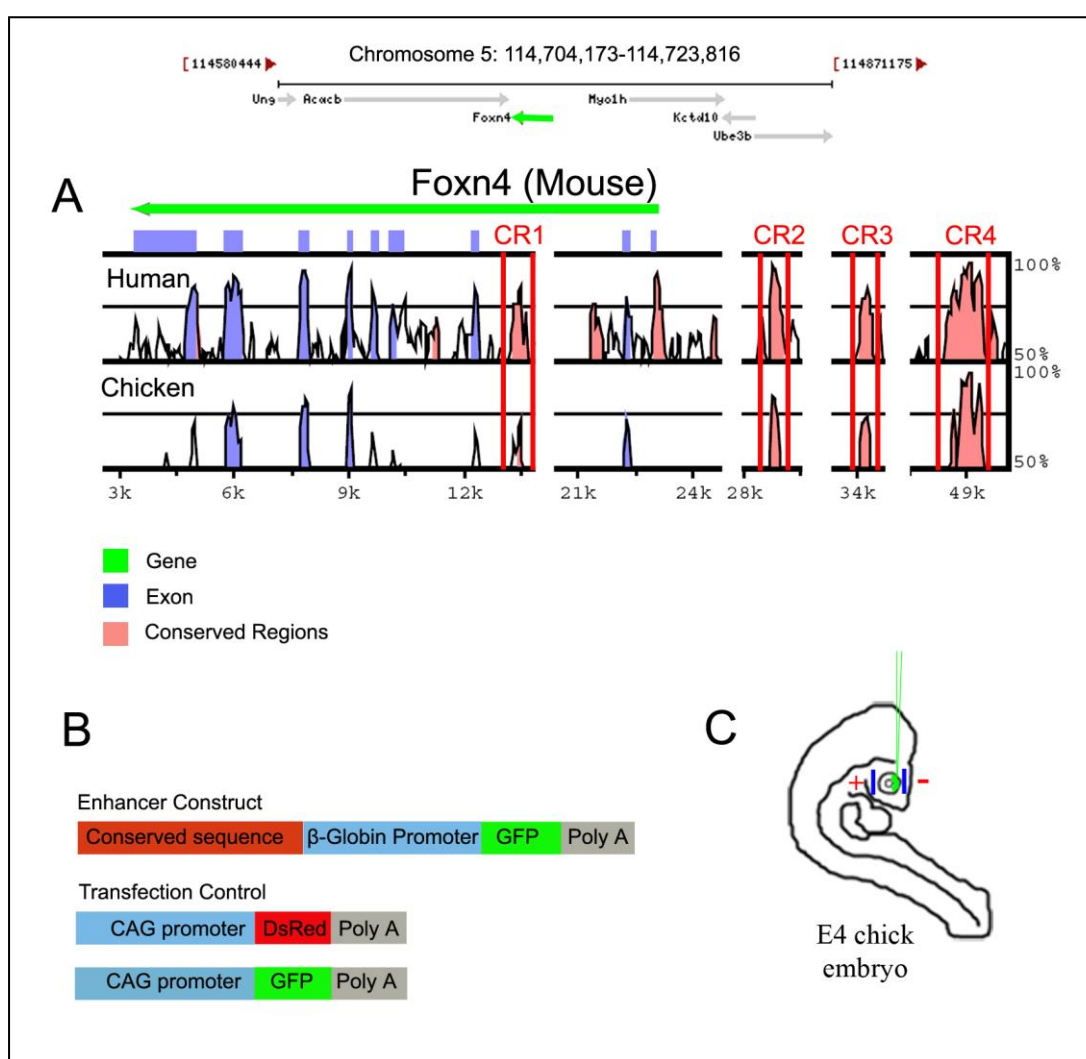
**Figure 6. Computational prediction of highly conserved regions (CR) that surround the mouse *Foxn4* gene.**

The sequences of *Foxn4* genes locus from the various genomes including human, cow, dog, rat, opossum, chick, zebrafish, and tetradon were retrieved using NCSRS (Doh et al., 2007). These sequences were then aligned using multi-LAGAN (Brudno et al., 2003) to identify sequences of at least 75% identity over a 100 bp span. The percent identity and the length of the conserved sequence was used to calculate a score for each CR (score = percent identity + (length/60)). Each alignment corresponds to the same region of the mouse genome. The percent identity of the alignment is between 50% and 100%. Any conserved regions with at least 75% identity are shaded in pink.

## 4.2 Generation of Reporter Constructs for Enhancer Activity Assay

In order to experimentally verify and characterize the function of a potential gene regulatory element (GRE), a reporter assay system containing an enhancer element was designed. This system utilizes a minimal basal promoter,  $\beta$ -globin promoter ( $\beta$ GP) and the reporter gene, green fluorescent protein (GFP). To identify potential enhancers for *Foxn4*, we generated 4 enhancer constructs with computational predicted *Foxn4* enhancer candidates. Each of the 4 conserved regions was individually PCR amplified (Table 1) and cloned into a  $\beta$ GP-GFP reporter construct (Fig. 7B). The primers and the size of PCR products are summarized in Table 2. Previously it has been shown that the negative controls,  $\beta$ -globin promoter ( $\beta$ GP) without any enhancer or with a random sequence showed inability to drive gene expression *in vivo*. However a positive control,  $\beta$ -globin promoter ( $\beta$ GP) with a known enhancer, the RER for Rhodopsin gene (Nie et al., 1996) was able to direct photoreceptor-specific GFP expression confirming the ability of the reporter construct to generate GFP, based on the spatial and temporal function of the enhancer.

For the transfection control, a strong ubiquitous CAG promoter (chicken  $\beta$ -actin promoter with CMV enhancer) is in place of the  $\beta$ -globin minimal promoter (Fig. 7B). For cotransfections, CAG-DsRed was used instead of CAG-GFP along with enhancer constructs to verify the correct transfection (Fig. 8). As shown in the preliminary studies, this transfection control construct CAG-GFP was able to drive reporter GFP expression (Fig. 4 and Fig. 5). A mixture of plasmid DNA constructs including enhancer constructs or/and transfection control, pCAG-DsRed were injected and electroporated into the chick retina at embryonic day 4 (E4) to transfect the retinal progenitor cells (Fig. 7C).





**Figure 7. Four highly conserved regions (CR) near the mouse *Foxn4* gene were isolated and tested for enhancer function.**

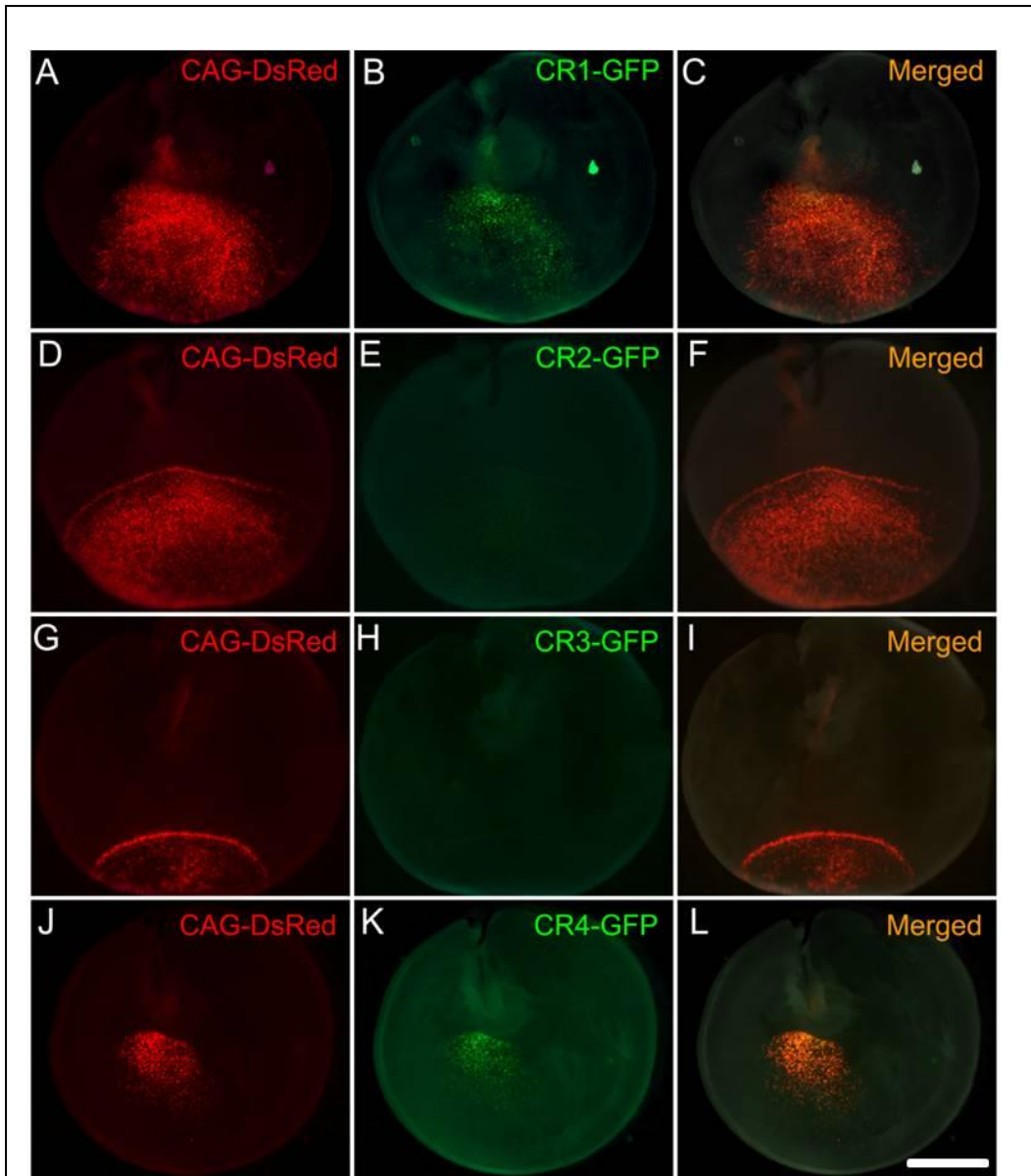
(A) Comparative sequence analysis between mouse and 9 other vertebrate *Foxn4* loci revealed 4 evolutionary conserved regions (CR). For simplicity only mouse, human and chicken alignment is shown here. Blue regions represent exons while pink regions represent conserved non-coding sequence. (B) Schematic presentation of the design of enhancer reporter constructs and transfection control constructs in plasmid. In enhancer constructs conserved sequence is cloned upstream of a basal  $\beta$ -globin promoter and a reporter GFP. In transfection control a ubiquitous CAG promoter (chicken  $\beta$ -actin promoter with CMV enhancer), is placed before a reporter gene (GFP, DsRed, etc.). (C) A mixture of plasmid DNA constructs including enhancer constructs and transfection control, pCAG-DsRed were injected and electroporated into the chick retina at embryonic day 4 (E4) to transfect the retinal progenitor cells.

**Table 2. List of evolutionarily conserved regions at Foxn4 locus and PCR primers for amplifying these regions.**

Conserved Region	Chr Start Position	Chr End Position	PCR product length (bp)	Primer	Sequence
Foxn4CR1	727,879	728,559	681	forward	TCAAACCAGTGTGTGACAGGGTCT
				reverse	TTAAGGGCATCTCGGTAAGTGGGA
Foxn4CR2	743,405	744,155	751	forward	AGCACTGGTTAGCATGGGAACTCT
				reverse	AACATCAGGAAAGCCAAGGAATTT
Foxn4CR3	748,691	749,342	652	forward	TCTGGGCATTTGGCAACTCTGTCT
				reverse	CCAACTGCCAGGGATTGTGATATT
Foxn4CR4	763,291	764,177	887	forward	TGTAAGAAGTGGCCTTGGAGCTGT
				reverse	GCTTCTCTTTCCCAAACCCAAAGTI

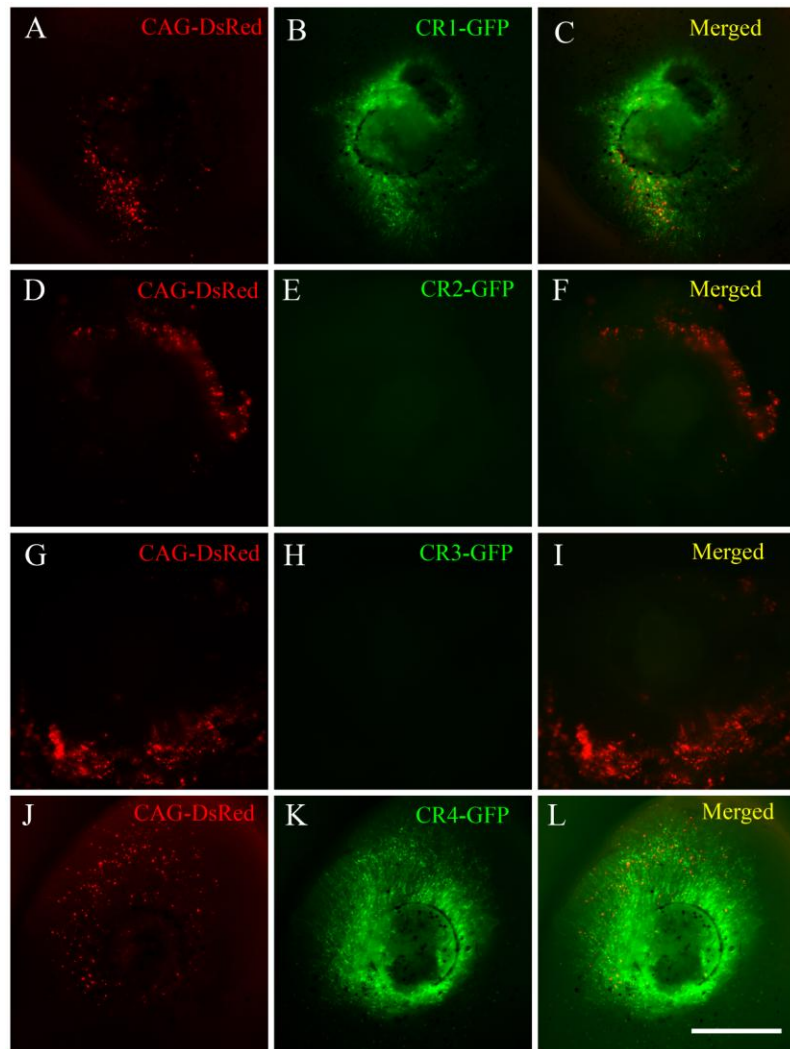
### **4.3 CR1 and CR4 possess enhancer activity in the developing chick and mouse retina**

To examine the enhancer activity of these 4 enhancer candidate sequences (CR1 to CR4), we tested their ability to direct tissue-specific gene expression with the use of a reporter assay system in both mouse and chick using *ex vivo* and *in ovo* electroporation methods. The corresponding mouse sequences of 4 highly conserved regions were individually cloned upstream of a human  $\beta$ -globin minimal promoter (Yee and Rigby, 1993) coupled to a reporter gene, green fluorescent protein (GFP), and injected and electroporated into the developing mouse retina (about embryonic day 15, E15) and into the chick retina Hamilton-Hamburger stage 22 (about embryonic day 4, E4), respectively, to transfect the developing retina. Reporter gene expressions were examined in transfected retinas at various stages during early retina development. Among 4 conserved sequences, *In ovo* electroporation of the embryonic chick retina with CR1- $\beta$ GP-GFP and CR4-  $\beta$ GP-GFP resulted in GFP expression in the transfected retina (Fig. 8, A-C and J-L respectively). Not surprisingly, *ex vivo* electroporation of the mouse retina with CR1- $\beta$ GP-GFP and CR4-  $\beta$ GP-GFP at E15 resulted in GFP expression as well (Fig. 9, A-C and J-L, respectively). Neither CR2 nor CR3 were observed to result in GFP expression in the retina of chick as well as mouse. This indicates that not all conserved sequences might not be functional tissue-specific enhancers. Another possibility is that CR2 and CR3 might be involved in gene regulation, but not sufficient to drive gene expression individually. Since, CR4 is more conserved than CR1; we selected CR4 for further analysis and characterization.



**Figure 8. CR1 and CR4 direct GFP expression in the embryonic chick retina.**

Chick retinas were injected and electroporated with a mixture of pCAG-DsRed (transfection control) and experimental constructs individually containing CR1 – CR4 on embryonic day 4 (E4). Transfected retinas were harvested at embryonic day 6 (E6) for reporter gene expression. Successful electroporation was confirmed by DsRed expression (A, D, G, and J). Imaging through individual channel shows that CR1 (B) and CR4 (K) has the ability to drive GFP expression. However, CR2 (E) and CR3 (H) do not show any ability to drive gene expression.

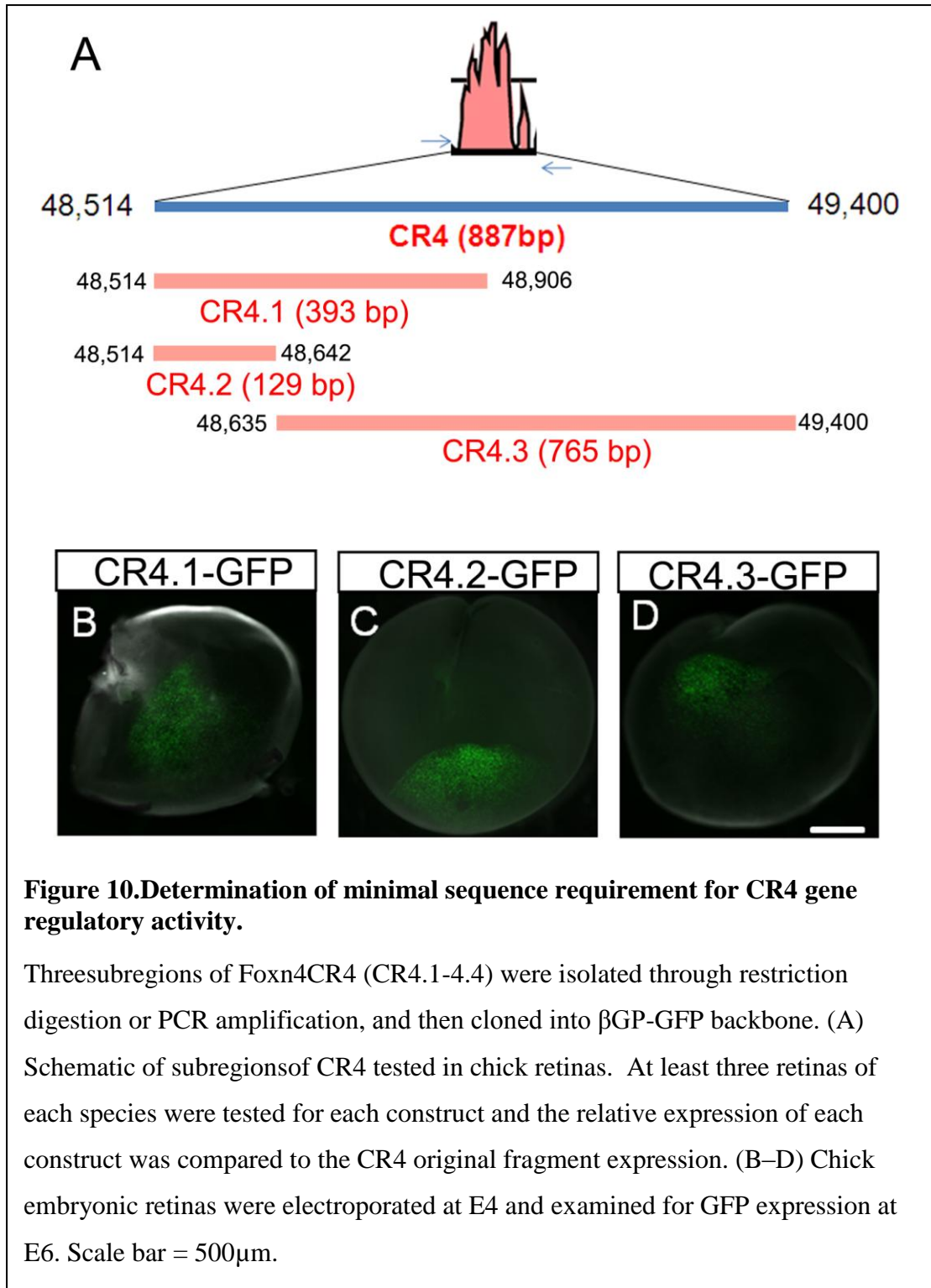


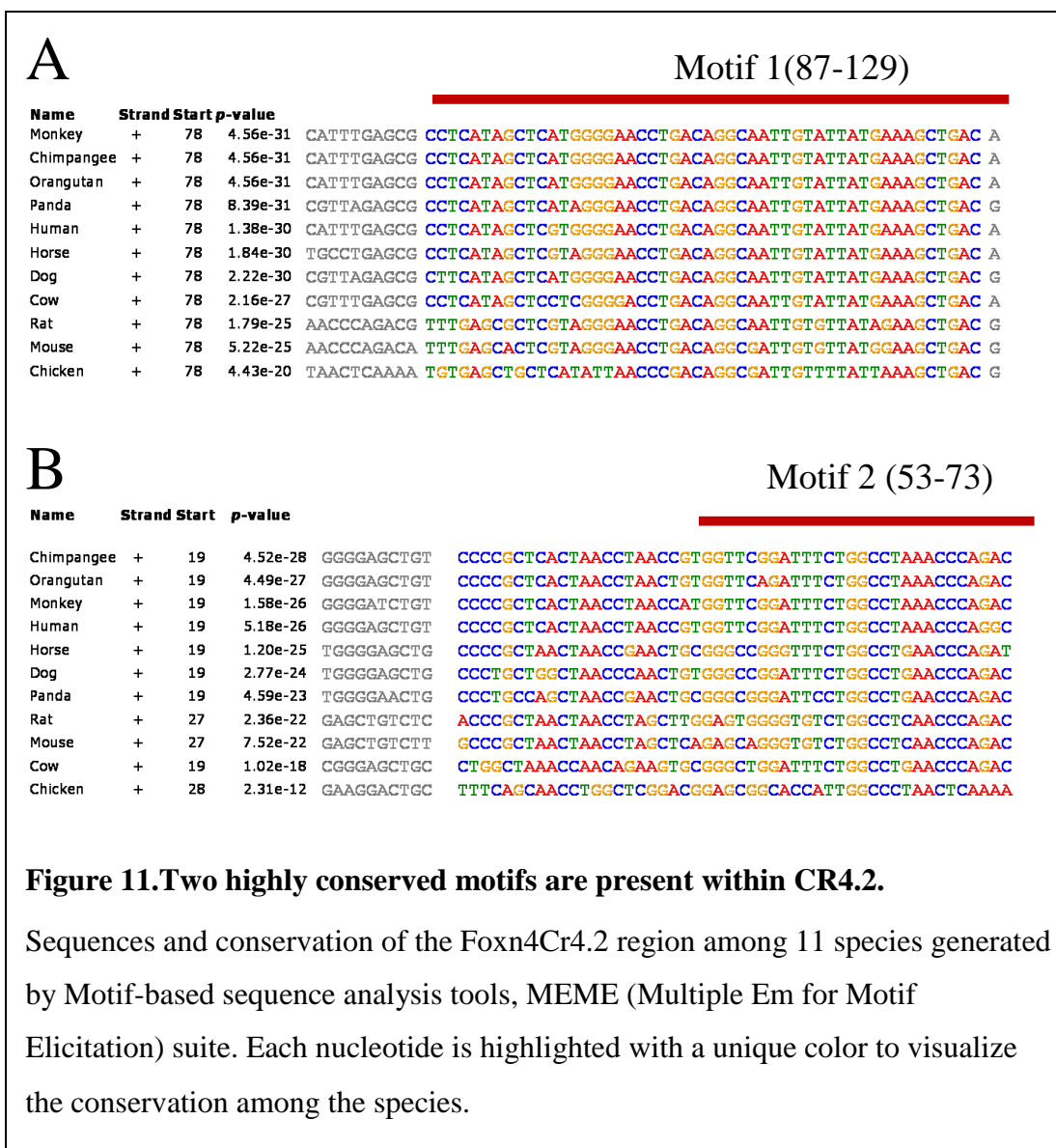
**Figure 9. CR1 and CR4 direct reporter GFP expression in embryonic mouse retina.**

Mouse retinas were transfected with a mixture of pCAG-DsRed (transfection control) and various enhancer constructs at embryonic day 15 (E15) through ex vivo electroporation method. Transfected retinas were examined 65 hours after the electroporation (E 17.5) for reporter gene expression. Successful electroporation was confirmed by DsRed expression (A, D, G, and J). Imaging through individual channel shows that CR1 (B) and CR4 (K) has the ability to drive GFP expression. However, CR2 (E) and CR3 (H) do not show any ability to drive gene expression. Scale bar = 500 $\mu$ m.

#### **4.4 Identification of a functional CR4 fragment which is evolutionarily conserved across phylogeny**

To identify functional subregions we dissected CR4 into pieces and tested individually for their ability to drive gene expression (Fig. 10). The DNA element that encompasses CR4 is an 887 base pair fragment with highly conserved blocks of sequence. Several smaller subregions encompassing various parts of the CR4 were then individually subcloned (Fig. 10) into vector containing  $\beta$ GP-GFP. Each subclone was tested for its ability to drive gene expression in the chick retina (Fig. 10). After careful analysis it was concluded that there are at least 2 active subregions within CR4. All of the fragments that included first 129 bp (CR4.2) showed the ability to drive expression of the reporter gene in chick retinas (only 2 are shown in Fig. 10, A-C). Interestingly, the other portion of CR4 (CR4.3), which is 765 bp long, was also able to drive GFP expression in the retina. However, CR4.2 was the strongest and most minimal element among all fragments of CR4 tested. Moreover, sequence alignment of CR4 region between 11 related species showed that 2 highly conserved motifs across phylogeny are present in CR4.2 (Fig. 11, A & B). Therefore, Foxn4CR4.2 was selected for further analysis.

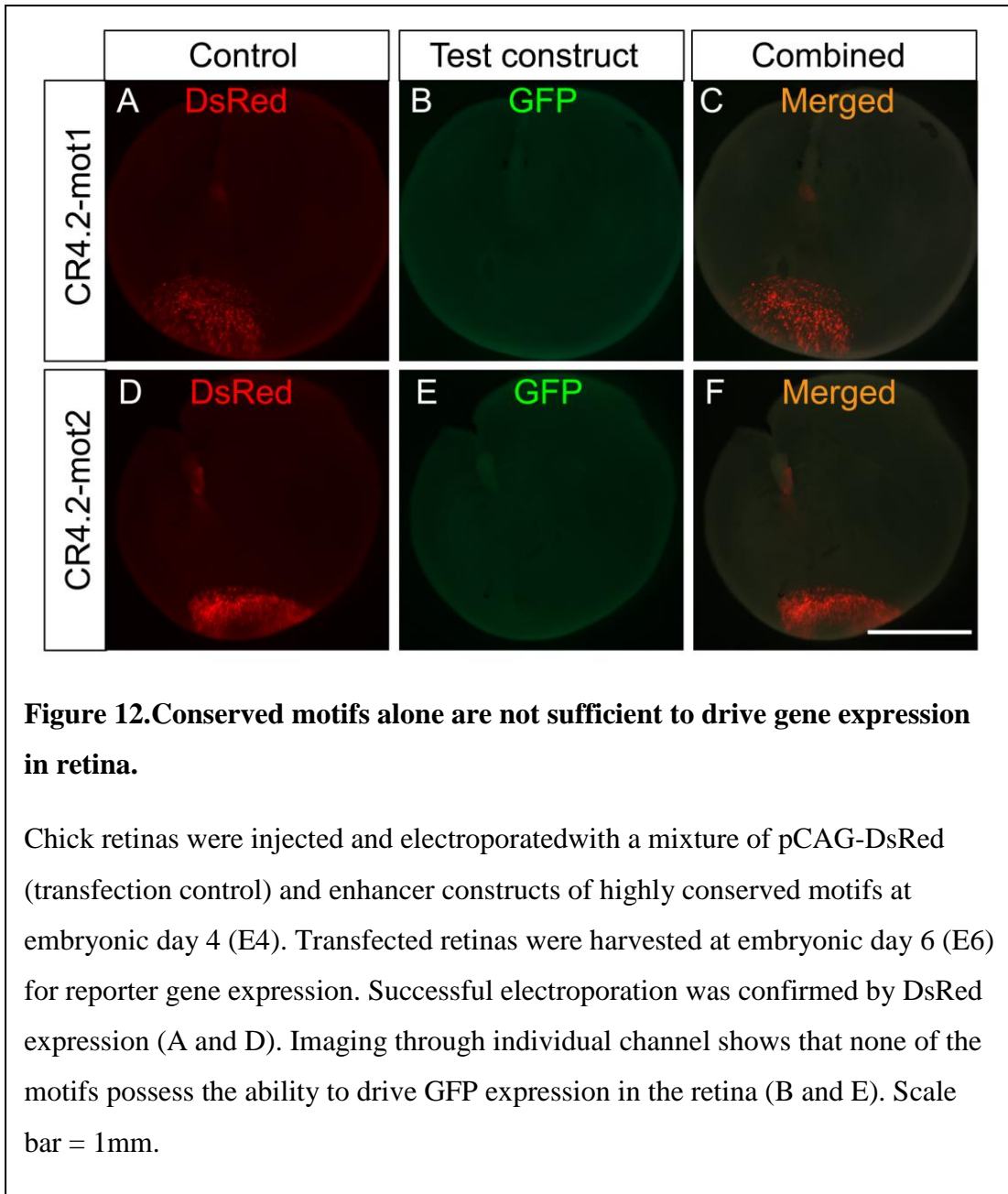






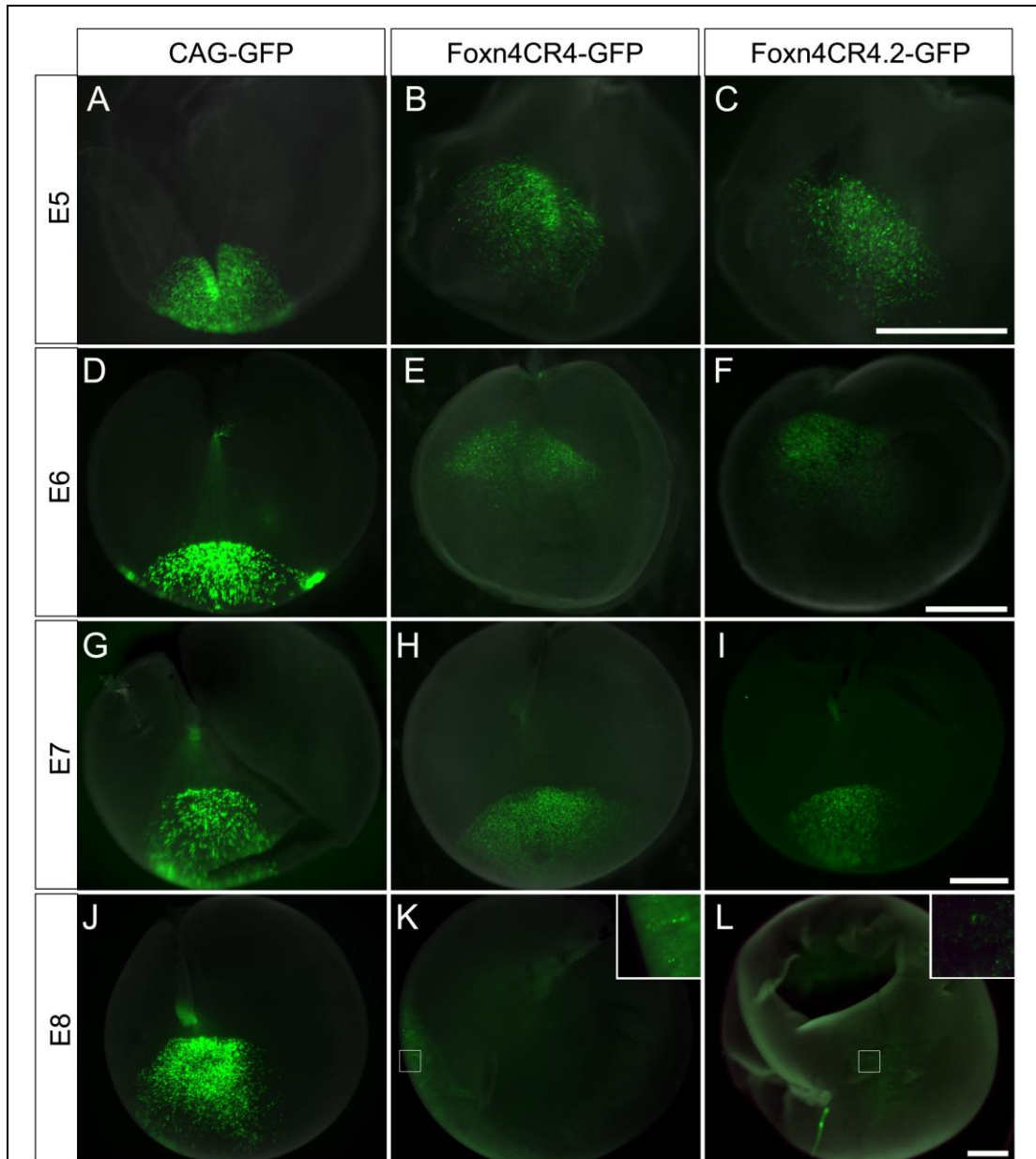
#### 4.4.1 Highly conserved motifs of CR4.2 alone is not sufficient to drive gene expression

Next we wanted to further narrow down CR4.2 to smaller functional sequences based on evolutionarily conserved motifs. We have synthesized 2 motifs, CR4.2-mot1 and CR4.2-mot2 then subcloned into  $\beta$ GP-GFP to individually test their ability to drive gene expression. Neither of the constructs of conserved motifs showed ability to drive reporter gene expression in chick embryonic retina (Fig. 12). This finding indicates that these motifs might be essential but not sufficient to enhance gene expression. Therefore, Foxn4CR4.2-  $\beta$ GP-GFP was selected for further characterization and all further experiments were carried out with this novel enhancer.



#### **4.5 Analysis of spatial and temporal expression patterns of Foxn4CR4.2-GFP in the developing retina**

Even though the expression of Foxn4 in chick retina starts at E2, the majority of the expression occurs between E6 and E9. Expression starts falling sharply after E9 and completely diminishes by E18 (Boije et al., 2008). To confirm that CR4.2 is a true enhancer of Foxn4, first we analyzed the temporal expression pattern of GFP driven by this enhancer in developing chick retina. Transfected retinas were harvested on various stages starting from E5. GFP expression driven by CR4.2 construct was observed in the transfected retina from E5 until E8 (Fig. 13, C, F, I, L). While the expression was strong at E6 and E7 stage (Fig. 13 F, I), by E8 it got very weak (Fig. 13, L) and no expression was observed after E9. Timing of the GFP expression driven by CR4.2, very much follows the same pattern of Foxn4 expression during retina development in chick (Boije et al., 2008). This suggests that CR4.2 could be an enhancer for driving Foxn4 expression in the retina. Moreover, similar expression pattern was observed in the retinas that were transfected by CR4 enhancer constructs (Fig. 13, B, E, H, and K). Interestingly, GFP intensity was much weaker in the enhancer construct transfected retinas compared to CAG driven GFP expression (Fig. 13, A, D, G, J). This could be an indication that CR4.2 might drive gene expression in selective cell types that express Foxn4.



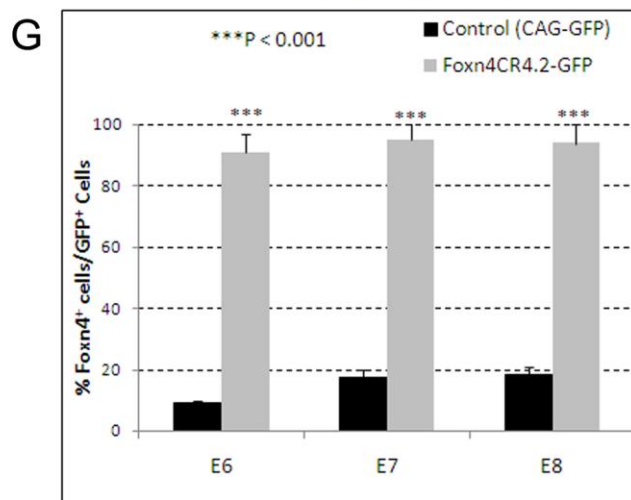
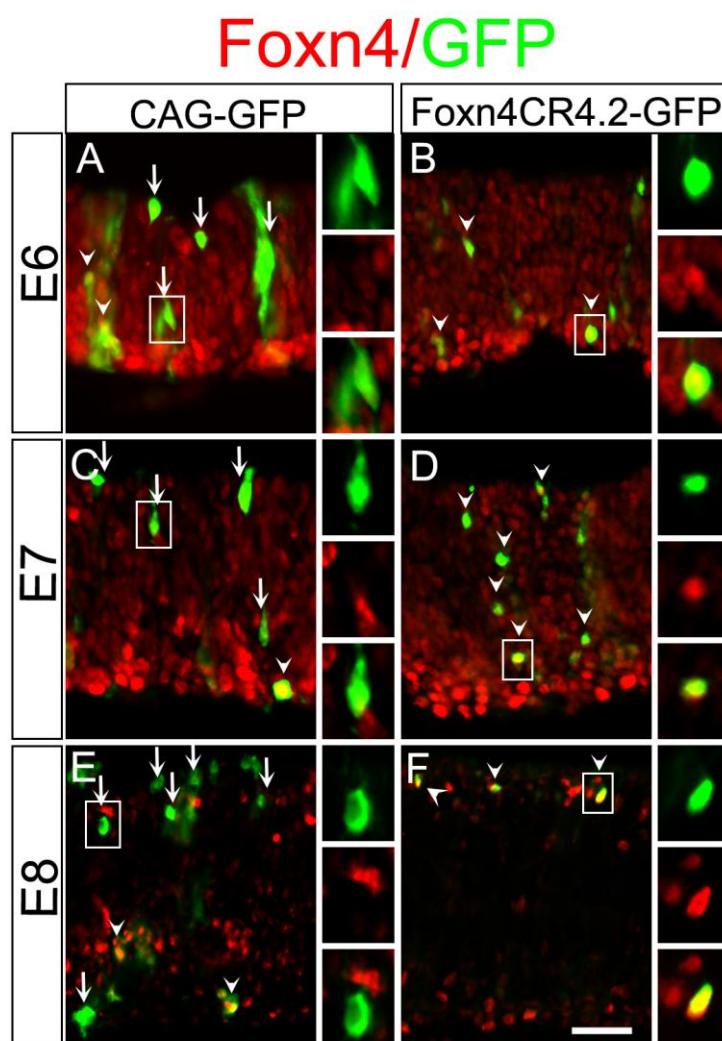
**Figure 13. Foxn4CR4.2-GFP expression pattern in chick retina.**

Chick retinas were injected and electroporated with control pCAG-GFP (A,D,G, and J) , pFoxn4CR4-βGP-GFP (B,E,H, and K) and pFoxn4CR4.2-βGP-GFP (C, F,I, and L) enhancer constructs separately at embryonic day 4 (E4). Transfected retinas were harvested at various embryonic stages from E5 to E8 for reporter gene expression. Scale bar = 1mm.

#### **4.6 CR4.2 is preferentially active in Foxn4 positive cells in the developing chick retina**

The retina does not produce different cell types from retinal progenitor cells dedicated to making only one cell type, with a few exceptions (Godinho et al., 2007; Rompani and Cepko, 2008; Turner and Cepko, 1987). Thus, cells that express a defining cell fate transcription factor can be overlapped among non-expressing cells. To determine if the CR4.2 enhancer accurately recapitulated some or all of the Foxn4 expression in the retina, GFP expression driven by the CR4.2 was compared with Foxn4 protein expression at the cellular level. Upon sectioning the retinas, it became apparent that, in contrast to GFP expression driven by a broadly active control promoter, CAG that was expressed in a number of cells, CR4.2 drove GFP reporter expression in subsets of cells with distinct morphology and laminar locations (Fig. 14). GFP and Foxn4 colocalization in the electroporated population of cells was analyzed as described in Fig. 14. The CR4.2 element plasmid was capable of labeling almost 65- 80% of the Foxn4-positive population (Fig. 14G). In contrast, the CAG promoter was only capable of labeling approximately 10-20% of the Foxn4-positive population. In the electroporated Foxn4-negative population, very few GFP-positive cells were detected with the CR4.2 fragment. The ability of the CR4.2 fragment to drive reporter expression in the Foxn4-positive population of cells, but not the Foxn4-negative population, demonstrates that this element does recapitulate at least some critical aspects of endogenous Foxn4 regulation. It is noteworthy that not all Foxn4-positive cells that were electroporated with CR4.2-GFP were positive for GFP. It is likely that there are other enhancers that drive expression of Foxn4 in this population, such as CR1 or CR4.3. Alternatively, the reporter may not be sensitive enough to capture this expression.





**Figure 14. Foxn4CR4.2-GFP+ cells express Foxn4 in the developing chick retina.**

Chick E4 retinas were electroporated either with the broadly active CAG-GFP plasmid or with CR4.2- $\beta$ GP-GFP enhancer construct. Transfected retinas were harvested at earlier stages (E6-E8) during development, sectioned, and immunostained for GFP (green), Foxn4 protein (Red) and DAPI (not shown). (A-F) The regulatory element present in the construct is identified on each panel. The scleral surface of the retina is at the top of the section. For each set of images (A-F) the entire retina cross-section is shown to allow for the laminar location to be easily visualized. The image on the right shows a merged high magnification image. The white-boxed region is shown in higher magnification on the right. Double labeled cells are indicated by arrowheads while arrows point to Foxn4-negative cells. (G) Workflow diagram to calculate the percentage of Foxn4-positive cells that were co-labeled with GFP. Each cell was scored for two variables — Foxn4 protein immunoreactivity and GFP immunoreactivity. The percentage of cells that were GFP-positive was calculated for the Foxn4-positive electroporated population of cells and plotted in G. The Foxn4-positive population in enhancer construct is represented by gray bars and in control population is represented by the black bars. Error bars represent standard error of the mean. Scale bar: 20  $\mu$ m.



#### **4.7 In ovo chick retinal electroporations of CR4.2-GFP labels horizontal and amacrine cells but not ganglion cells or photoreceptors**

Loss of function mutations of Foxn4 in the mouse leads to a loss of horizontal cells and a large decrease in amacrine cells (Li et al., 2004). This suggests that endogenous Foxn4 is unlikely to be expressed in RGCs and PRs and these cells should not express CR4.2-GFP. As it is easier to access the early retina in chick embryos than in mouse embryos, we examined in ovo electroporated embryonic chick retinas for the expression of CR4.2 enhancer activity in HC or AC. To characterize the enhancer function CR4.2 in the chick retina, GFP expression patterns of Foxn4CR4.2-βGP-GFP were compared to those of a transfection control CAG-GFP at E6, E7 and E8. To determine the cell specific expression of GFP in the chick retina, cell type-specific antibodies, such as Lim1/2 for horizontal cells(Boije et al., 2008; Edqvist et al., 2006; Margeta, 2008; Poche et al., 2007; Suga et al., 2009), Brn3a for ganglion cells (Badea et al., 2009; Huang et al., 2001; Liu et al., 2000; Nadal-Nicolas et al., 2009), NeuN for RGCs and ACs (Mullen et al., 1992), Pax6 for RGCs, HCs, ACs (Ericson et al., 1997) and Visinin for cone photoreceptors (Yamagata et al., 1990); were used to stain retina sections harvested at various time points during retinal development from E6 to E8 (Fig. 15-19).

##### **4.7.1 Foxn4CR4.2 activity is significantly increased in retinal horizontal cells**

Transcription factor Lim1/2 is essential for horizontal cell development and its laminar position in the retina (Boije et al., 2009; Edqvist and Hallbook, 2004; Poche et al., 2007; Poche and Reese, 2009; Suga et al., 2009). At E6, (Fig. 15, A & B) the majority of Lim1/2 positive cells are near the edge of INBL, while some are migrating through the inner nuclear layer (INL) towards the outer portion of the INL where mature horizontal cells reside. Migration of Lim1/2 positive cells continues through E8 (Fig. 15, E & F) and nears completion by E10 (Doh et al., 2010). Interestingly, GFP positive cells in the CR4.2 transfected retinas follow a very similar pattern of laminar location (Fig. 15 B,D and F). Not surprisingly, 35- 45% percent of electroporated cells were positive for HCs in CR4.2 (Fig. 15 B,D and F), which is significantly higher than in CAG-GFP transfected retinas (5-10%, Fig.15, A, C & E). This suggests that the CR4.2 enhancer and Foxn4 protein are active in RPCs that will give rise to HCs. In other words, the GFP may effectively fate map some of the HCs that require Foxn4 for their development.

#### **4.7.2 Foxn4CR4.2 is not active in retinal ganglion cells**

The expression of a retinal ganglion cell marker Brn3a start in the GCL around E6 (Doh et al., 2010). Brn3a positive cells are organized into 3-4 cell layers in thickness by E8. Brn3a expression increases significantly in both intensity and in number of cells that express Brn3a at E8, and its expression is restricted to the GCL (Doh et al., 2010). Among the retinal cells that were transfected with CR4.2-GFP, only about 1% were positive for Brn3a while about 5-20 % of GFP positive cells in control group were also positive for Brn3a (Fig. 16). This result clearly indicates that the CR4.2 element is not active in RGCs.

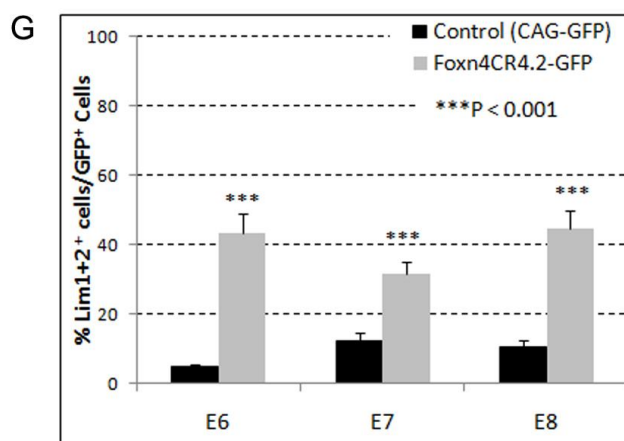
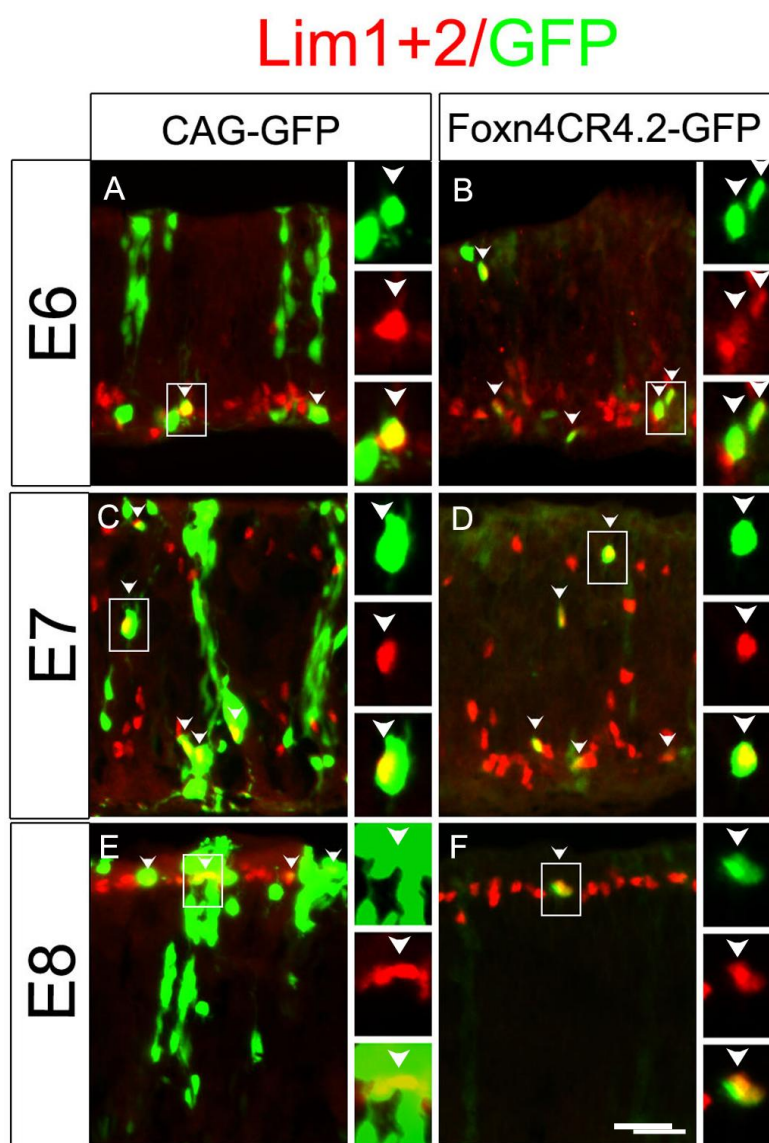
### **4.7.3 Foxn4CR4.2 is also functional in amacrine cells**

NeuN is a marker of early neurons and in the retina labels amacrine and ganglion cells. Use of NeuN antibodies in the mouse retina showed immunoreactivity in the GCL and to a much lesser extent in the INL (Mullen et al., 1992). In the chicken retina, NeuN labeling has been shown in the INBL beginning at E6 and in the INL at E8 (Doh et al., 2010). Even though Brn3a failed to colabel CR4.2 driven GFP positive cells, about 10-15 % of those cells were colabeled with NeuN at E6 and E7 stage, which is slightly higher than control (Fig. 17). However this number of colabeled cells went down to 5% at E8 stage in enhancer group, significantly lower than control group. This suggests that Foxn4CR4.2 might be active in ACs as it is not active in RGCs demonstrated by Brn3a staining.

Pax6 is a nuclear marker for ganglion, amacrine, and progenitor cells that is required for multipotency in retinal cells. Pax6 also labels Horizontal cells in chick retina at a later stage. Interestingly, about 30-40% of GFP positive cells in CR4.2 transfected chick retina also showed to be positive for Pax6 (Fig. 18). Percent of colabeled cells in enhancer transfected retina is much higher than CAG-GFP transfected retina. Together, the immunostaining results from Brn3a, NeuN, and Pax6 suggests that Foxn4CR4.2 is active in Retinal Progenitor cells and Amacrine cells in addition to Horizontal cells.

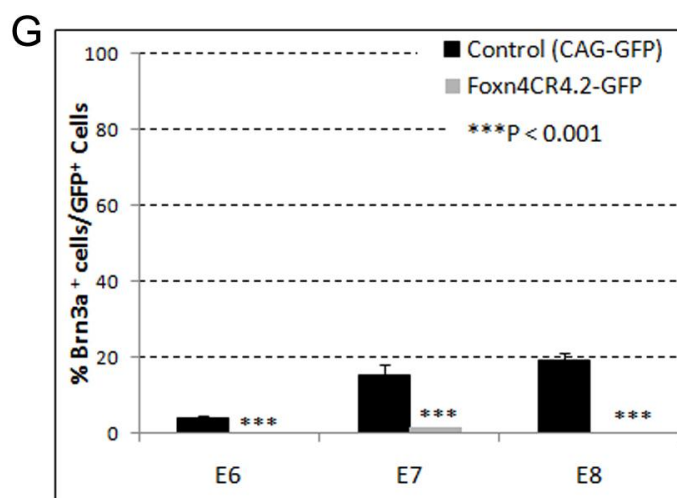
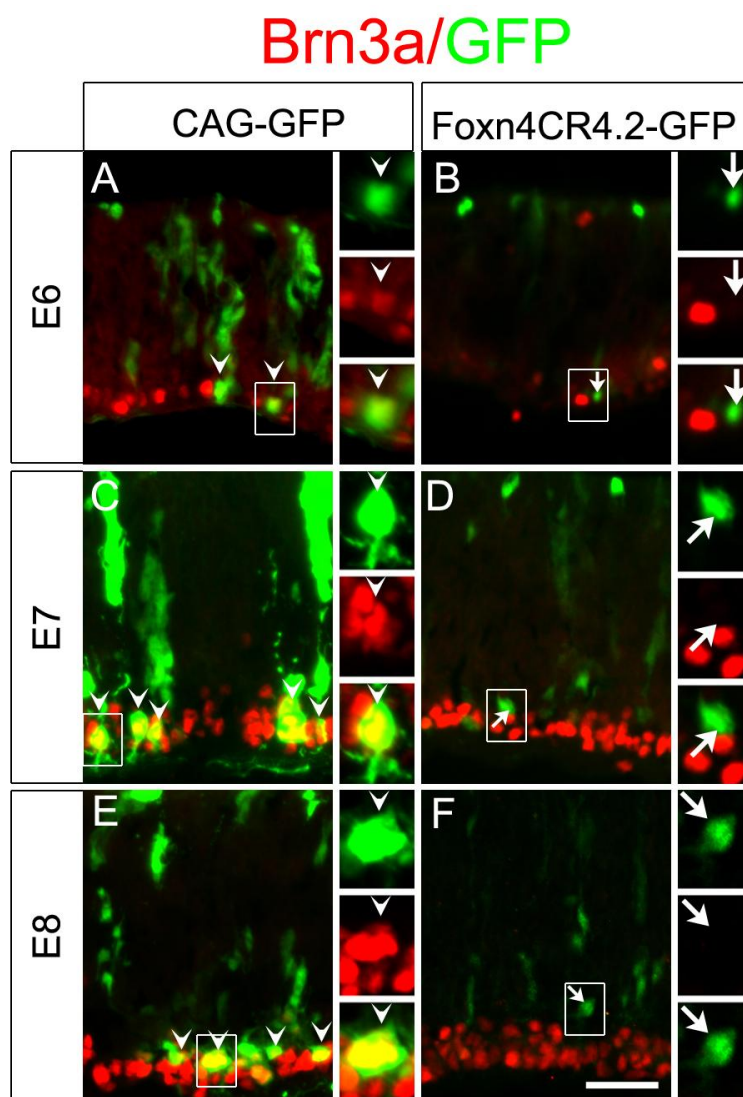
### **4.7.4 Foxn4CR4.2 is not active in cone photoreceptors cells**

The expression of photoreceptor marker Visinin, a retinal photoreceptor protein which is believed to be cone specific, starts around E4. At E6, its expression increases in intensity but individual cells are still distinguishable (Fig. 19). Visinin labeling then increases in intensity and the labeled cells composed a significant portion of the ONL at E8. Like RGCs, only about 1-2 % GFP positive cells were observed to be cone photoreceptor in CR4.4-GFP transfected cells (Fig. 19, B,D, F, and G), which is much higher in control transfected cells (about 10-15%, Fig. 19, A,C,E, and G). It should be noted that because of the high intensity some overlapped cells appear to be pseudo positive. However, based on laminar location and cell morphology and with the help of confocal microscopy, it was found that only very few of those Photoreceptor cells were also GFP positive as well (Fig 19). This result suggests that Foxn4CR4.2 is not active in photoreceptor cells.



**Figure 15. CR4.2 is preferentially active in horizontal cells.**

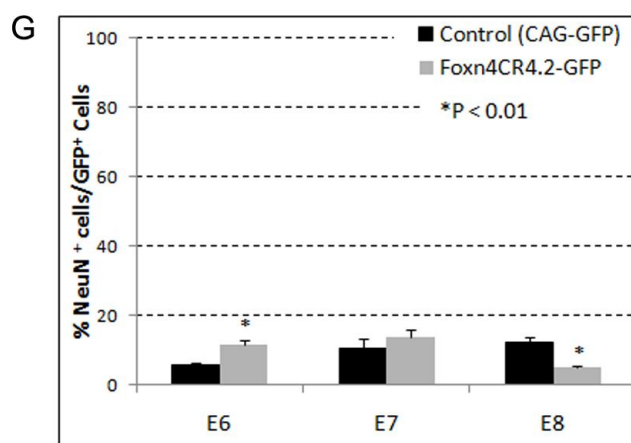
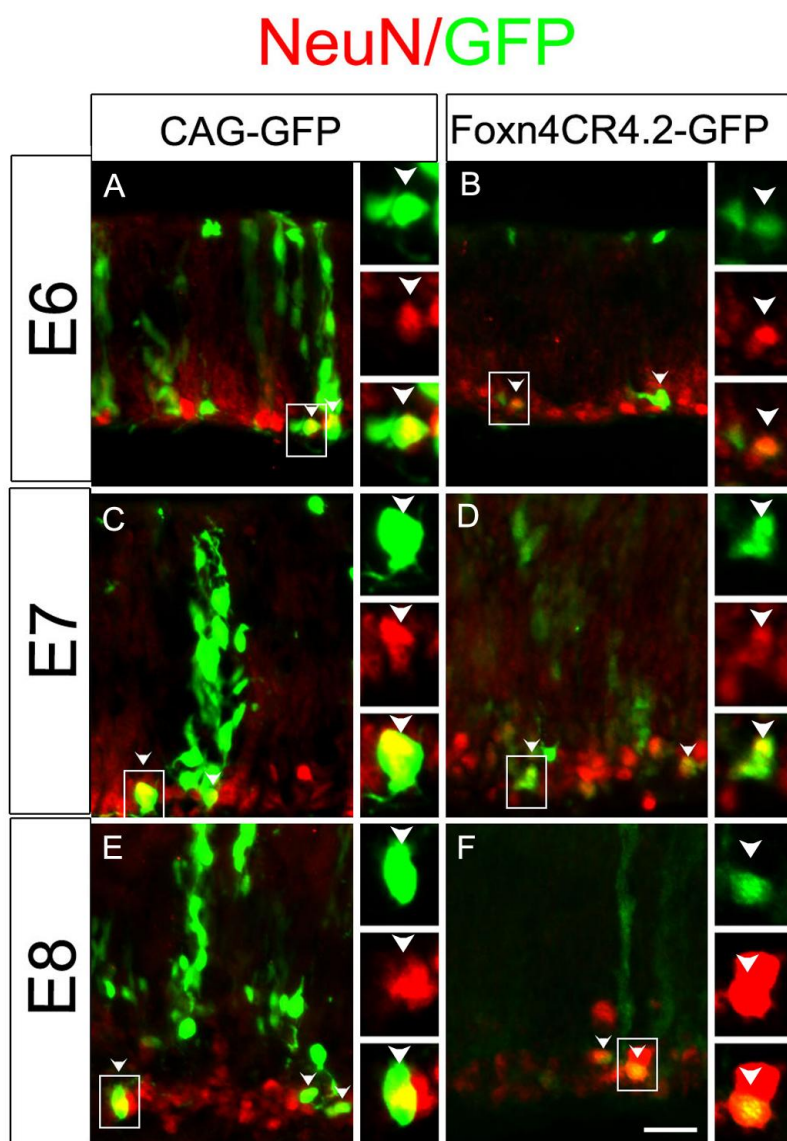
Chick E4 retinas were electroporated either with the broadly active CAG-GFP plasmid or with CR4.2- $\beta$ GP-GFP enhancer construct. Transfected retinas tissue from chicken embryos were harvested at earlier stages (E6-E8) during development, sectioned, and immunostained for GFP (green), HC specific Lim1+2 protein (Red) and DAPI (not shown). (A-F) The regulatory element present in the construct is identified on each panel. The scleral surface of the retina is at the top of the section. For each set of images (A-F) the entire retina cross section is shown to allow for the laminar location to be easily visualized. The image on the right shows a merged high magnification. The white-boxed region is shown in higher magnification on the right. Double labeled cells are indicated by arrowheads. (G) Workflow diagram to calculate the percentage of Lim1+2-positive cells that were co-labeled with GFP. Each cell was scored for two variables — Lim1+2 protein immunoreactivity and GFP immunoreactivity. The percentage of cells that were GFP-positive was calculated for the Lim1+2-positive electroporated population of cells and plotted in G. The Lim1+2-positive population in enhancer construct is represented by gray bars and in control population is represented by the black bars. Error bars represent standard error of the mean. Scale bar = 20  $\mu$ m.



**Figure 16. CR4.2 is not active in retinal ganglion cells.**

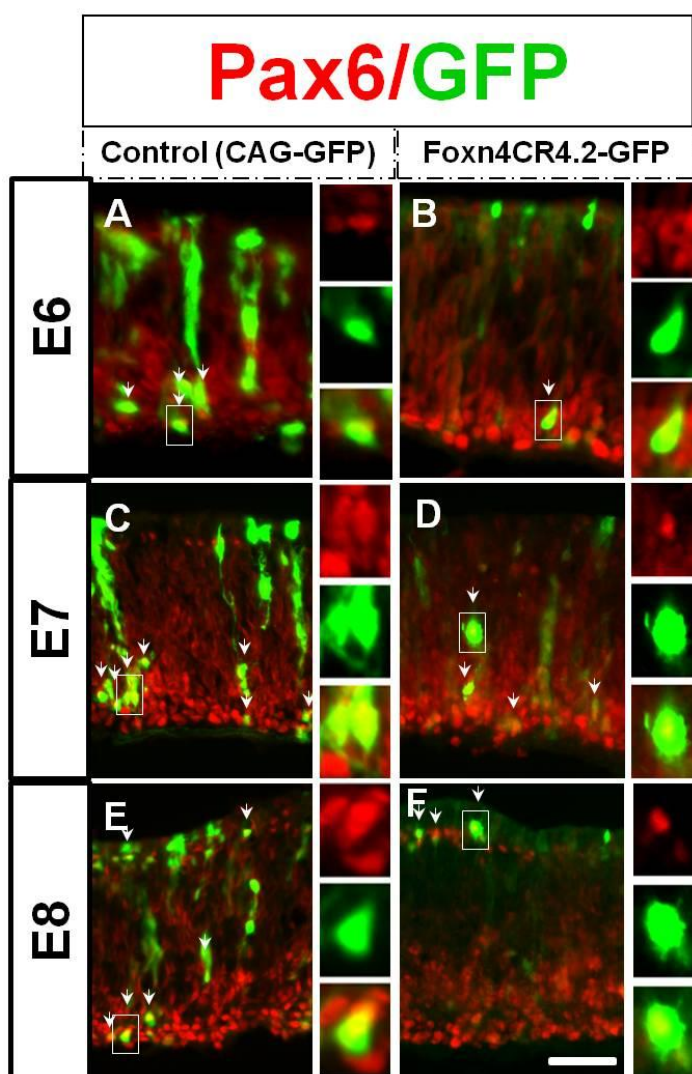
Chick E4 retinas were electroporated either with the broadly active CAG-GFP plasmid or with CR4.2- $\beta$ GP-GFP enhancer construct. Transfected retinas were harvested at earlier stages (E6-E8) during development, sectioned, and immunostained for GFP (green), RGC specific Brn3a protein (Red) and DAPI (not shown). (A-F) The regulatory element present in the construct is identified on each panel. The scleral surface of the retina is at the top of the section. For each set of images (A-F) the entire retina cross-section is shown to allow for the laminar location to be easily visualized. The image on the right shows a merged high magnification image. The white-boxed region is shown in higher magnification on the right. Double labeled cells are indicated by arrowheads while arrows point GFP positive cells that are not co-labeled with Brn3a. (G) Workflow diagram to calculate the percentage of Brn3a positive cells that were co-labeled with GFP. Each cell was scored for two variables — Brn3a protein immunoreactivity and GFP immunoreactivity. The percentage of cells that were GFP-positive was calculated for the Brn3a-positive electroporated population of cells and plotted in G. The Brn3a-positive population in enhancer construct is represented by gray bars and in control population is represented by the black bars. Error bars represent standard error of the mean. Scale bar = 20  $\mu$ m.



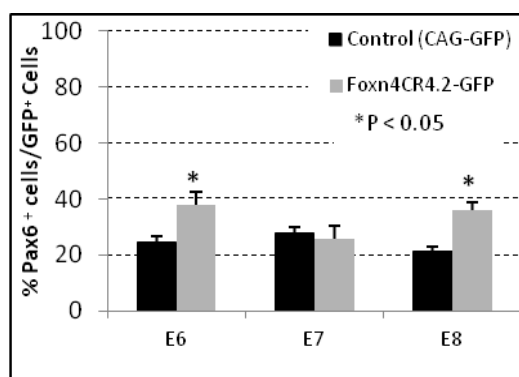


**Figure 17. CR4.2 is active in amacrine cells.**

Chick E4 retinas were electroporated either with the broadly active CAG-GFP plasmid or with CR4.2- $\beta$ GP-GFP enhancer construct. Transfected retinas were harvested at earlier stages (E6-E8) during development, sectioned, and immunostained for GFP (green), NeuN (Red) and DAPI (not shown). (A-F) The regulatory element present in the construct is identified on each panel. The scleral surface of the retina is at the top of the section. For each set of images (A-F) the entire retina cross-section is shown to allow for the laminar location to be easily visualized. The image on the right shows a merged high-magnification image. The white-boxed region is shown in higher magnification on the right. Double-labeled cells are indicated by arrowheads. (G) Workflow diagram to calculate the percentage of NeuN positive cells that were co-labeled with GFP. Each cell was scored for two variables — NeuN protein immunoreactivity and GFP immunoreactivity. The percentage of cells that were GFP-positive was calculated for the NeuN-positive electroporated population of cells and plotted in G. NeuN-positive population in enhancer construct is represented by gray bars and in control population is represented by the black bars. Error bars represent standard error of the mean. Scale bar: 20  $\mu$ m.



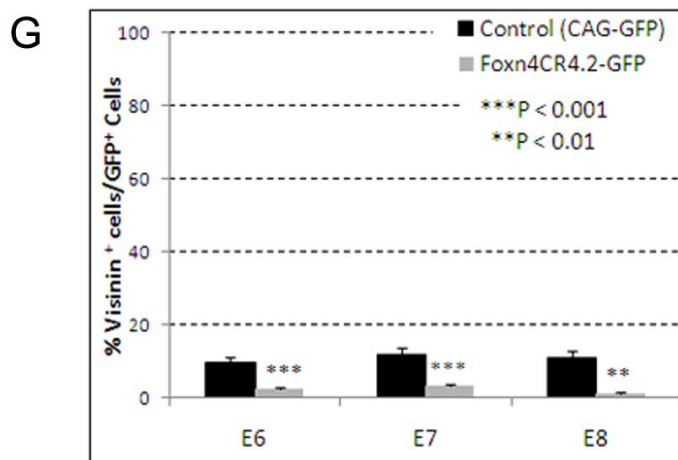
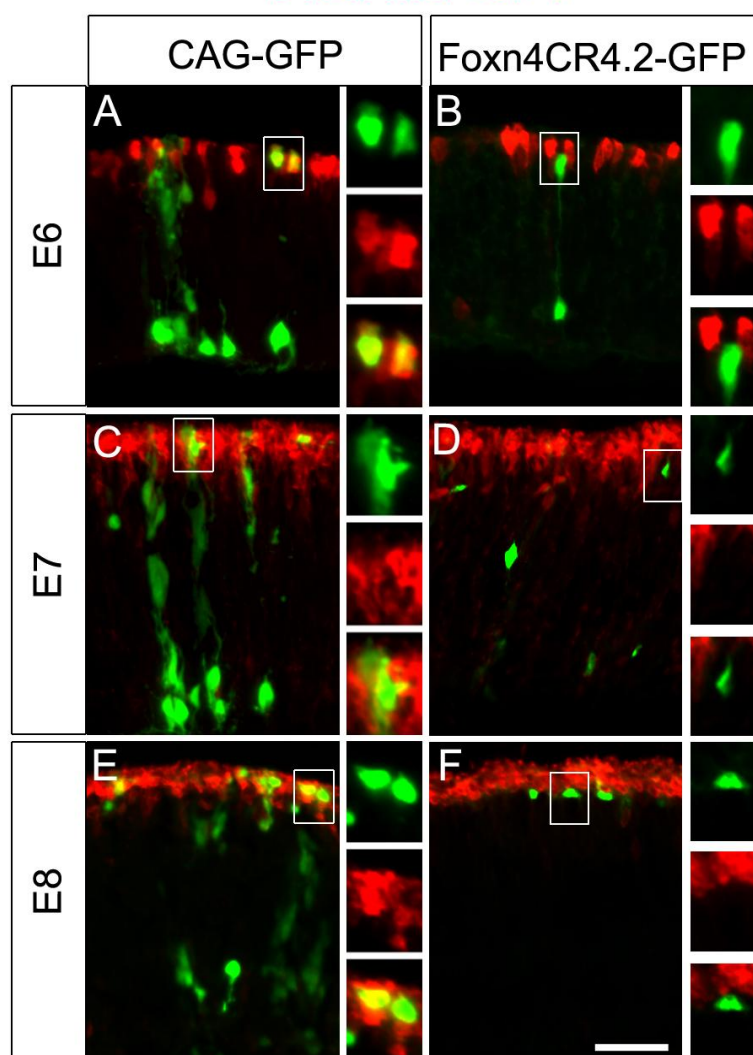
G



### **Figure 18. CR4.2 is active in horizontal and amacrine cells**

Chick E4 retinas were electroporated either with the broadly active CAG-GFP plasmid or with CR4.2-βGP-GFP enhancer construct. Transfected retinas were harvested at earlier stages (E6-E8) during development, sectioned, and immunostained for GFP (green), Pax6 (Red) and DAPI (not shown). (A-F) The regulatory element present in the construct is identified on each panel. The scleral surface of the retina is at the top of the section. For each set of images (A-F) the entire retina cross-section is shown to allow for the laminar location to be easily visualized. The image on the right shows a merged high-magnification image. The white-boxed region is shown in higher magnification on the right. Double-labeled cells are indicated by pointed arrows. (G) Workflow diagram to calculate the percentage of Pax6 positive cells that were co-labeled with GFP. Each cell was scored for two variables — Pax6 protein immunoreactivity and GFP immunoreactivity. The percentage of cells that were GFP-positive was calculated for the Pax6-positive electroporated population of cells and plotted in G. Pax6-positive population in enhancer construct is represented by gray bars and in control population is represented by the black bars. Error bars represent standard error of the mean. Scale bar: 20 μm.

# Visinin/GFP



**Figure 19. CR4.2 is not active in photoreceptor cells.**

E4Chick retinas were electroporated either with the broadly active CAG-GFP plasmid or with CR4.2-βGP-GFP enhancer construct. Transfected retinas were harvested at earlier stages (E6-E8) during development, sectioned, and immunostained for GFP (green), Visinin (Red) and DAPI (not shown). (A-F) The regulatory element present in the construct is identified on each panel. The scleral surface of the retina is at the top of the section. For each set of images (A-F) the entire retina cross-section is shown to allow for the laminar location to be easily visualized. The image on the right shows a merged high-magnification image. The white-boxed region is shown in higher magnification on the right. Double-labeled cells are indicated by arrowheads. (G) Workflow diagram to calculate the percentage of Visinin-positive cells that were co-labeled with GFP. Each cell was scored for two variables — Visinin protein immunoreactivity and GFP immunoreactivity. The percentage of cells that were GFP-positive was calculated for the Visinin-positive electroporated population of cells and plotted in G. Visinin-positive population in enhancer construct is represented by gray bars and in control population is represented by the black bars. Error bars represent standard error of the mean. Scale bar = 20 μm.

## 4.8 Identification of protein factors that bind to CR4.2

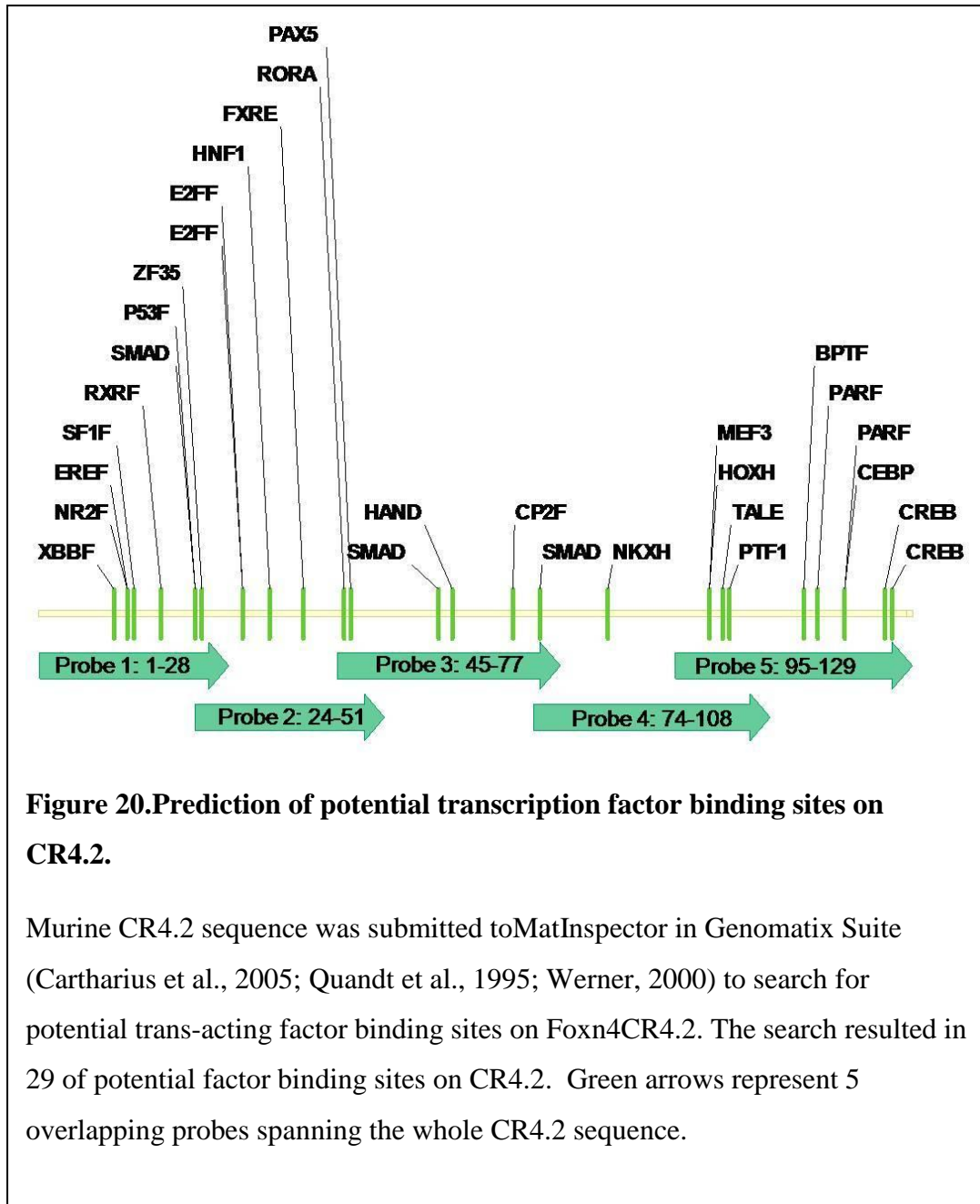
The ability of Foxn4CR4.2 to direct reporter GFP expression is most likely to be associated with its binding to protein factors. To identify these binding factors that may interact with CR4.2, we used MatInspector in Genomatix Suite (München, Germany) (Cartharius et al., 2005; Quandt et al., 1995; Werner, 2000) to search for potential *trans*-acting factor binding sites on Foxn4CR4.2. The search resulted in 29 of potential factor binding sites on CR4.2 (Fig. 20).

### 4.8.1 Regions of CR4.2 show sequence specific binding

Electrophoretic mobility shift assay (EMSA) was used to test for binding of characterized or uncharacterized protein factors; it provides a simple, rapid, and sensitive method for detecting sequence-specific DNA-binding proteins. Protein factors that bind specifically to an end-labeled DNA probe retard the mobility of the DNA during polyacrylamide gel electrophoresis, resulting in discrete bands corresponding to protein-DNA complexes of a particular molecular weight. To identify the specific regions with enhancer activity within Foxn4CR4.2, short double stranded DNA probes (< 40 bp) were designed for use in EMSA. These short probes span the whole sequence of CR4.2. Nuclear extracts were prepared from retina tissues from chick embryo at various ages between E5 and E8. However, as we saw very small differences in protein-probe binding pattern within these developmental stages, data from only E6 stage are reported and discussed here. Initially 5 overlapping probes spanning the whole CR4.2 sequence were screened. Probe-2, Probe-3 and Probe-5 (Table 3 and Fig. 20) showed sequence specific binding with nuclear extracts prepared from chick embryonic retinal tissue (Fig. 21). However, follow-up

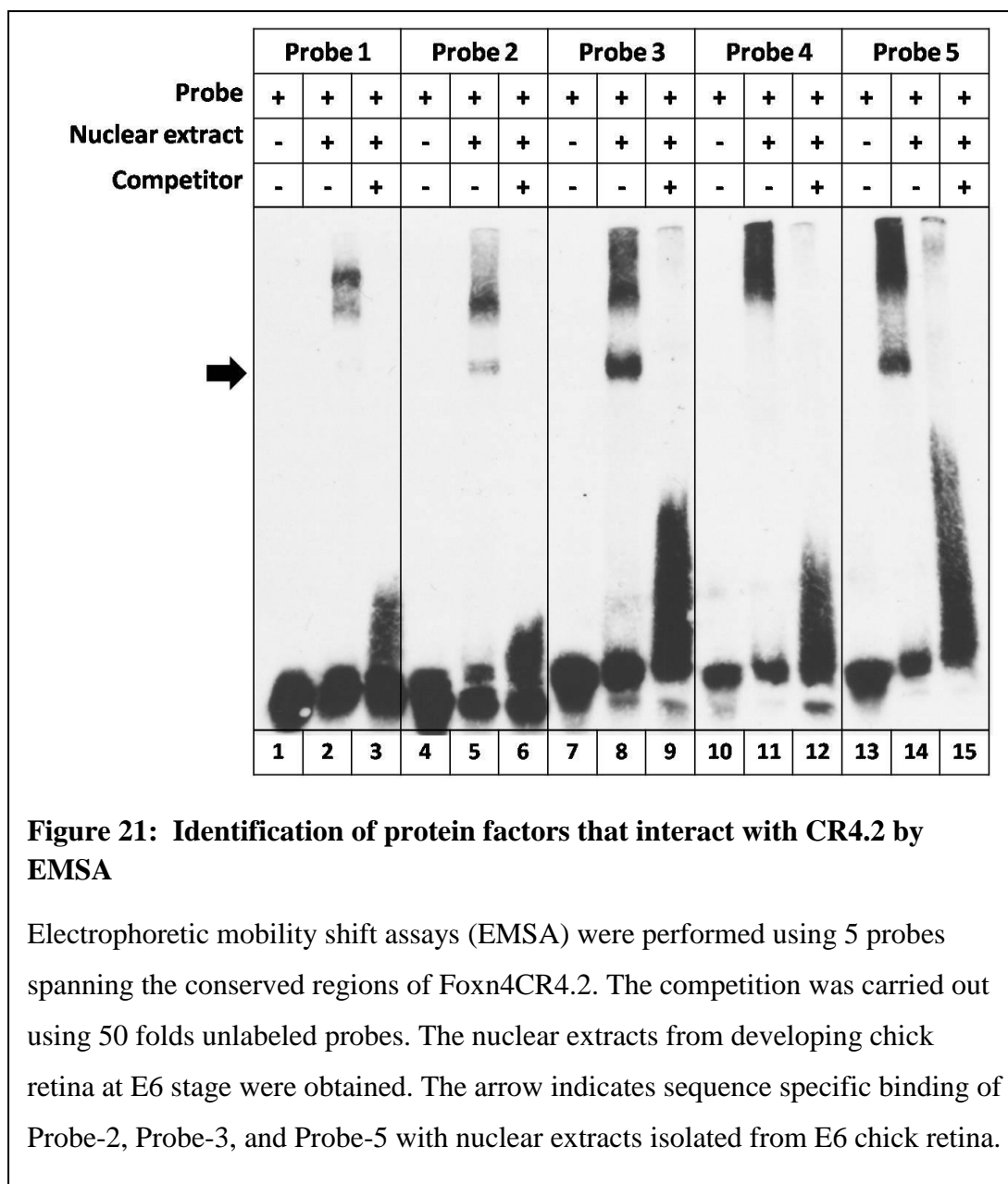
EMSA experiments with Probe-2 did not reveal any significant binding. Interestingly both Probe-3 and Probe-5 reside within the 2 most highly conserved motifs of CR4.2 across phylogeny (Fig. 22 and Fig. 11). Therefore we selected these 2 probes for further analysis.





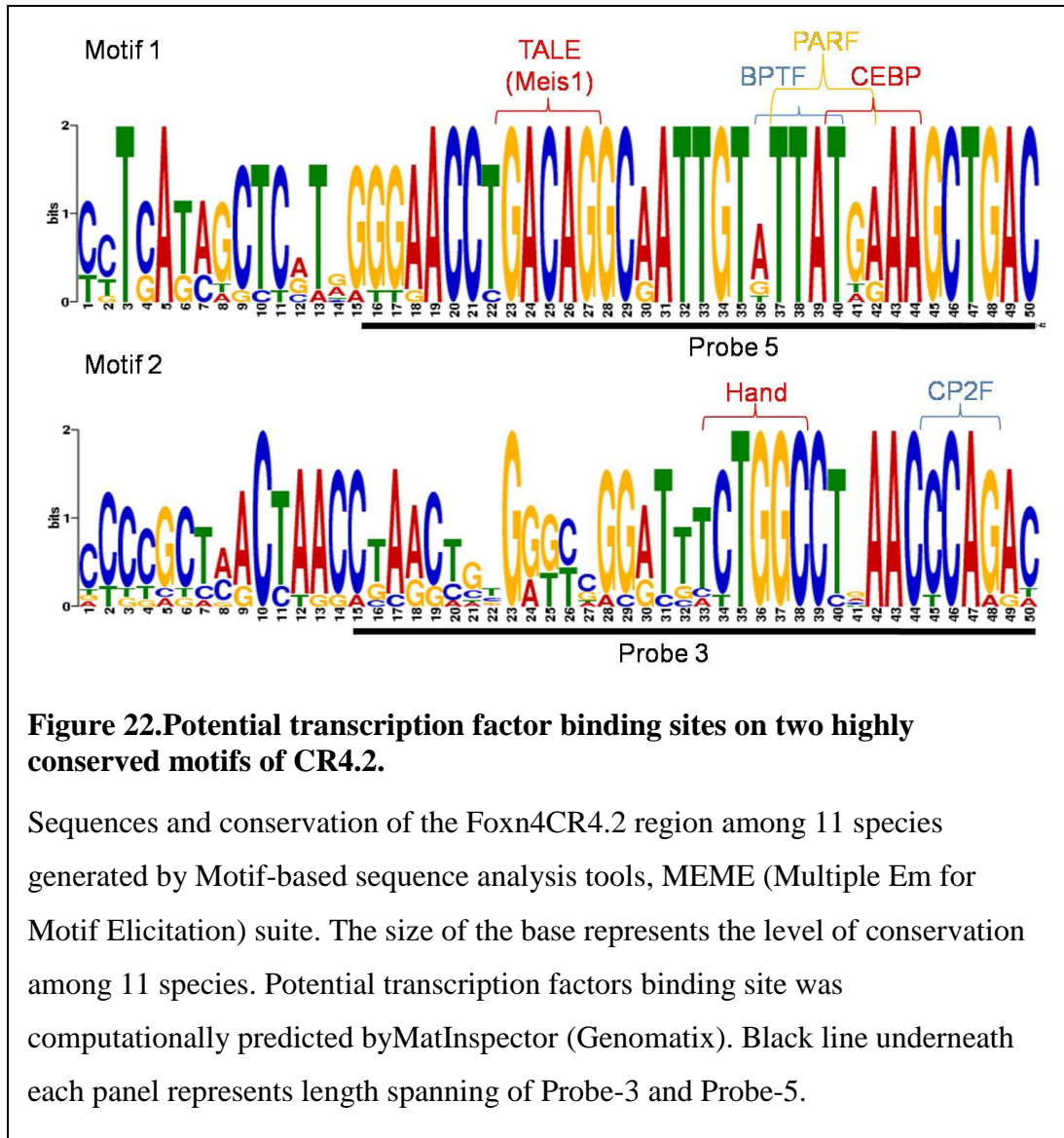
**Figure 20. Prediction of potential transcription factor binding sites on CR4.2.**

Murine CR4.2 sequence was submitted to MatInspector in Genomatix Suite (Cartharius et al., 2005; Quandt et al., 1995; Werner, 2000) to search for potential trans-acting factor binding sites on Foxn4CR4.2. The search resulted in 29 of potential factor binding sites on CR4.2. Green arrows represent 5 overlapping probes spanning the whole CR4.2 sequence.



**Figure 21: Identification of protein factors that interact with CR4.2 by EMSA**

Electrophoretic mobility shift assays (EMSA) were performed using 5 probes spanning the conserved regions of Foxn4CR4.2. The competition was carried out using 50 folds unlabeled probes. The nuclear extracts from developing chick retina at E6 stage were obtained. The arrow indicates sequence specific binding of Probe-2, Probe-3, and Probe-5 with nuclear extracts isolated from E6 chick retina.

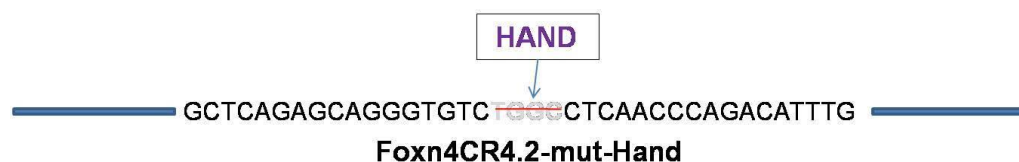


#### **4.8.2 Hand transcription factor showed specific binding to CR4.2**

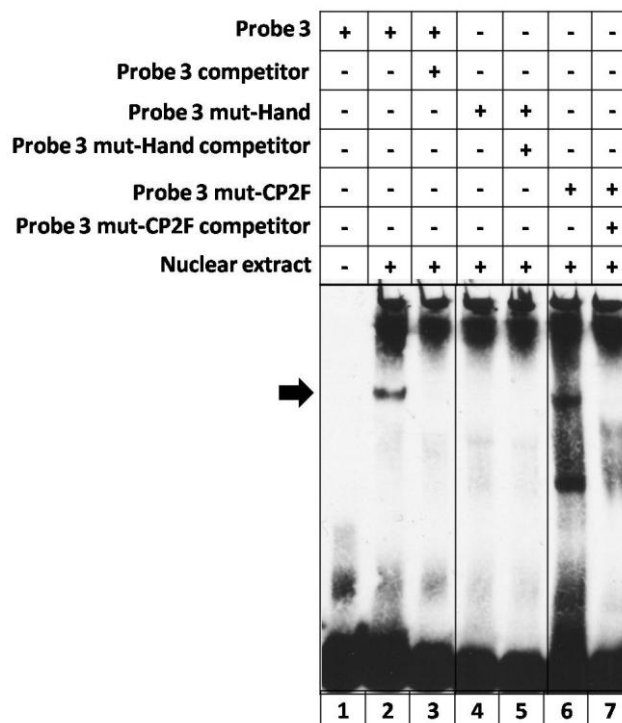
Since, probe-3 and probe-5 showed sequence specific binding, next we wanted to find out the specific site and the factor(s) involved in this protein-DNA interaction.

Computational prediction by MatInspector, showed that 2 factors, HAND (Heart- and Neural crest Derivatives) and CP2F (CP2-erythrocyte Factor) has binding site on Probe-3. To test whether the protein-DNA interaction observed is sequence specific, we synthesized 2 mutant probes for each Probe-3 and Probe-5 by deleting 4 bp at the predicted protein factor binding site (Table-3). This prediction was done using MatInspector (Genomatix) and MEME suite (Fig. 22). Electrophoretic mobility shift assays (EMSA) were performed using Probe-3 and mutation probes spanning 45-77 bp region of CR4.2. Hand and CP2F mutant probes were synthesized with a deletion of 4 bp at the Hand and CP2F binding sites respectively from probe-3. Interestingly, sequence specific binding to probe-3 was abolished when Hand-mutant was used instead of probe 3 (Fig. 23, lane-4). Mutation at the CP2F binding site did not affect the protein binding to Probe-3 (Fig. 23, lane 6). This indicates that Hand transcription factor might interact with CR4.2 at the binding site on Probe-3. For further verification of Hand binding with Probe-3, we wished to perform EMSA supershift assay, but could not do so, due to unavailability of chick-specific Hand Ab (data not shown).

A



B

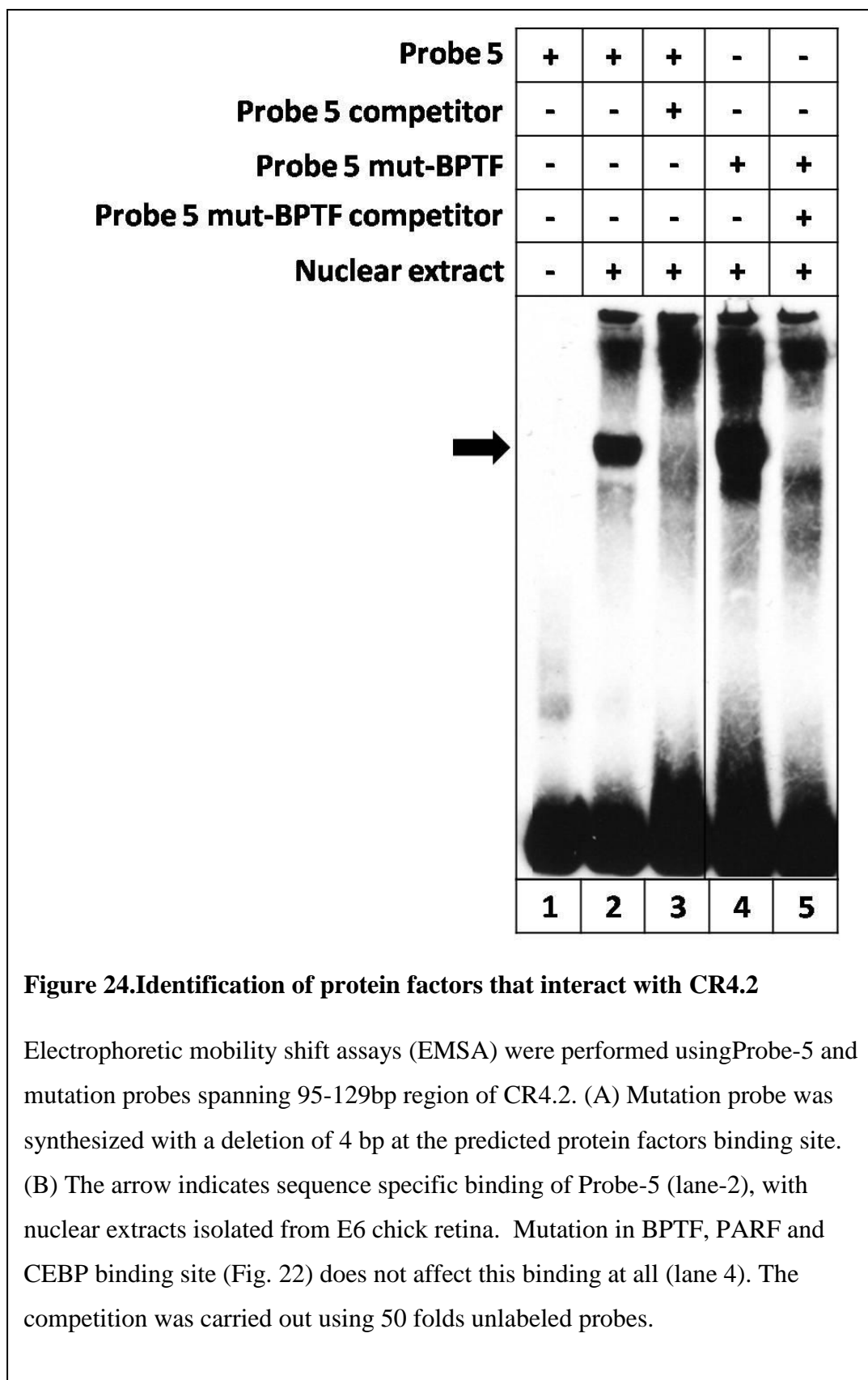


**Figure 23. Identification of protein factors that interact with Probe 3.**

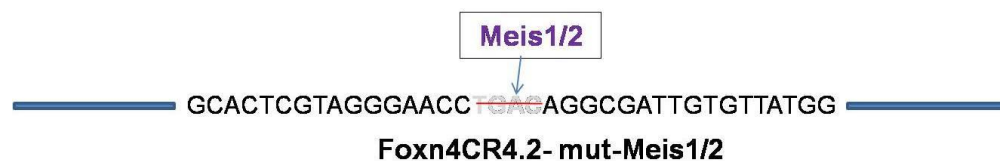
Electrophoretic mobility shift assays (EMSA) were performed using Probe 3 and mutation probes spanning 45-77 bp region of CR4.2. (A) Mutation probe was synthesized with a deletion of 4 bp at the predicted protein factors binding site. (B) The arrow indicates sequence specific binding of Probe 3 (lane-2), with nuclear extracts isolated from E6 chick retina. This binding was abolished when Hand-mutant was used instead of probe 3 (lane-4). Mutation in CP2F binding site (Fig. 22) does not affect this binding rather increase binding affinity for other protein (lane 6). The competition was carried out using 50 folds unlabeled probes.

#### 4.8.2 Meis1 transcription factor bind to CR4.2

Four factors, Meis1 (Myeloid Ecotropic viral Integration Site1), BPTF (Bromodomain and PHD domain transcription factors), PARF (PAR/bZIP family), and CEBP (Ccaat/Enhancer Binding Protein) are computationally predicted to have binding site on Probe-5. To identify protein factors specifically bind to Probe-5, BPTF- mutant and Meis1-mutant probes were synthesized with a deletion of 4 bp at the BPTF and Meis1/2 binding sites respectively from Probe-5. As the binding site for BPTF overlaps with 2 other factors, PARF and CEBP, mutation at this site abolish binding of all 3 factors (Fig. 22). Electrophoretic mobility shift assays (EMSA) were performed using probe 5 and BPTF-mutant probes spanning 95-129 bp region of CR4.2. Mutation at the BPTF and other 2 factors binding site did not affect the sequence-specific binding of probe-5 (Fig. 24, lane 4). Interestingly, mutation at Meis1 binding site greatly reduced the protein binding to Probe-5 (Fig. 25, lane 3). Unlabeled competitor for Probe-5 was able to diminish the binding (Fig. 25, lane 4) while mutant unlabeled competitor failed to do the same (Fig. 25, lane 5). Band supershift using chick specific primary Ab against Meis1 significantly shifted the band towards downward instead of upward (Fig. 25, lane 6). This may indicate that addition of Meis1Ab blocked Meis1 proteins from binding to probe-5. Together these results from Probe-5 binding assays suggest that Meis1 might interacts with CR4.2 in vitro.




A

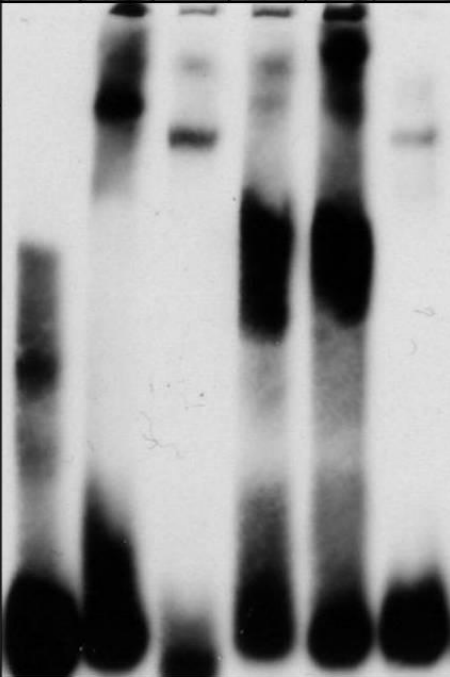


B

<b>Probe 5</b>	+	+	-	+	+	+
<b>Probe 5 competitor</b>	-	-	-	+	-	-
<b>Probe 5 mut-Meis</b>	-	-	+	-	-	-
<b>Probe 5 mut-Meis competitor</b>	-	-	-	-	+	-
<b>Anti-Meis Ab</b>	-	-	-	-	-	+
<b>Nuclear extract</b>	-	+	+	+	+	+





1	2	3	4	5	6
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**Figure 25: Meis1 transcription factor interacts with CR4.2.**

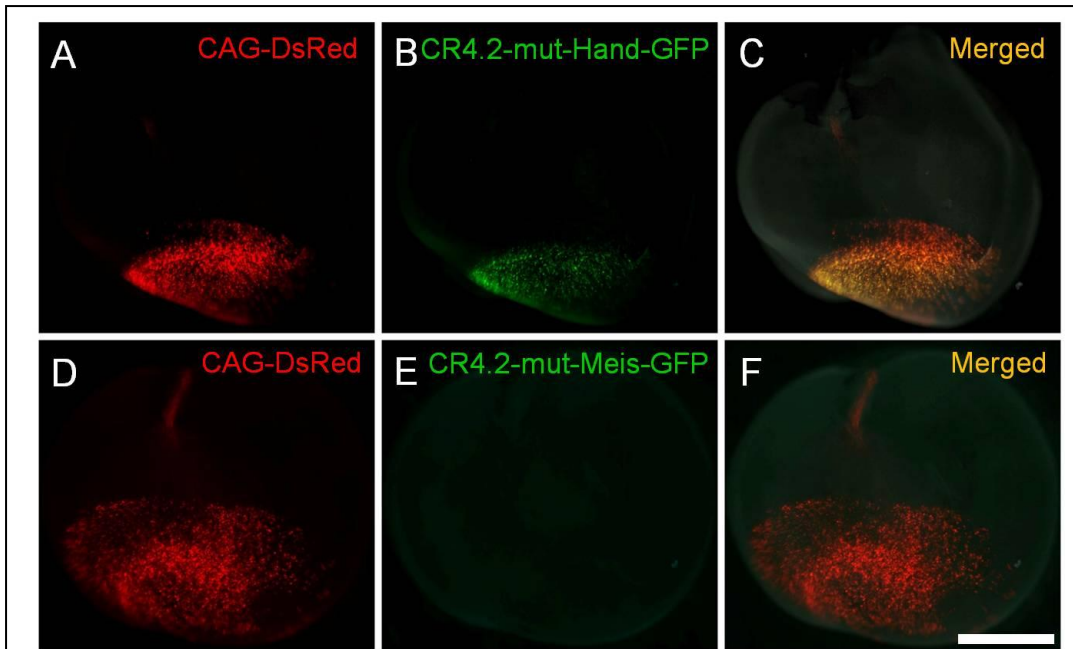
Electrophoretic mobility shift assays (EMSA) were performed using probe 5 and Meis1/2 mutant probes spanning 95-129bp region of CR4.2. (A) Mutation probe was synthesized with a deletion of 4 bp at Meis1/2 binding site. (B) The top arrow indicates sequence specific binding of Probe 5 (lane-2), with nuclear extracts isolated from E6 chick retina. Mutation at Meis1/2 binding site greatly reduced and shifted protein binding (lane 3 & 5). Unlabeled competitor was able to diminish the binding (lane 4) while mutant unlabeled competitor failed to do the same. Primary Ab against Meis1/2 was added in lane 6 while secondary Ab against Meis1/2 primary Ab was added in lane 7. Addition of Ab significantly shifted the band towards downward instead of upward. Adding secondary Ab did not make any significant difference. All of the competition were carried out using 50 folds unlabeled probes.

**Table 3: List of tested probes used in EMSA for CR4.2.**

<b>EMSA Probes</b>	<b>Forward Sequence</b>
Probe 1	tgtaagaagtggccttggagctgtcttg
Probe 2	tcttgcccgctaactaacctagctcaga
Probe 3	gctcagagcagggtgtctggcctcaaccagac
Probe 4	agacatttgagcactcgtagggaaacctgacaggcg
Probe 5	gaacctgacaggcgattgtgttatggaagctgacg
Probe 3 mut-Hand	gctcagagcagggttaagggcctcaaccagac
Probe 3 mut-CP2F	gctcagagcagggtgtctggcctcaactgaac
Probe 5 mut-Meis1	gaaccaggcgattgtgttatggaagctgacg
Probe 5 mut-BPTF	Gaacctgacaggcgattgtgtgaagctgacg

#### **4.9 Identifying Minimal Functional Sequence Elements of CR4.2**

EMSA protein binding assay shows the potential for DNA-protein interactions in vitro, these may not be indicative of the in vivo mechanisms regulating the function of the enhancer activity. As EMSA results indicated that Hand and Meis1/2 would be potential transcription factors bind to CR4.2, we wanted to test the relevance of these in vitro binding events driving gene expression in vivo. Therefore, we synthesized 2 mutant CR4.2 constructs using site directed mutagenesis method by deleting 4bp motif specific to Hand and Meis1/2 binding (Fig. 23 & 25). Mutant constructs, CR4.2-mut-Hand- $\beta$ GP-GFP and CR4.2-mut-Meis- $\beta$ GP-GFP were tested for their enhancer functions, using in ovo electroporation method as described earlier. Chick retinas electroporated with CR4.2-Hand-mutant constructs showed similar GFP expression pattern as it was observed in CR4.2-GFP transfected retinas (Fig. 26, A-C)). Interestingly, mutation at Meis1/2 binding site completely abolished the ability of CR4.2 enhancer to drive reporter gene expression (Fig. 26, D-F)

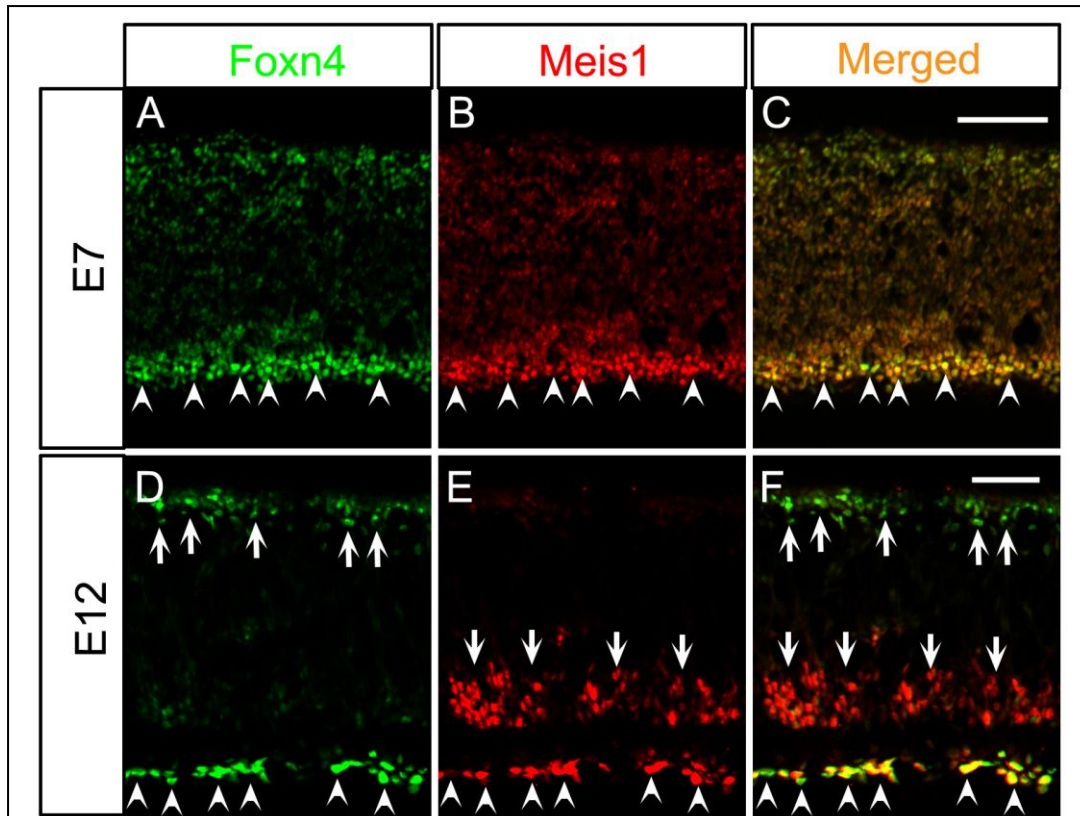


**Figure 26. Mutation at Meis1 binding site abolishes CR4.2-GFP expression.**

Chick retinas were injected and electroporated with a mixture of pCAG-DsRed (transfection control) and a mutant enhancer construct on embryonic day 4 (E4). Transfected retinas were harvested at embryonic day 6 (E6) for reporter gene expression. Successful electroporation was confirmed by DsRed expression (A and D). Imaging through individual channel shows that mutation at Hand binding site does not affect the gene driving ability of CR4.2 (B). Mutation at Meis1 binding site completely abolished the gene regulatory ability of CR4.2. Scale bar = 1mm.

#### **4.10 Meis1 expression correlates with horizontal and amacrine cell development**

Since, Meis1 was identified as the protein factor bind to CR4.2, next we wanted to examine if the Foxn4 positive cells also express Meis1. Previously, spatial and temporal expression pattern of Foxn4 and Meis1 in chick retina were examined by using in-situ hybridization method (Boije et al., 2008; Heine et al., 2008a). Foxn4 expression was shown to start at E3 and declined after E9, while no expression was observed after E14 (Boije et al., 2008). In chick retina, Meis1 was reported to express from E2 and continue through E9 (Heine et al., 2008a). Here we used antibodies against of Foxn4 and Meis1 to examine the coexpression of these proteins. Retinal sections were harvested and stained at 2 different time points, E7 and E12 (Fig. 27). At E7, majority of the cells that express Foxn4, also observed to express Meis1 (Fig. 27, A-C). Interestingly, at E12 both Foxn4 and Meis1 were observed to coexpress only in the ganglion cell layer (progenitor cells). Foxn4 expression was also observed in horizontal cells, but not in the amacrine cells (Fig. 27, D). Interestingly, Meis1 expression was seen in amacrine cell layer, but not in the horizontal cells. Like other vertebrate retina, in chick retina horizontal cells are differentiate earlier than that of amacrine cell (Doh et al., 2010). These results may indicate that Meis1 is involved in the regulation of both horizontal and amacrine cells in chick retina.



**Figure 27. Coexpression of Foxn4 and Meis1 in developing chick retina**

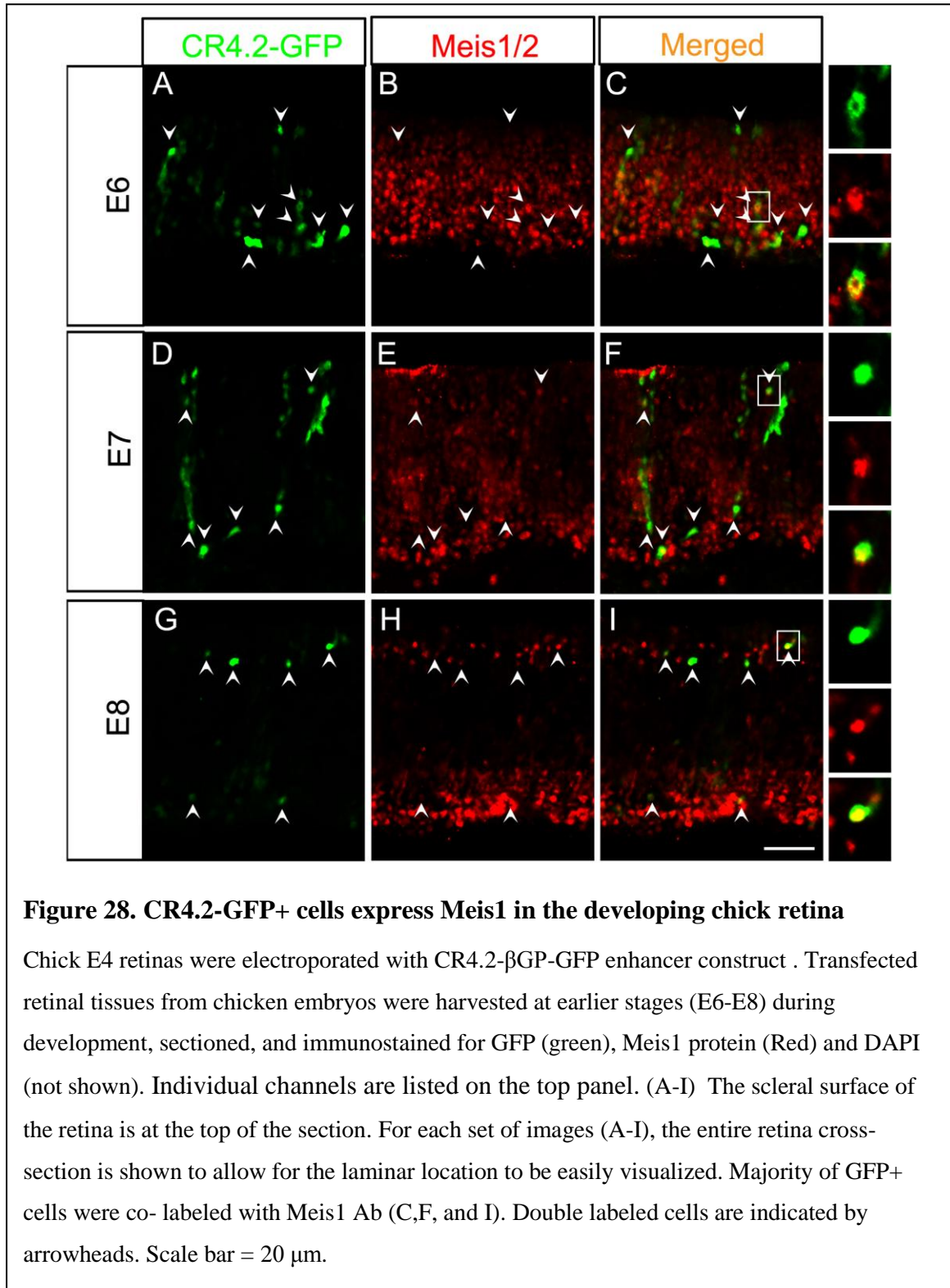
Normal retinal tissues from chicken embryos were harvested at 2 stages E7 and E12 during development. After fixation these tissues were sectioned and immunostained for Foxn4 (green), Meis1 protein (Red) and DAPI (not shown).

(A-F) The scleral surface of the retina is at the top of the section. For each set of images (A-F) the entire retina cross section is shown to allow for the laminar location to be easily visualized. Double labeled cells are indicated by arrowheads, while arrows point to the cells that are not co-labeled by both Abs .

Scale bar = 20  $\mu$ m.

#### **4.11 CR4.2-GFP<sup>+</sup> cells express Meis1**

To determine if the Foxn4CR4.2 activity accurately fate map some or all of the Meis1 expressing cells in the retina, GFP expression was compared with Meis1 protein expression at the cellular level. GFP and Meis1 colocalization in the electroporated population of cells was analyzed as described in Fig. 28. The CR4.2 element plasmid was capable of labeling almost 80- 90% of the Meis1-positive population (Fig. 28). This result supports the interaction between Foxn4Cr4.2 and Meis1 transcription factor in these cells.



#### 4.12 Confirming the role of Meis1 transcription factor in regulating Foxn4 expression using loss-of-function assays

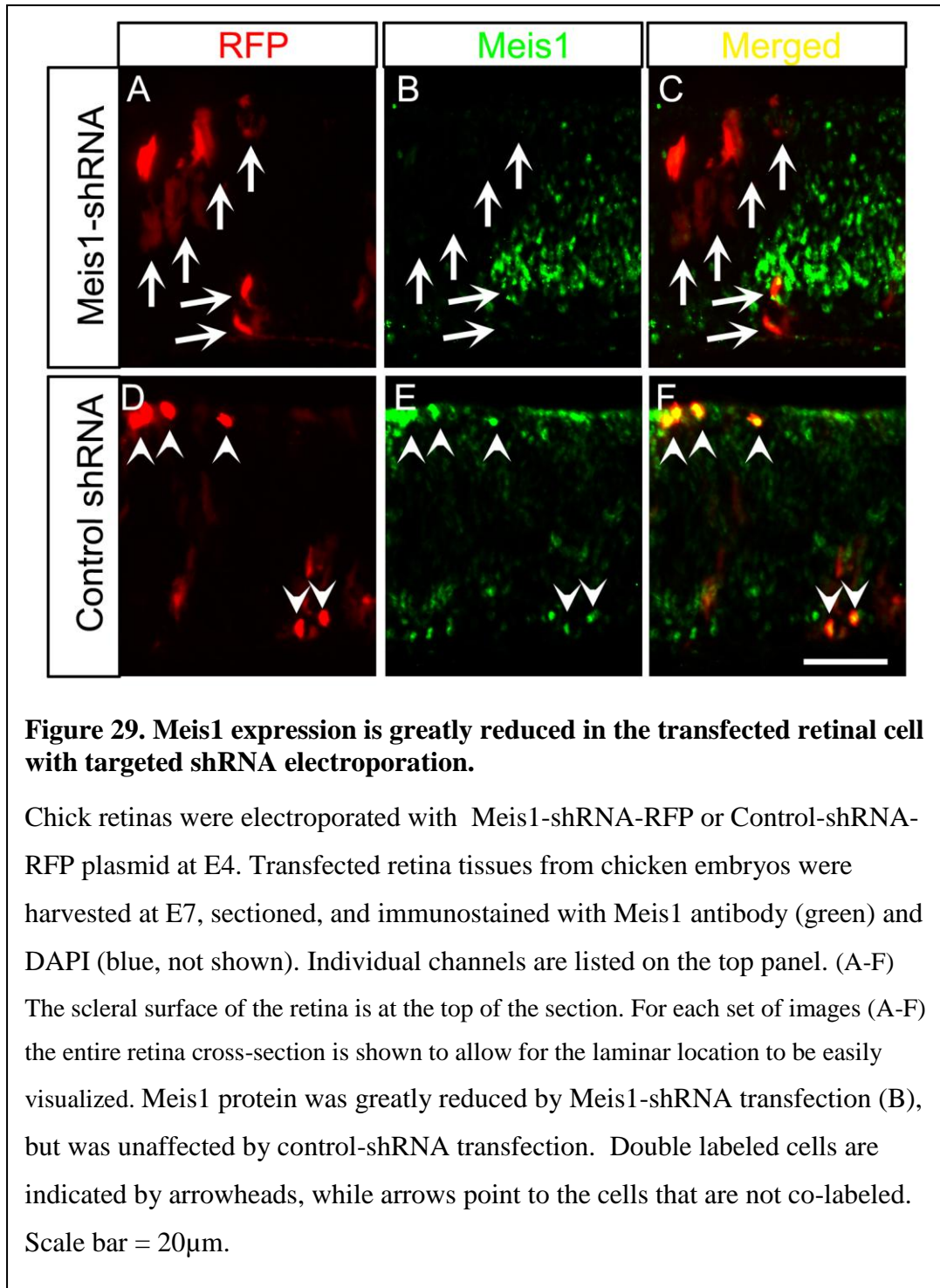


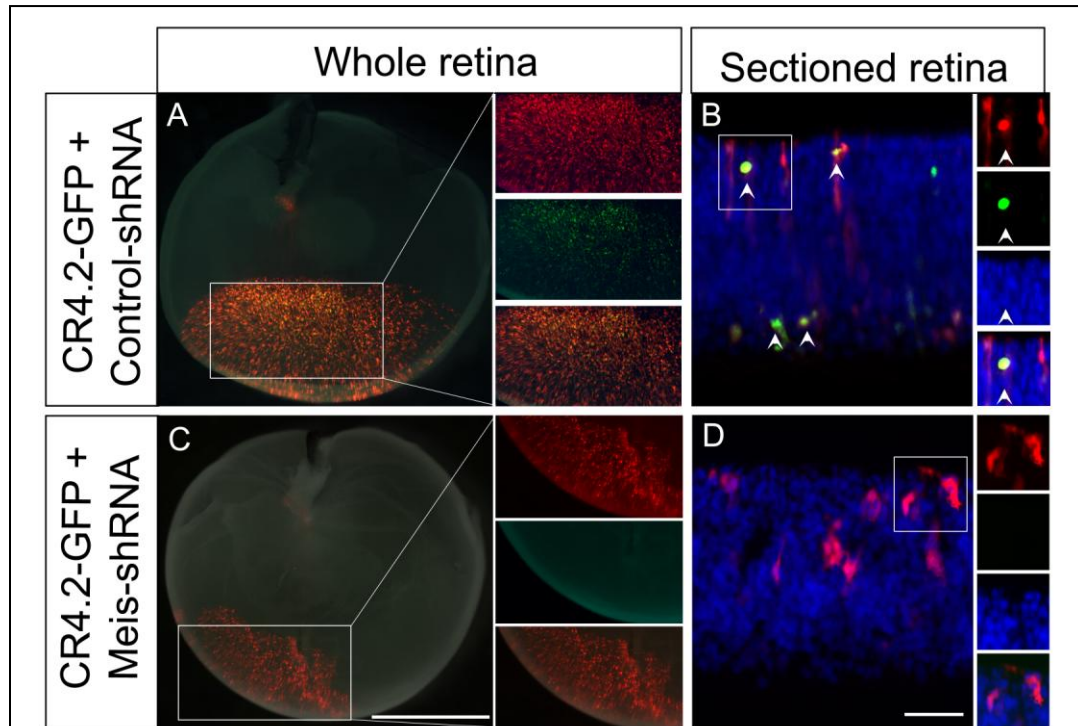
As Meis1 binding site was proven to be essential for gene driving ability of CR4.2, next we wanted to determine the role of Meis1 in Foxn4 expression. RNA interference method was used to repress Meis1 expression in retina through transfecting plasmid vector containing short hairpin RNA (shRNA) sequence designed to target specifically at the Meis1 with red fluorescence protein as a reporter (Meis1-shRNA-RFP, OriGene Technologies, Inc., Rockville, MD).

#### **4.12.1 Knocking-down of Meis1 abolish gene driving ability of the novel enhancer Foxn4CR4.2**

Since, Meis1 and Meis2 in chicken are closely related and 93% homologous to each other, it was very difficult to design effective shRNA, exclusively against a single Meis mRNA. Moreover, both Meis proteins active in RPCs binds to same exact DNA motif. However, it was reported that in chick retina Meis2 expression starts at E1 and diminish by E3 (Heine et al., 2008b). Therefore, we designed and synthesized a shRNA, which is specific against Meis1 and Meis2 mRNA but not expected to have any effect on Meis2 protein. Meis1 targeting shRNA constructs were electroporated into chick retina at E4 and Meis1 expressions were analyzed via immunostaining methods using anti-Meis1 Ab, 3 days after electroporation (E7, Fig. 29). It was observed that Meis1 expression was greatly reduced in the shRNA-transfected cells in E7 retinas (Fig. 29).

To test that knocking down of Meis1 indeed affect the enhancer activity of CR4.2, we cotransfected chick retina at E4 with Meis1-shRNA-RFP and CR4.2- $\beta$ GP-GFP constructs. Transfection with Meis1 specific shRNA completely abolished the GFP expression by Foxn4CR4.2-GFP (Fig. 30).



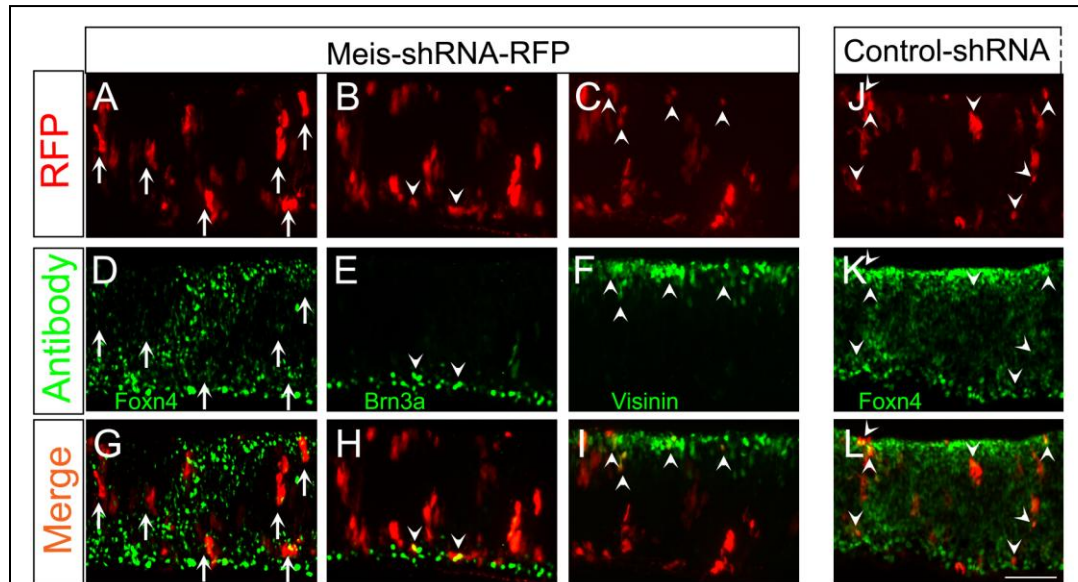


**Figure 30. Knockdown of Meis1/2 abolishes the gene regulatory ability of CR4.2 in chick retina**

Chick retinas were injected and electroporated with a mixture of enhancer construct CR4.2- $\beta$ GP-GFP and either control-shRNA (A,B) or Meis targeting shRNA, Meis1-shRNA (C,D) on embryonic day 4 (E4). Transfected retinas were harvested at embryonic day 7 (E7) for reporter gene expression. Successful electroporation was confirmed by RFP expression. Higher magnification imaging through individual channel on right panels shows that control-shRNA does not affect the gene regulatory function of CR4.2 (A, B). Transfection with Meis1 specific shRNA completely abolished the gene regulatory ability of CR4.2. Double labeled cells are indicated by arrowheads. Scale bar on (C) = 1mm and (D) = 20  $\mu$ m.

#### **4.12.2 Knockdown of Meis1 affects Foxn4 expression in chick retina.**

Chick retinas were electroporated with Meis1-shRNA-RFP plasmid at E4 and harvested at E7. Transfected retinal tissues were sectioned, and immunostained for cell specific antibodies; Foxn4, Brn3a and Visinin, and DAPI. To examine the effect of Meis1/2 knockdown on Foxn4 expression, we immunostained transfected RFP+ samples and determined the percentage of Foxn4+ cells in RFP+ population. Not surprisingly, Foxn4 expression was greatly reduced in the transfected cells (Fig. 31, D). Interestingly, Brn3a and Visinin expression was unaffected by Meis1 knock-down (Fig. 31, E,F). Together these results suggest that Meis1 transcription factor is involved in retinal horizontal cell differentiation process by regulating *Foxn4* expression.



**Figure 31. Knockdown of Meis1 reduces Foxn4 expression in chick retina.**

Chick retinas were electroporated with Meis-shRNA-RFP or Control-shRNA-RFP plasmid at E4. Transfected retina tissues from chicken embryos were harvested at E7, sectioned, and immunostained for cell specific antibodies; Foxn4, Brn3a and Visinin (green), and DAPI (blue, not shown). (D, E, F, and K) Immunostaining with cell specific markers are identified on the middle row. The scleral surface of the retina is at the top of the section. For each set of images (A-L) the entire retina cross-section is shown to allow for the laminar location to be easily visualized. Foxn4 expression was greatly reduced in the Meis1-shRNA transfected cells (D) but not in the control-shRNA transfected cell (K). Brn3a (E) and Visinin (F) expression was unaffected by Meis1 knock-down. Double labeled cells are indicated by arrowheads, while . Scale bar = 20  $\mu$ m.

## **CHAPTER 5**

### **DISCUSSION, SUMMARY AND FUTURE DIRECTIONS**

## 5.1 Discussion

In this work, we report for the first time the identification of a novel *cis*-element, Foxn4CR4.2 capable of regulating gene expression in the developing retina. We demonstrated that the highly evolutionarily conserved CR4.2 was preferentially active in the horizontal and amacrine cells.

The activity of the CR4.2 element correlates with Foxn4 protein expression in progenitor cells and newly postmitotic cells. Using the technique of electroporation, this study sought to identify enhancer elements for the Foxn4 gene that are involved in progenitor cell differentiation into horizontal and amacrine cells during retina development. The expression of GFP driven by the CR4.2 element closely matched that of endogenous Foxn4 protein in the developing retinas of the chicken. This labeling was seen at a time when RPCs were present in fair numbers, and cells identified with GFP often had long processes suggestive of RPCs or migrating precursor cells. These observations demonstrate that the CR4.2 element is active in some Foxn4-positive RPCs of the chicken, as was seen in assays for endogenous Foxn4 mRNA (Boije et al., 2008). GFP expression controlled by the CR4.2 element was thus limited to a developmental time window encompassing cycling RPCs and early postmitotic cells, suggesting that its activity may match that of endogenous Foxn4 in the specification of HCs and ACs. The Foxn4CR4.2 element is active specifically during the development of HCs and ACs. Exploration of the role of Foxn4 in the retina has largely focused on HCs, with evidence suggesting that Foxn4 is necessary for the genesis of HCs (Li et al., 2004). Evidence here strongly suggests that the CR4.2 fragment is in fact linked with the developmental role of Foxn4 in HC and AC genesis. In the chicken retina, CR4.2- GFP labeled a large number

of HCs (as identified by Lim1+2 and Pax6 immunostaining) in the experiment carried out with in ovo electroporation.

Further studies focused on understanding the regulation of this highly conserved element will shed light on the mechanism by which horizontal cells are specified. Approximately 35-45% of HCs were also labeled by CR4.2-GFP when introduced into chicken cells by in ovo electroporation. The activity of the CR4.2 might then suggest that Foxn4 is expressed in early, unidentifiable HCs, and/or the RPC that gives rise to HCs. However, the majority of HCs were not labeled by CR4.2-GFP. This could be due to heterogeneity among the HCs of the chick (Genis-Galvez et al., 1981; Tanabe et al., 2006), wherein some types may not have a history of CR4.2 expression. Alternatively, CR4.2 activity might occur in an early phase of HC development and we could only detect a fraction of these cells before it was turned off. In contrast to HCs and ACs, several populations of cells did not have CR4.2 activity, demonstrating the specificity of this element. These included cells such as RGCs and PRs, which do not normally express Foxn4 protein.

In addition, we uncovered a novel role of Meis1 (myeloid ecotropic viral integration site) proteins in regulating Foxn4 expression during retinal progenitor cells differentiation.

Meis1 is a member of TALE (Three Amino acid Loop Extension) homeodomain transcription factors involved in many processes of vertebrate development and morphogenesis. Meis1 specifies positional information in the retina and tectum to organize the zebrafish visual system (Erickson et al., 2010). Meis1 marks RPCs throughout the period of neurogenesis in the retina, whereas Meis2 is specific for RPCs prior to the onset of retinal differentiation (Heine et al., 2008a). Even though many studies have shown the involvement of Meis1 protein in retina development, no studies have



been reported on its role in the regulation of Foxn4 expression during horizontal cell differentiation process. In this study, we found that the knockdown of Meis1 resulted in a spontaneously reduced expression of Foxn4. This result supports a role of Meis1 in regulating Foxn4 expression. Our findings from EMSA results also suggest that there might be other factors bind to CR4.2. Numerous studies have shown that Hox, Pbx and Meis families of transcription factors form heteromeric complexes and bind DNA through specific homeobox domains to regulate gene expression (Ferretti et al., 2006; French et al., 2007; Heine et al., 2008a). Together, these findings may suggest that Meis1 played a role in Foxn4 gene expression via its interaction with Hoxa9 and Pbx1.

## 5.2 Summary and Conclusions

A better understanding of how genes are regulated and the identification of both *cis* and *trans* functional elements in this regulation is massively important to understanding the mechanisms behind development, disease, repair, and regeneration. As is seen in the case of Foxn4 and many other developmentally significant genes, expression during development must be carefully orchestrated to control the order and duration of events such as proliferation, differentiation, and apoptosis. Reverse engineering the body's methods for development, repair, and regeneration may hold the key to developing therapies. Enhancers play a central role in this orchestration and may hold the key to new drug targets sites, drug delivery modalities, and understanding the biochemical mechanisms behind diseases. Enhancer based therapies may someday enable drug delivery methods that are capable of recognizing specific micro-environments such as a disease state or specific cell types to explicitly control the activation, dosage, or release of a drug. Enhancers also may give tissue engineers the tools necessary for making stem cell

based therapies a reality by allowing them to control the expression of developmental genes.

We have demonstrated a two staged system. First computational analysis was performed to predict gene regulatory elements and second the biological function of identified sequences was experimentally verified using *in ovo/ex vivo* electroporation methods.

Genome alignments were performed on noncoding sequences flanking *Foxn4* gene. This resulted in the identification and analysis of 4 highly conserved regions (CR), which were computationally predicted to have a high likelihood of gene regulatory function.

In order to verify enhancer activity of the conserved regions, chick retina was chosen as the preferred model system. Previously developed *in ovo* electroporation method was modified to increase the success rate . This method has advantages over conventional methods by changing the time of electroporation to embryonic day 4. This change allowed for retinal progenitor cell specific electroporation and label all types of cell in the retina.

In order to experimentally verify the enhancer function of predicted CRs a reporter construct based enhancer activity assay was performed. This construct system allows for the direct visualization of the spatio-temporal regulation of gene expression by an enhancer through the detection of reporter GFP expression. This system was then applied to the mouse and chick models using *ex vivo/in ovo* electroporation for fast and efficient analysis of enhancer function. Four highly conserved non-coding region located proximally to the *Foxn4* gene were verified for enhancer activity in this manner. Two highly conserved regions, CR1 and CR4 showed to have gene driving ability both in

mouse and chick retina. CR4 was chosen for further analysis as this region is most conserved among all regions. The enhancer Foxn4CR4.2, a subregion of CR4 was of particular interest as it was found to have enhancer function in retinal progenitor cells, horizontal cells, and amacrine cells of embryonic chick retinas.

In addition, we have identified a widely known transcription factor, Meis1 that interacts with Foxn4CR4.2 in regulating Foxn4 expression. These findings provide additional molecular insight into the progenitor cell differentiation process via transcription factors. Our study also provides a useful and efficient method for regulatory sequence verification and functional study of DNA binding proteins in gene regulation during development.

### **5.3 Future Direction**

In order to further study this enhancer and characterize its ability to drive gene expression at earlier time points and in other tissues, transgenic animals that contain the Foxn4CR4.2- $\beta$ GP-GFP sequence need to be generated. Our preliminary studies with chicken embryos suggest that this sequence has also enhancer activity in the developing brain. Foxn4CR4.2 transgenic mouse model should confirm the gene regulatory activity in the brain. It will be interesting to determine whether the enhancer activity of Foxn4CR4.2 is inhibited or otherwise downregulated in the retina concurrently with the down regulation of Foxn4 expression.

It needs note that the other 2 functional regions of Foxn4, CR1 and CR4.3, both have demonstrated their ability to direct gene expression. Future studies should be conducted

to determine how these two sequences involved in the regulation of *Foxn4*, whether independently or in a combinatorial manner.

Site specific mutagenesis and gene knockdown assays confirmed that Meis1 transcription factor is essential for the regulation of *Foxn4* expression. However, data from EMSA suggests that there might be other *trans*-acting factors bind to CR4.2. Numerous studies have found that Hox, Pbx and Meis families of transcription factors form heteromeric complexes and bind DNA through specific homeobox domains to regulate gene expression (Ferretti et al., 2006; French et al., 2007; Heine et al., 2008a). We have already designed shRNAs specific against HoxA9 and Pbx1 for knockdown assays. It is essential to extend this study to confirm the involvement of other *trans*-acting factors in this important process of gene regulation during development.

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