Exploring the roles of rax1 among genetic networks involved in vertebrate eye development

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Exploring the roles of *rax1* among genetic networks involved in vertebrate eye development

by

Mohamad Fakhereddin

A Thesis

Submitted to the Faculty of Graduate Studies through Biological Sciences in Partial Fulfillment of the Requirements for the Degree of Master of Science

University of Windsor

Windsor, Ontario, Canada

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Exploring the roles of *rax1* among genetic networks involved in vertebrate eye development

by

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February 17, 2017
DECLARATION OF ORIGINALITY

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ABSTRACT

Three homeobox transcription factors were studied for their importance in eye development - *rax1*, *pax6*, and *six3*. *Rax1* encodes transcription factors that regulate genes necessary for the development of the eye. I show that perturbation of *rax1* expression via morpholino mediated translational knockdown has no affect on the shape of *pax6* and *six3* expression domains at early stages. Significant *pax6* expression changes were only seen at later stages of development. This inverts the hierarchal model of these transcription factors in early eye development. Furthermore, to confirm the position of these genes in our model we examined the relationship of significant eye field markers - namely, *foxe3*, *γ-crystallin*, *pitx3*, *mafA*, and *otx2*. By knocking down *rax1* and observing the outcome on the eye field markers I established that *rax1* is a major contributor but downstream to the overall process of eye development.
ACKNOWLEDGEMENTS

First, I would like to thank my close friends and family for their support and encouragement during the time spent completing my Masters. They were always there to offer help and advice.

I am very happy to have Dr. John Hudson and Dr. Siyaram Pandey as my committee members and would like to thank them very much for their advice and support with my project, as well as throughout my graduate career.

I met a lot of very nice and welcoming people in the Biology Department and formed good friendships with a fair few. Everyone in the department, including faculty, staff members, undergraduate and graduate students are all great people who I will always be thankful for due to the amount of support and help they offered me. The friends I made in the Crawford lab will never be forgotten as I had a wonderful experience with each and every one of them and remain grateful for what they have done for me and my graduate career.

I feel extremely proud and privileged to have met and worked with my supervisor, Dr. Michael Crawford. His Embryology course introduced me to developmental biology and his passion for the field fascinated me. I am so thankful for all of his support, advice, and encouragement with my project and all other life matters. To me, he is a true mentor, role model, and friend.

Lastly, and most importantly, I want to thank my wife Amne. I am truly blessed to have someone in my life that motivates me everyday to do and be better. Her patience, empathy, and help during my Masters allowed me to finish my project and gain a better understanding of what it is to be a good student, husband and human being. I love you.
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Xenopus laevis

Rax1 Rescue Clone - Plasmid 298 (pm298)

Morpholinos

Microinjections

Whole-mount in situ hybridization

Results

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<tbody>
<tr>
<td>ACC</td>
<td>Animal Care Committee</td>
</tr>
<tr>
<td>ANP</td>
<td>Anterior Neural Plate</td>
</tr>
<tr>
<td><em>ap</em></td>
<td><em>apterous</em></td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
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<tr>
<td>CDK</td>
<td>Cyclin Dependant Kinase</td>
</tr>
<tr>
<td>CDKI</td>
<td>Cyclin Dependant Kinase Inhibitor</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>CO</td>
<td>Control</td>
</tr>
<tr>
<td>CO MO</td>
<td>Control morpholino</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>EFTFs</td>
<td>Eye Field Transcription Factors</td>
</tr>
<tr>
<td>EnR</td>
<td>Engrailed Repressor</td>
</tr>
<tr>
<td>Ey</td>
<td>Eye field</td>
</tr>
<tr>
<td><em>ey</em></td>
<td><em>Eyeless</em> gene</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>HCG</td>
<td>Human Chorionic Gonadotrophin hormone</td>
</tr>
<tr>
<td>HMG</td>
<td>High mobility group</td>
</tr>
<tr>
<td>Hox genes</td>
<td>Homeotic genes</td>
</tr>
<tr>
<td>Maf</td>
<td>Musculoaponeurotic fibrosarcoma oncogene homolog</td>
</tr>
<tr>
<td>MBS</td>
<td>Modified Barth’s solution</td>
</tr>
<tr>
<td>MO</td>
<td>Morpholino oligonucleotide</td>
</tr>
<tr>
<td>ng</td>
<td>nanograms</td>
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NR Neural retina
NT Neural tube
Otx2 Othrodenticle homeobox homolog 2
\textit{Pax} genes \textit{paired-box} genes
pg picograms
PLE Presumptive lens ectoderm
pm298 Plasmid 298
\textit{Rax} genes Retinal \textit{homeobox} genes
RPC Retinal progenitor cells
RPE Retinal pigmented epithelium
RPL Retinal pigmented layer
RT-PCR Reverse transcriptase polymerase chain reaction
S14 Stage 14 of embryonic development in \textit{Xenopus laevis}
S19 Stage 19 of embryonic development in \textit{Xenopus laevis}
S27 Stage 27 of embryonic development in \textit{Xenopus laevis}
sey small eye
\textit{Six} genes \textit{Sine oculis} homeobox genes
so \textit{sine oculis}
\textit{Sox} genes \textit{SRY - related HMG} box genes
UTR Untranslated region
CHAPTER 1

Introduction

Embryogenesis requires genes to turn on in a systematic manner and to interact with each other in order to properly assemble cells, tissues, and organs. *Xenopus laevis* serves as a magnificent model for studying organ development at the embryonic level. For example, *Xenopus* provides an abundance of eggs that can be simultaneously fertilized externally, allowing for systematic observation of processes through the early stages of embryonic development. There is a century-long tradition of eye studies in amphibians (Reviewed in Fabler, 1996), and therefore an abundance of techniques and reagents available to study the genes that regulate eye development.

Specifically, this model organism has been helpful in studying induction of the eye. The eye is the product of inductive interactions between underlying neural tissue and the presumptive head ectoderm, ultimately leading to the formation of the optic cup and the lens (Henry et. al., 2002). In *Xenopus*, lens induction occurs in two phases. The first phase is known as the early phase and takes place during gastrulation, from stages 10-12 of development (Nieuwkoop & Faber). The ectoderm is capable of responding to neural cues during stage 11. The second phase of lens induction is known as the late phase, and it occurs when the optic vesicles bulge sufficiently to come into contact with the overlying head ectoderm at around stage 19 (Henry et. al., 2002). During this phase, the site of lens formation is specified in the head ectoderm consequently allowing for
induction and differentiation from both lens ectoderm and retina neural tissue (Henry et. al., 2002).

**Eye Development**

The first morphological sign of eye development in the vertebrates is a bilateral evagination of the diencephalon. As the evagination of the optic primordia proceeds, optic vesicles are formed and extend towards the non-neural surface ectoderm. Interposed between the surface ectoderm and neural structures of most of the head, mesenchyme presents a physical barrier to interaction in all places except the areas of presumptive lens and cement gland. When optic vesicles (neural) and ectoderm come into direct contact, inductive signals are exchanged between them (Chow and Lang, 2001).

The first morphological sign of the presumptive lens is formation of the lens placode (Figure 1.1). The lens placode involves columnarization of the surface ectodermal cells immediately above the optic vesicle. The optic vesicle and lens placode induce invagination behaviours in each other causing the lens vesicle and a double-layered optic cup to form. This produces the first indication of the final shape of the eye in vertebrates (Chow and Lang, 2001). The pigmented retina is formed by differentiation of the outer layer of the optic cup, whereas the inner layer gives rise to the neural retina (Lang, 1999). The ectoderm contributes to the lens placode and overlying ectoderm, and differentiates into lens and cornea, respectively.
Figure 1.1: An overview of eye development. A) Neural tube closure leads to direct contact between the optic vesicles and pre-lens ectoderm that thickens to form a placode (red). B) The lens placode invaginates and induces the optic cup (orange). C) The lens vesicle separates and is placed in the cavity formed by the invagination of the optic cup. D) Lens, neural retina, and cornea are essentially formed and as the retinal cup pinches off from the diencephalon, an optic stalk is formed that encloses the optic nerve (adapted and modified from the Brown et. al., 2011).

Differentiation and Proliferation in Eye Development

During early eye development, a population of proliferative, undifferentiated precursors is necessary prior to differentiation of the organ. Neural differentiation in *Xenopus* begins immediately after gastrulation, however, at this point, only the anterior neural plate is specified. Subsequently the neural plate rolls up to form a tube, and at the anterior end, it begins to balloon to form presumptive brain. It is important that the retinal progenitor cells of the neuro-ectoderm that are located in the optic vesicles undergo several cycles of proliferation to provide a population sufficient to differentiate into diverse populations of cells within the retina. We know that differentiation events
occurring in retinal progenitor cells (RPCs) are tightly linked to proliferation (Nelson et al., 2009).

During the G1 phase of the cell cycle, the retinal progenitor cells face a decision to continue with proliferation or to differentiate (Ohnuma et al., 1999). The decision to differentiate is one-way, causing the cell to enter the G0 phase; cells are prevented from re-entering the cell cycle. During the G1 phase there are specific, active Cyclin/Cyclin-Dependent Kinase (CDK) enzyme complexes, namely CyclinD:CDK4/6 and CyclinE:CDK1/2, that help to mediate whether proliferation or differentiation is to occur (Duparc et al., 2007). However, there are also CDK inhibitors present that may influence the activity of the CDK enzyme complexes. In *Xenopus*, only the Cip/Kip family of CDK inhibitors has been identified (Su et al., 1995).

For eye morphogenesis to occur, a minimal threshold of proliferating cells is required in the anterior neural plate (Nelson et al., 2009), and this requires active suppression of differentiation. Expression of *hairy2* and *zic2* are typically positively regulated by the *rax1* gene, and both act as anti-neurogenic transcription factors (Ando et al., 2005). *p27Xic1* is a cell cycle inhibitor that is also suppressed by *rax1* which ultimately promotes proliferation. Furthermore, *rax1* represses the expression of pro-neural genes *xdelta-1* and *xngnr-1*, preventing the differentiation of competent cells. Suppression of *rax1* confirms these observations: activation of an *engrailed* repressor chimera. *Rax1-enR*, causes inhibition of *rax1* targets which leads to the expansion of
the pro-neural genes in the anterior neural plate. This reduces proliferation and induces neuralization, ultimately reducing the size of the eye field (Andreazzoli et al., 2003).

Studies have shown that level of cyclinD1, which is a major cyclin enzyme in the retinal progenitor cells, are significantly increased when there is an over-expression of \textit{rax1} by mRNA injections. Furthermore, the expression of \textit{p27Xic1} is simultaneously inhibited by \textit{rax1} over-expression (Casarosa et al., 2003). The other eye field transcription factors, \textit{six3} and \textit{six6}, both bind to the promoter of \textit{p27Kip1}, ultimately inhibiting its activity and inducing proliferation (Li et al., 2002). Furthermore, \textit{six3} regulates \textit{cyclinD1} and \textit{p27} expression levels (Gestri et al., 2005). Therefore, the assortment of eye field transcription factors involved in eye development regulate several cell cycle and differentiation parameters during eye development.

**Dorsoventral Patterning of the Eye by \textit{BMP4}**

Dorso-ventral patterning of the eye is under the influence of \textit{bmp4}, a ventralizing agent that serves as an antagonist to the activation/transformation of ectoderm to neuroectoderm (Schmidt et al., 1995). When \textit{bmp4} is over-expressed in \textit{Xenopus} embryos, ventralization results which leads to suppression of neuroectoderm. This conversion from neural to epidermal fates is caused by a reduction in the competence of tissues to respond to neuralizing cues from the underlying mesoderm (Nakayama et al., 1998).
Inhibition of *bmp4* is essential for neural plate induction and, ultimately, for the development of the neural component of the eye field. This is demonstrated by explant studies in mice where over-expression of *bmp4* in the presumptive lens ectoderm can suppress lens development (Furuta and Hogan, 1998). In *bmp4* null mutant mice, explants of presumptive lens ectoderm forms normal lens and induces retina when transplanted on wild-type optic vesicles, but when *bmp4* is present the isolated ectoderm fails to form lens (Furuta and Hogan, 1998). Furthermore, *bmp4* null mutant mice do not survive past E10.5, demonstrating how important this gene is to dorsal-ventral axis maintenance (Furuta and Hogan, 1998).

Not surprisingly then, *bmp4* also represses the expression of major neural markers. These neural markers, *rax1*, *pax6*, and *otx2*, are repressed when *bmp4* coated beads are implanted surgically into the anterior neural plate region during mid-neural stages (Hartley et al., 2001). Furthermore, *bmp4* that is expressed ectopically using a *pax6* promotor suppresses *rax1* and *otx2*. Additionally, *bmp4* was found to repress the dorsalizing pathway that is required for the optic cup and lens formation (Hartley et al., 2001).

**Genes Involved**

As described above, there are several genes involved in the regulation of eye development in *Xenopus*, some of which commence expression during gastrulation at stage 10.5. Among these genes are *otx2* and *six3* which play a major role in eye field
expression, as opposed to *rax1*, *pax2*, and *pax6* sub-domains which are confined to areas within the larger domains of *otx2* and *six3*. The domains of these early eye field transcription factors overlap during expression: *sox2* is expressed along the neural tube and in the eye field domain where *rax1*, *pax6*, and *six3* overlap (Figure 1.2).

**Figure 1.2:** A schematic summarizing the expression of Eye Field Transcription Factors involved in early invertebrate eye development.

**Homeobox Factors**

The most studied genes involved in eye development are the homeobox genes that demonstrate the most spectacular mutant phenotypes including *rax1*, *pax6*, *pitx3*, *otx2*, and *six3*. The homeobox sequence found within these genes encodes a protein
domain known as the homeodomain. This protein domain is 60 amino acids long and contains motifs that recognize a TAAT consensus target sequence (Beebe et al., 1994).

**Rax1**

*Rax1* is an eye field transcription factor that has a homeodomain sequence which shares a high homology with the *paired*-like genes (Kamijyo et al., 2014). The core homeodomain sequence of *rax1* binds to a TAAT consensus motif (Terada et al., 2006). *Rax1* RNA is expressed in the forebrain region where the optic vesicles are formed (Giudetti et al., 2014). *Rax1* expresses prior to *pax6* expression and in coordination with *six3* (Oliver et al., 1995). The *rax1* gene is involved in the development of the retina, pineal gland, pituitary gland, and hypothalamus (Kamijyo et al., 2014).

*Rax1* is heavily involved in eye development in *Xenopus* where it begins its expression during the stages following gastrulation. It is initially expressed at stage 10 of development (Zuber et al., 2003), but is especially prominent at stage 13 and continues to express throughout, until stage 45, well after eye development completes and expression begins to decline. Expression of *rax1* is detectable during the development of the optic vesicle, before contact is made with the overlying ectoderm. This takes place around stages 16 and 17.

The significance of *rax1*’s role in eye development has been highlighted in research due to the phenotypes that result from mutations in the *rax1* gene. These
mutations lead to defects in the eye which have been characterized in mice and humans. In mice, the *eyeless (ey1)* mutant phenotype lacks eyes and/or optic tract. This naturally occurring phenotype is due to a mutation in the start codon of murine *rax1* gene (Tucker et al., 2001). In humans, mutations found in the homeodomain of the *RAX1* gene have led to microphthalmia and anophthalmia. Individuals who suffer from microphthalmia are characterized by reduced eye size, whereas those who suffer from anophthalmia show a lack of eyes (Voronina et al., 2004). Conversely, in *Xenopus*, over-expression of the *rax1* gene has been shown to cause an enlarged retinal pigment epithelia (Mathers et al., 1997).

*Rax1* interaction with the *pax* gene family is heavily researched due to the similarity of their respective mutant phenotypes, as well as the overlapping patterns and timing of their expression domains (Zuber et al., 2003). The *pax6* gene is involved in eye development and, like *rax1*, is expressed in the retinal field and the anterior neural plate (Mathers & Jamrich, 2000). In the later stages of eye development, *pax6* expression leads to lens and retina development while expression of *rax1* appears to be more specifically responsible for retinal development. An interesting interaction between *rax1* and *pax6* has been observed in which individuals expressing a *rax1* mutant phenotype lack the structures of the lens even though *rax1* is not expressed in the lens (Swindell et al., 2008). According to research conducted by Zuber and his colleagues, *rax1* activates *pax6* expression, resulting in the development of lens structure (Zuber et al., 2003). However, by co-injecting *pax6* mRNA with *six3* morpholino, Saqib Sachani
found that \textit{pax6} rescues the expression of \textit{rax1} (Sachani, 2011; Current study). This leads us to speculate that \textit{rax1} expression is driven by \textit{pax6}, not the reverse.

\textbf{Pax6}

The \textit{pax6} gene is a paired-like transcription factor belonging to the \textit{Pax} family. It encodes two DNA binding motifs, a homeodomain and the \textit{paired} box (Stuart et al., 1994). \textit{Pax6} is commonly known as the master regulator of eye development and is vital for retina and lens development. Mutations of the \textit{PAX6} gene leads to the human disorder known as aniridia. Mutations of the \textit{Pax6} gene are also found in mice and \textit{Drosophila}, where a mutation can lead to \textit{small eye} (sey) in mice and an eyeless phenotype in \textit{Drosophila} (Chow and Lang, 2001). The \textit{Pax6} heterozygous mutant observed in mice is characterized by the \textit{small eye} (sey) phenotype, ultimately resulting in hypoplasia of the iris, cataracts, and microphthalmia (Hill et al., 1991). However, mice that are \textit{Pax6} null mutants tend to be anophthalmic and generally die at birth (Grindley et al., 1995).

\textit{Pax6} plays a major role in \textit{Xenopus} eye development. It is first expressed at stage 10 (Zuber et. al., 2003) and is then later expressed in the retina and presumptive lens. \textit{Pax6} is also involved in development of the brain and is expressed in the neural tube. It is expressed in the neuroepithelial cells at stage 14, where these cells eventually give rise to pigmented and neural retinas. During eye development, \textit{pax6} is expressed throughout stages 12.5 to 28, where the lens begins to thicken and becomes
distinct from the overlying epithelium after stage 28. Expression of pax6 in the lens remains relatively high up to stage 33. Once stage 42 of development is complete, pax6 expresses solely in the inner nuclear and the ganglion cell layers of retina (Harris & Hirsch, 1997). Ectopic pax6 expression in Xenopus leads to formation of a fully differentiated ectopic eye, as well as enhanced expression of eye field markers, namely rax1, six3, and otx2 (Chow et al., 1999).

**Six3**

The six3 gene is a transcription factor found in Xenopus that is homologous to the sine oculis (so) gene in Drosophila. It is part of the Six family and has cloned homologues in human, avian, mouse, and fish (Oliver et al., 1995). Six3 expression in mice is first detectable in lens placode, followed by expression in the lens epithelium during further developmental stages (Oliver et al., 1995). The six3 sequence encodes the DNA binding homeodomain, as well as a Six domain that mediates interaction with other transcription factors (Oliver et al., 1995). Six3 itself lacks the ability to activate these genes in the absence of a partner (Otho et. al., 1999). Six3 is a crucial gene in Xenopus eye development, beginning as a maternal transcript onward at stage 10.5 during gastrulation (Zuber et. al. 2003). The expression domain for six3 is located in the anterior end of the neurula in neuroectoderm, at stage 14. During stage 20, the mid-region of the ventral diencephalon shows heavy expression of six3 which then migrates anteriorly towards the telencephalon. Expression is then limited mainly to the eye region and the ventral diencephalon between the eyes at stage 32 (Ghanbari et al., 2001).
Mutations of the \textit{six3} gene and its homologues is of common interest in \textit{Xenopus} and other species. Mutations of the homologous fly \textit{so} gene leads to retinal degeneration as well as premature development of the optic lobe (Serikaku & O’Tousa, 1994). In medaka fish, over-expression of \textit{six3} causes enlarged optic vesicles and expansion of the presumptive midbrain (Wittbrodt et al., 1999). Gene knockdown experiments were performed on medaka fish by use of \textit{six3} morpholinos and abnormalities were found in the development of craniofacial, forebrain, and eye structures. When morpholino concentrations were increased, small eyes, cyclopic eyes and, eventually, complete absence of eyes resulted (Wittbrodt et al., 2002). In mice, \textit{Six3} null mutant embryos show a loss of telencephalic regions and are characterized by craniofacial anomalies (Oliver et al., 2008). In both human and mouse mutants, holoprosencephaly occurs to different degrees leading to microphthalmia or cyclopia (Lacbawan et al., 2009).

\textit{Pitx3}

\textit{Pitx3} is a member of the \textit{paired}-like homeodomain family of genes, and it is expressed in several areas in \textit{Xenopus}, including the brachial arches, otic vesicles, pituitary, the presumptive lens ectoderm, the somites, and the heart and gut. It is expressed in the mid-blastula, around stages 9-11.5, as well as during early gastrulation. Expression increases after the mid-neural stages, expressing through stages 12-19, which covers the late phase of induction (Khosrowshahian et al., 2005).
Pitx3 is believed to express before the lens placode thickens, and it is maintained in the lens placode, as well as the lens vesicle and the lens pit. Expression of pitx3 in the pre-lens and lens-tissue is high from stage 19-34, where the strongest expression is viewed at stage 24 in competent ectoderm when it is in contact with the optic vesicle (Khosrowshahian et al., 2005). At this stage the expression is marked at the presumptive lens ectoderm, but once the presumptive lens ectoderm develops into the lens placode, expression of pitx3 is strongest in the lens placode. Eventually, expression in eye is restricted to the lens epithelial layer as development reaches later stages (Pommereit, Pieler, & Hollemann, 2001).

Experiments of pitx3 inhibition in Xenopus embryos via morpholino knockdown impaired eye development, resulting in reduced eyes, or even inhibiting them entirely when the concentration of morpholino was high (Khosrowshahian et al., 2005). When pitx3 is over-expressed, however, the pax6 domain expands, suggesting that pax6 may be under the control of pitx3 in the lens. Conversely, during pitx3 knockdown experiments in whole embryos, expression of pax6 tends to be down-regulated. It is also believed that pitx3 regulates expression of rax1, foxe3, and otx2 (Khosrowshahian et al., 2005). Mutations of Pitx3 in mice and humans have also been observed. PITX3 mutation in humans cause abnormal cornea displacement, underdeveloped iris, pupil displacement, multiple pupillary openings, and the development of congenital cataracts (Semina et al., 1998; Espinoza et. al., 2002). Deletion of Pitx3 in mice leads to aphakia which is characterized by small eyes without lenses (Semina et al., 2000).
**Otx2**

The *otx2* gene is a homeobox gene that is related to the orthodenticle family of genes (Simeone et al., 1993). *Otx2* in *Xenopus* embryos is detectable as a maternal transcript. Expression levels of *otx2* during blastula stages are low but detectable nonetheless. *Otx2* expression is visible in the eye field at stage 10.5 in the mid-gastrula embryo and is activated before most of the other eye field transcription factors (Simeone et al., 1993). After gastrulation, expression of *otx2* is also limited to the anterior dorsal region of the embryo, where it restricts to the mesendoderm and the anterior ectoderm regions after gastrulation has taken place (Boncinelli et al., 1995).

*Otx2* and *rax1* have been shown to have some interplay in eye development, but *in situ* hybridization experiments determined that their early expression domains were quite different (Andreazzoli et al., 1999), suggesting the interplay must occur at later stages or be indirect. *Otx2* is inhibited by over-expression of *rax1*, indicating that *otx2* is not needed for eye field specification in early stages of development since *rax1* over-expressing embryos still form eyes. Possibly, *six3* is active and can carry on this role, although it is necessary for the specification of the anterior neural domains (Andreazzoli et al., 1999).

Following morpholino-mediated *otx2* knockdown experiments in *Xenopus*, unusual anterior development and deformed eyes were observed (Carron et al., 2005). By contrast, when *otx2* is over-expressed, the result is induction of the ectopic cement glands as well as abnormally large eyes (Gammil and Sive, 1997). Homologs of the
*otx2* gene are found in humans and mice and are vital for early specification of the neuroectoderm when it transitions to mid- and forebrain (Simeone, 1998). Deletion of the *Otx2* gene in mice causes lethal defects during gastrulation. Mice that are *Otx2* null mutants are characterized by missing midbrain and forebrain structures, most likely due to abnormal neural induction (Pannese et al., 1995).

**Pax2**

*Pax2*, like *pax6*, contains the highly conserved DNA binding domain known as a *paired* domain which is encoded by the *paired-box* (Pichaud and Desplan, 2002). Interestingly, *pax2* also has an octapeptide domain which *pax6* does not posses, categorizing it as a multi-functional transcription factor (Eccless et. al., 2002). Although *pax2* does not hold the same evolutionarily conserved role in eye development as *pax6*, it still has an important role in the process. *Pax2* is first detectable in the developing optic cup and then, following invagination, is limited to glial cells which extend to form the optic stalk (Macdonald and Wilson, 1998). Other than being expressed in the eyes, *pax2* is also expressed in the otic vesicle primordium and specifies regions in the central nervous system (CNS) and kidney (Quinn et. al., 1996; Tavassoli et. al., 1997).

**Optx2**

*Optx2* is a homeobox gene and a member of the *six* family. *Optx2* plays an important role in early regulation of retinal development as well as interacting with other
transcription factors in regulating cell proliferation of the retina. The \textit{optx2} homolog in \textit{Xenopus} is the \textit{xoptx2} gene and is first detected at stage 15 as a single band on the anterior edge of the developing neural plate until it reaches around stage 17, when expression begins to extend laterally. At stage 18, \textit{xoptx2} expression separates into two regions that are consistent with the eye field locations. Stages 20-22 sees the expression become limited to the developing eye primordia. Expression of \textit{xoptx2} is detected in the cells of the protruding primary optic vesicles (stage 24). The vesicles invaginate at stage 28 to form the optic cup where expression is still strong in the developing retina, but is not visible in the thickening ectodermal lens placode. \textit{xoptx2} expression is still detectable at stages 33 and 34 in the developing neural retina, but slowly begins to diminish thereafter (Zuber et al., 1999).

\textit{Optx2} plays a vital role in the overall size of the developing eye and brain of \textit{Xenopus} embryos. Over-expression also caused an enlargement of the eye field which is believed to be due to an increase in the progenitor population, which is expected if the eye bud is enlarged. \textit{Xoptx2} also increased the size of \textit{rax1}'s expression domain in embryos at stage 17. This was also the case with \textit{Pax6} and \textit{tbox3} (formerly known as \textit{ET}), the domains of which increased within the eye field at stage 17. Ectopic over-expression of \textit{xoptx2} induced an enlarged eye, where folding of the neural retina was also visible. \textit{Xoptx2} knockout experiments resulted in \textit{Xenopus} embryos with significantly reduced eyes, and sometimes eliminated eye formation. Reducing the expression of \textit{xoptx2} also led to a significant reduction in the expression domain of \textit{rax1}.
These effects are similar in other vertebrates and their homolog genes (Zuber et al., 1999).

**Lhx2**

*Lhx2* is a member of the *LIM*-homeodomain transcription factor family which is structurally characterized by a carboxy terminal homeodomain, as well as two amino terminal zinc-finger motifs. *lhx2* is one of the various eye field transcription factors that is essential for eye development. In *Xenopus*, *lhx2* expression is detected at stage 12.5 in the neuroectodermal layer of the anterior plate in the location of the eye field. At this stage, the expression is visible as a single band but diverges into the two eye primordia at stage 18 when the optic vesicles begin to form. At stage 24, *lhx2* expression is detected in the presumptive retinal epithelium, the neural retina, and the optic stalk. However, once the lens begin to form around stage 28, expression is absent from the lens placode but is still visible in the neural retina and the presumptive retinal pigment epithelium. *Lhx2* continues to express in all cells of the developing retina until retinal progenitors start to differentiate, which occurs at around stage 41. At this stage, expression becomes limited to the ciliary marginal zone and a selected group of cells in the inner nuclear layer (Viczian et al., 2006).

Homologues of *Lhx2* are found in mice and *Drosophila*. In mice, the *Lhx2* gene is essential for eye development. *Lhx2* mutant mice lack eyes even though the optic prominences evaginate normally from the brain. The optic vesicles never form, thus no
eye forms. In *Drosophila*, the *lhx2* homologue is likely the selector gene *apterous (ap)*. The expression pattern of *ap* is very similar to *lhx2*. In fact, experiments have shown that human *lhx2* can rescue the fly mutant *ap* phenotype (Rincon-Limas et al., 1999). Typically, the inactivation of eye field transcription factors in *Drosophila* leads to eyeless flies.

**Sox Transcription Factors**

The *sox* genes are part of the High Mobility Group (HMG) proteins which are transcription factors that have the HMG domain, a 75 amino acid DNA-binding domain. These genes play a significant role in eye development in *Xenopus*, mice, and chick. First to express is the *sox1* gene in the lens placode area, later limiting expression to the lens fibre cells. *Sox2* is first expressed in the anterior neural plate region at around stage 14 in *Xenopus*, but is then found to express along the developing neural tube and eventually in the anterior dorsal head region at stage 19. *Sox2* expression is also noticeable during the later phase of eye development in the PLE region, as well as the optic cup. It’s expression is significantly higher during thickening of the lens placode (Kamachi et al., 1998). Expression of *sox3* takes places in the lens placode region as well, occurring during induction, playing a role in the activation of *crystallins* (Kamachi et al., 1998). It has been found that *de novo* mutations of the *Sox2* gene lead to the absence of eyes in mammals (Ragge et al., 2005).
MafA Leucine Zipper Transcription Factors

*MafA* is part of the *maf* family of leucine zipper transcription factors and plays a role in lens induction, placode thickening and differentiation. There are several members of the *Maf* family including *mafA*, *mafB*, and *c-maf*. *MafA* is mainly seen to be expressed in the lens ectoderm as opposed to *mafB* which is expressed primarily in the optic vesicle anlagen (Ishibashi and Yasuda, 2001). *MafA* is expressed around stage 24 of development in the lens placode during presumptive lens ectoderm induction and differentiation. It is also observed to up-regulate expression of the *crystallin* genes. In *Xenopus* embryos, as well as chick embryos, *mafA* over-expression causes ectopic induction of *crystallin* in the presumptive lens ectoderm. In contrast to *mafA*, *mafB* is expressed at stage 20, during induction by the optic vesicle. It is believed to induce the expression of *mafA* in the presumptive lens ectoderm. Both *mafA* and *mafB* can substitute for the other’s function if need be: if *mafB* is knocked down in embryos, *mafA* mRNA expression rescues the activation of the *crystallins* (Ishibashi and Yasuda, 2001).

Forkhead Factors

The family of *forkhead* (or *winged-helix*) transcription factors display a wide variety of functional diversity with respect to developmental processes. They are involved in cell growth, lens progenitor cell proliferation, and cell cycle regulation, amongst many other cellular processes (Carlsson & Mahlapuu, 2002). *Forkhead*
transcription factors have a DNA binding domain called the *forkhead* box which allows them to bind to DNA as monomers (Kaestner et. al., 2000).

**Foxe3**

*Foxe3*, also commonly known as *lens1*, is a transcription factor which is part of the sub-family of the *forkhead* family and is notably recognized as a *winged-helix* motif (Kaufmann & Knochel, 1996). Generally speaking, the expression pattern of *foxe3* is limited to the lens lineage (Blixt et. al., 2000). In *Xenopus*, *foxe3* acts to promote proliferation and sustain an undifferentiated state prior to lens specification. Shortly after lens specification is completed, *foxe3* increasingly limits expression from the presumptive lens ectoderm to the lens placode and then to the epithelium of the differentiating lens. When *foxe3* is ectopically over-expressed in *Xenopus* there is complete suppression of lens differentiation which is reflected by a total loss of γ-crystallin expression. *Foxe3* appears to maintain the ectoderm when expression levels are high, but this is occurring in an undifferentiated manner. Once the differentiation of the lens takes place, *foxe3* expression drops while *sox2* and *sox3* are positively regulated in the presumptive lens ectoderm, followed by progressive differentiation.

**Crystallins**

*Crystallins* are structural proteins of the lens and are key members of the heat shock protein superfamily (Ghosh et. al., 2005). They are involved in stress response
and cellular protection, and their expression signals the terminal differentiation of lens fibre cells (Wistow & Piatigorsky, 1998). Crystallin proteins are abundant in the lens, constituting over 90% of lens proteins (Clark, 2004). They have structural qualities that are important for transparency and refracting light (Jaffe & Horwitz, 1992). There are a few categories of crystallins, but the most commonly discussed ones are $\alpha$-crystallins, $\beta$-crystallins, and $\gamma$-crystallins (Wistow & Piatigorsky, 1998).

$\alpha$-crystallins are a family comprised of $\alpha$-A-crystallin and $\alpha$-B-crystallin, which are present in the lens in a 3:1 ratio. These two make up roughly 40% of the crystallins found in the lens. $\beta$-crystallins and $\gamma$-crystallins both derive from a common ancestor and have two types of Greek key motifs, which are anti-parallel $\beta$-sheets fused together (Bax et. al., 1990; Blundell et. al., 1981). $\gamma$-crystallins exist only as monomers because a compact complex forms between domains. This tight, condensed structure allows $\gamma$-crystallin to provide transparency by folding in a regulated state (Lubsen et. al., 1998). However, the expression levels of both $\beta$-crystallins and $\gamma$-crystallins vary among different species regardless of their structural similarities.

In *Xenopus* embryos, both $\alpha$-crystallins and $\gamma$-crystallins are expressed in the developing lens vesicle and show high levels of expression throughout the differentiation of the lens fibre (Van Leen et. al., 1987). Further in development, $\beta$-crystallin expression is evident in the lens fibre cells. The crystallins are in active form
through primary lens fibre differentiation to secondary lens fibre formation (Treton et. al., 1991).

The genes discussed above are listed below in Table 1, summarizing their functions in eye development. There is an overlapping pattern of expression characterized by these eye field transcription factors, but their functional expression is divided into subunits of the eye. These subunits are: the optic stalk, pigmented retina, neural retina, and the lens (see Figure 1.3).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
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</thead>
<tbody>
<tr>
<td>Rax1</td>
<td>Development of retina and optic vesicles</td>
</tr>
<tr>
<td>Pax6</td>
<td>Lens epithelium maintenance</td>
</tr>
<tr>
<td>Six3</td>
<td>Inhibitor of BMP4</td>
</tr>
<tr>
<td>Sox2</td>
<td>Regulator of crystallin expression</td>
</tr>
<tr>
<td>Otx2</td>
<td>Inhibitor of BMP4</td>
</tr>
<tr>
<td>BMP4</td>
<td>Promotes ventral fate</td>
</tr>
<tr>
<td>Foxe3</td>
<td>Proliferation</td>
</tr>
<tr>
<td>Ptx3</td>
<td>Retina and lens induction</td>
</tr>
<tr>
<td>MafA</td>
<td>Crystallin activation</td>
</tr>
<tr>
<td>γ-crystallin</td>
<td>lens differentiation and development</td>
</tr>
<tr>
<td>Pax2</td>
<td>RPC proliferation</td>
</tr>
</tbody>
</table>

**Table 1:** A summary of genes involved in lens and eye development with their important functions.
**Figure 1.3:** A schematic showing the various transcription factors expressing in different tissues of the eye. The proximal end (left) shows the early expressed markers, the distal end (right) shows the late expressed markers; NR - Neural Retina (Adapted and modified from Saqib Sachani).
My Project

Eye development has been extensively studied over the years and is still a growing field with plenty of research required. Zuber and his colleagues developed a model for eye field specification and lens induction where otx2 primarily drives neural patterning (Figure 1.4). His research also suggests that there is a feedback loop between secondary eye field transcription factors such as pax6, rax1, and six3 in which these genes are expressed after the early expression of otx2. From their developed model they concluded that rax1 inhibits expression of otx2 and that rax1 is upstream of six3 and pax6.

Figure 1.4: The model proposed by Zuber and colleagues showing a summary of the eye field induction in the anterior neural plate in which rax1 is driving the expression of both pax6 and six3 (adapted and modified from Zuber et. al., 2003).
Zuber’s early model requires revision and further research on the eye field transcription factors involved in eye development. *Six3* and *otx2* have been found to be the earliest expressed genes during late gastrulation and both are expressed before *rax1* prior to anterior neural plate definition (Chow and Lang, 2001; Zuber et al., 2003). Therefore, it is not yet clear how *rax1* in Zuber’s model can serve as the primary initiator in the hierarchy since it appears to express so late. Furthermore, *six3* and *otx2* mutants have more severe phenotypes in the eye and other brain structure. In contrast, *rax1* mutant phenotypes are localized more specifically in the eye region. *Otx2* null mutants in mice displayed missing forebrain and midbrain structures, most likely due to abnormal neural induction (Pannese et al., 1995). In *Xenopus*, morpholino-mediated knockdown of *otx2* led to severe eye and anterior development abnormalities (Carron et al., 2005). Knocking down *six3* in medaka fish led to abnormalities found in the development of craniofacial, forebrain, and eye structures. Increasing morpholino concentrations resulted in small eyes, cyclopic eyes and, eventually, complete absence of eyes (Wittbrodt et al., 2002). *Six3* null mutant mice exhibited a loss of telencephalic regions and were characterized by craniofacial anomalies (Oliver et al., 2008). In *Xenopus*, *six3* perturbation also resulted in abnormal craniofacial development such as improper neural closure, reduced or lost forebrain, and a complete loss of eye structures (Sachani, 2011). These findings contradict Zuber’s model and suggest that *rax1* is downstream of *otx2* and *six3* in eye development.

Our lab has developed results which contradict Zuber’s intelligent model through knockdown, rescue, and over-expression experiments on *six3* and *pax6* followed by *in*
situ hybridization to examine the effect on other eye field transcription factors and eye marker genes. Six3 morpholino-mediated knockdown inhibits pax6 expression at early and late stages of eye development. Conversely, over expression six3 results in enhanced pax6 protein levels (Sachani, 2011). Furthermore, six3 knockdown led to complete inhibition of rax1 expression at stage 19, when rax1 is normally expressed in the anterior neural plate. Knocking down pax6 did not effect six3 expression domain at early stages of development, but did reduce the eye field domain of six3 at late stages (Sachani, 2011). Ectopic pax6 expression expanded the expression domain of six3 at early to mid neurula stages, suggesting pax6 feeds back on six3 at those stages. This might be a direct interaction, but it is also possible that the effect is indirect. Enhancement/enlargement of the eye field by ectopic pax6 might increase the number of cells present in the organ, thereby increasing six3 domain.

Similar to six3, pax6 morpholino-mediated knockdown results in complete abolishment of rax1 expression in the anterior neural plate and presumptive eye field (Sachani, 2011). Conversely, pax6 over-expression resulted in expanded rax1 expression domain. Interestingly, when six3 morpholino is co-injected with pax6 mRNA, rax1 expression is rescued to normal and is even slightly over-expressed on the injected side. This indicates pax6 is likely operating upon rax1 downstream of six3 and it is also consistent with the finding that in the absence of six3, pax6 is inhibited and rax1 is then down-regulated as a result (Sachani, 2011). Morpholino-mediated knockdown experiments for six3 also revealed deleterious effects on other eye genes: otx2, pitx3, foxe3, mafA, γ-crystallin, pax2, and sox2 all exhibited a significantly reduced expression
domains or complete inhibition of expression. *Pax6* knockdown yielded the same result except that it did not have any affect on *otx2* expression (Sachani, 2011). These findings led our lab to propose model which is still under investigation (Figure 1.5). Nonetheless, it situates *six3* upstream of *pax6* and *rax1* in contrast to Zuber’s model which shows *rax1* being upstream of *pax6* and *six3*, in that order (Figure 1.4).

My project will focus primarily on the role of *rax1* in eye development and its interaction with other previously studied eye field transcription factors. In order to evaluate and understand the functional role of *rax1*, knockdown experiments will be conducted by use of microinjected morpholino oligonucleotides that is directed against *rax1* mRNA. The effect *rax1* knockdown has on *six3* and *pax6* will help confirm that *rax1* is downstream of *six3* and *pax6* and reveal more about the relationship between *rax1* and *foxe3*, *pitx3*, γ-crystallin, *mafA* and *otx2*. Over-expression and rescue experiments will also be conducted. Over-expression will involve co-injecting *rax1* mRNA with a lineage marker GFP and determining the effect on *foxe3*, *pitx3*, and γ-crystallin expression. Three rescue experiments will be attempted: 1) anti- *rax1* morpholino mRNA (*pm298*) and GFP will be co-injected with a *rax1*-specific morpholino, 2) *six3* mRNA will be co-injected with GFP and *rax1* morpholino, and 3) *pax6* mRNA will be co-injected with GFP and *rax1* morpholino. The rescue experiments are to determine if expression of *foxe3*, *pitx3*, and γ-crystallin is rescuable by *pm298*, *six3*, and *pax6*. Whole-mount in situ hybridizations will be conducted to characterize the phenotypes that result and to study the interaction associated with *rax1* and these eye field genes.
Figure 1.5: The proposed model derived by Crawford and colleagues (unpublished; Sachani, 2011) and from relevant literature showing gene interaction in eye development. This model demonstrates that *pax6* expression is driven by *six3* and that *rax1* expression is driven by *pax6* directly and *six3* indirectly.
References


Su J.Y., Rempel R.E., Erikson E., Maller J.L. Cloning and characterization of the


CHAPTER 2

Rax1 is crucial for normal eye morphology and is downstream of six3 and pax6

In the past, gain- and loss-of-function, as well as animal cap and whole embryo RT-PCR assays were employed to determine the hierarchal role of three Eye Field Transcription Factors during eye development. Based upon studies in Xenopus, rax1 is presently understood to stand upstream of six3 and pax6 (Zuber et. al., 2003). By contrast, members of our lab have shown that six3 appears to work upstream of both pax6 and rax1 (Sachani, 2011). The outcome of rax1 knockdown experiments on eye field markers - foxe3, pitx3, γ-crystallin, mafA, and otx2 - will confirm rax1’s role in eye development. Furthermore, over-expression of rax1 and rescue experiments involving an anti-rax1 morpholino mRNA, as well as pax6 and six3 rescue experiments will solidify the hierarchal order of these genes. These findings will help us establish the roles of six3, pax6, and rax1 and understand their hierarchal order in eye development in Xenopus laevis.

Morpholino-mediated rax1 translation knockdown should faithfully reproduce the characteristic small-eye phenotype seen in mammals. However, rax1 knockdown is not expected to affect the expression of six3 or pax6 at early stages of development. Since pax6 is an intermediary of rax1 and six3, down-regulation of rax1 should only affect the expression pattern of pax6. Our studies show that six3 is upstream of both pax6 and rax1 (Sachani, 2011). Rax1 is rescued by ectopically administering pax6 mRNA upon six3 knockdown (Sachani, 2011). Therefore, six3 is expected to be unaffected by rax1
knockdown. In addition to translational knockdown, over-expression of \textit{rax1} will provide further understanding of the interactions \textit{rax1} has on eye field genes. Furthermore, it is necessary to perform rescue experiments and determine whether the expression of these genes can be restored upon \textit{rax1} morpholino-mediated knockdown. Since \textit{rax1}, \textit{six3}, and \textit{pax6} are the priority transcription factors under investigation for my project, using their mRNA to rescue expression of the other genes must be carried out. However, a plasmid that contains the \textit{rax1} insert but excludes the \textit{rax1} morpholino sites must be designed to avoid knocking down \textit{rax1} expression when performing the rescue experiment. \textit{Six3} and \textit{pax6} mRNA will be co-injected with \textit{rax1} morpholino to determine if they are able to rescue the expression of eye field genes. If \textit{rax1} is indeed downstream of \textit{pax6} and \textit{six3}, mRNA of \textit{pax6} and \textit{six3} shouldn't be able to rescue \textit{rax1} morpholino knockdown.

Before reporting the outcome \textit{rax1} knockdown produces, it is imperative to confirm the specificity of the \textit{rax1} morpholino used to rule out off-target and toxic effects. Two \textit{rax1} morpholinos - Rax1.1 and Rax1.2 - were unilaterally injected in \textit{Xenopus} embryos at 2-cell stage; the contralateral un-injected side serves as a control. Phenotypes were expected to resemble fish, murine, and human mutant phenotypes. To further confirm specificity, three control morpholinos were also used: Rax1.1b mismatch control, Rax1.2 mismatch control, and a Fluorescent Standard Control. Rax1.1b control and Rax1.2 control are similar in sequence to experimental morpholinos but with five nucleotide sequence mismatches. Unilateral injection of these control morpholinos should produce normal eye phenotypes. A synergistic injection which combines Rax1.1 and Rax1.2 morpholinos at lower concentrations was carried out to determine if the
same phenotype is produced which confirms the specificity of said morpholinos. Upon confirming specificity, the optimal morpholino dose required to produce a consistent phenotype needed to be determined. This was done by unilaterally injecting \textit{rax1} morpholino into a 2-cell embryo at a range of concentrations and observing what dosage produces the expected phenotype without resulting in off-target or toxic effects.
Materials and Methods

*Xenopus laevis*

African clawed frogs (*Xenopus laevis*) were delivered from Xenopus I, Inc. (Michigan, USA). Rearing of frogs followed the regulations of the University of Windsor’s Animal Care Committee (ACC), as well as Federal and Provincial regulations. To induce ovulation, an adult female frog was injected with 0.75 cc of Human Chorionic Gonadotrophin (HCG) hormone (Sigma-Aldrich, Ontario, Canada). Once eggs were obtained, fertilization and de-jellying eggs followed the protocol outlined by Drysdale and Elinson (1991). Embryos were staged according to Nieuwkoop and Faber and fixed in MEMPFA, followed by storage in 70% methanol at -20°C.

*Rax1 Rescue Clone - Plasmid 298 (pm298)*

The *rax1* cDNA minus the *rax1* morpholino binding sites was amplified using Phusion high fidelity DNA polymerase (NEB). The insert was cloned into the XhoI site of pCS2+. Initial denaturation was 94°C for 2 minutes, followed by denaturing at 94°C for 45 seconds. Annealing temperature was set at 67°C for 45 seconds followed by extension at 74°C for 30 seconds for 26 cycles. The sequence for the forward primer is: GATCCTCGAGAGGGTCCTCAATGCACCTGCACA and the sequence for the reverse primer is: GATCCTCGAGGCTCGAGAGGCCTTGAATTC. The amplicon was purified and digested with XhoI before being ligated into the XhoI site of pCS2+. *pm298* encodes the ORF of *rax1* but does not include the 5’ UTR binding sites for Rax1.1 and Rax1.1 morpholinos. Therefore, when the *pm298* transcript is co-injected a *rax1*
morpholino, it can still undergo translation, generating a normal phenotype. The rescue clone generated (pm298) was confirmed by bi-directional sequencing (Robarts Research Institute London, Ontario).

**Morpholinos**

Two fluorescein-tagged morpholino oligonucleotides (MO) (directed against *rax1*) were designed and ordered from Gene Tools, LLC (Orlando, Florida). The first morpholino is Rax1.1 and its sequence targeting the 5' UTR *rax1* region is 5' CTTGAGGTGTTCAATCACGATGATT 3'. The second morpholino is Rax1.2 and its sequence is 5’ CTTTATCTGATCGTGCTTAGTAGTC 3’.

Three fluorescent morpholino controls were used to confirm specificity of the *rax1* morpholinos; these three are Rax1.1b CO, Rax1.2 CO, and a Fluorescence Standard Control. The Fluorescence Standard Control is a generic 3'-Carboxyfluoroscein morpholino designed by the manufacturer which controls for morpholino and fluorescein effects (5’ CCTCTTACCTCAGTTACAATTATA 3’). The sequence for the Rax1.1b CO morpholino is: 5’ CTTGAGCTCTATCATGACCATCATT 3’. The sequence for the Rax1.2 CO morpholino is: 5’ CTTTATGTCATCGTCCTAACTAGTC 3’.

**Microinjection**

All injections made into the fertilized embryos were performed using a Drummond nano-injector. Whether it was a morpholino or mRNA being injected, the volume injected into each embryo was constant at 4.6nL and was injected into the animal pole of the embryos at either the 1-cell stage, or the 2-cell stage unilaterally,
targeting one of the blastomeres. During injection, the embryos were submerged in 2% Ficoll-400 and 0.3x MBS to allow them to heal. They were placed in the incubator at 12°C for a minimum of 60 minutes before transferring them to 0.1x MBS solution. Replacing the 0.1x MBS twice a day was crucial for embryos health. The concentration for the majority of morpholino injections was 10ng; rescue experiments were performed using 50pg transcription factor RNA and 150pg of GFP RNA as a tracer.

**Whole-mount *in situ* hybridization**

*In situ* hybridization techniques were performed essentially as per Smith and Harland (1991) using digoxigenin labeled probes synthesized by previous graduate students. Post *in situ* hybridization, images were captured using a camera attached to a Leica N2FL III stereoscope and Northern Eclipse software. See **APPENDIX B** for full protocol.
Results

*Rax1* plays a crucial role in eye development. *Six3* expression is independent of *rax1* at early-to-late stages of eye development.

In order to confirm the role of *rax1*, the translation of *rax1* mRNA was knocked down through morpholino injections. Several concentrations were tested for two *rax1* morpholinos, *Rax1.1* and *Rax1.2*. To control for off-target effects, morpholino specificity was tested by: 1) assessing if the same phenotype was achievable using two different morpholino target sequences; 2) determining if low concentrations of the two morpholinos synergized to mimic the phenotype of higher concentrations of either alone; 3) mismatch morpholino controls produced no phenotypic effects; 4) a commercially designed fluorescent control morpholino with no similarity to *Xenopus* sequences proved innocuous. Data characterizing the phenotypes displayed from *rax1* morpholinos, as well as controls, was collected (Tables 2 and 3). Based upon images in Figure 2.1 (A-C), we see that the knockdown of *rax1* causes an outcome where the eye produced is a *small eye* phenotype (B). At higher concentrations we see that the dorsoventral patterning of the eye, as well as the eye itself, is abolished (C).

*Rax1* morpholino was injected unilaterally into *Xenopus* embryos, allowing the un-injected side to serve as a control. Following a series of riboprobe *in situ* hybridization experiments where a *six3* probe was used, it appeared that the *six3* expression domain is unaffected by *rax1* being knocked down at earlier stages and slightly later stages of eye development (Figure 2.1: D-F). This solidifies the proposed model where *rax1* is downstream of *six3*. 
Table 2: Effect of rax1.1 morpholino mediated knockdown with percentages of phenotypes observed for different morpholino concentrations injected.

Morpholino specificity of rax1 morpholinos and rax1 control morpholinos tested at an optimal MO dose. Rescue experiments that combine pax6 mRNA with rax1 MO, six3 mRNA with rax1 MO, and pm298 mRNA with rax1 MO. All rescues included GFP mRNA as a tracer. Phenotypic consequences can compound and so percentages sum to more than 100%. All embryos were unilaterally injected and grown to stage 37/38 for examination.

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<td>1b CO MO (10ng)</td>
<td>1b CO MO (20ng)</td>
<td>1 5ng</td>
<td>1 10ng</td>
<td>1 15ng</td>
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<td>31%</td>
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Table 3: Effect of *rax1.2* morpholino mediated knockdown with percentages of phenotypes observed for different morpholino concentrations injected.

Morpholino specificity of *rax1* morpholinos and *rax1* control morpholinos tested at an optimal MO dose. All embryos were unilaterally injected and grown to stage 37/38 for examination.

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<tr>
<th>Morpholino Category</th>
<th>Fluor. Std. CO (10ng)</th>
<th>Rax1.2 CO MO (10ng)</th>
<th>Rax1.2 CO MO (20ng)</th>
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<th>Rax1.2 10ng</th>
<th>Rax1.2 15ng</th>
<th>Rax1.2 20ng</th>
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<td>6%</td>
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<td>35%</td>
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<td>3%</td>
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Figure 2.1: *Rax1* expression is important for normal eye morphology. *Six3* expression and maintenance is independent of *rax1* activity

A-C) Embryos were examined at stage 37/38 of development. When injected with 5ng of *rax1* morpholino, retinal development is only slightly affected as seen by the development of the retina (A). Increasing the concentration to 10ng shows a *small-eye* phenotype (B). At 20ng morpholino concentration we see abnormal dorsoventral patterning and an almost complete loss of retinal development (C).

D-F) Whole-mount *in situ* hybridization of *rax1*-MO injected embryos probed with *six3*. Three stages were examined: 14 (D), 19 (E), and 27 (F). The expression domain of *six3* is unaffected by *rax1* mediated knockdown at all three stages. White arrows in images indicate the injected side of the embryo and are highlighting the expression pattern of the injected side, specifically the eye field region. The un-injected side of the embryo is opposite of the dashed line and is showing normal expression of *six3*. 
*Pax6* is upstream of *rax1* but downstream of *six3*. *Pax6* is affected by *rax1* knockdown at a later stage in development.

After unilateral injections of *rax1* morpholino, embryos at stages 14, 19, and 27 were collected and examined by whole-mount *in situ* hybridization with a *pax6* probe. In *Figure 2.2 A* and *B*, (stages 14 and 19, respectively) *pax6* expression domains appear to be normal when compared to the un-injected (control) side. However, at later stages, in the eye field domain of *pax6* was slightly reduced (*Figure 2.2 C*). This suggests that late stage *pax6* is dependent on *rax1* expression for continued activation and maintenance. It also shows that *rax1* indirect-deficit alters the size and number of cells in which *pax6* expresses.
Figure 2.2: *Pax6* expression could be indirectly affected by *rax1* during later stages of eye development

**A-C)** Whole-mount *in situ* hybridization of *rax1*-MO injected embryos probed with *pax6*. Three stages were examined: 14 (A), 19 (B), and 27 (C). The expression domain of *pax6* is unaffected by *rax1* mediated knockdown at earlier stages of development (A,B). However, at stage 27 (C) the expression domain of *pax6* is slightly reduced where the arrow is pointing. Arrows in images indicate the injected side of the embryo and are highlighting the expression pattern of the injected side, specifically the eye field region. The un-injected side of the embryo is opposite of the dashed line and is showing normal expression of *pax6*. 
Rax1 regulates eye marker genes further downstream: knocking down rax1 results in decreased expression pattern for foxe3 (lens1)

Rax1 is a key marker in the early stages of eye development as it is expressed throughout the anterior neural plate, as well as expressing in the optic vesicles during later stages (Gestri et. al., 2005). Rax1 is crucial for specification at early stages of development which is followed by proliferation of the retinal progenitor cells (Andreazzoli et al., 1999). Foxe3 (commonly known as lens1) promotes proliferation and sustains an undifferentiated state prior to lens specification. After specification, foxe3 limits expression from the presumptive lens ectoderm to the lens placode and then to the epithelium of the differentiating lens.

Following unilateral rax1 morpholino injection, the expression pattern for foxe3 appeared to be completely abolished in the presumptive lens region (Figure 2.3 A). Furthermore, when rax1 mRNA was injected there was an increase in foxe3 expression (Figure 2.3 B). This relationship is better understood when pm298 is co-injected with rax1 morpholino. Due to the absence of rax1 morpholino binding sites in pm298, rax1 mRNA is available for translation. Consequently, foxe3 expression is rescued and even slightly over-expressed (Figure 2.3 C). Interestingly, this is not the case when either pax6 or six3 mRNA is co-injected with rax1 morpholino. Foxe3 expression remains highly perturbed (Figure 2.3 D,E). However, pax6 and six3 still rescue eye morphology as a whole according to our findings in Table 2.
Figure 2.3: *Rax1* alters expression of *foxe3* during eye development, specifically in the lens

A) Whole-mount *in situ* hybridization of *rax1*-MO injected embryos probed with *foxe3* at stage 27 of development. The expression domain of *foxe3* is clearly missing in the injected side by *rax1* mediated knockdown, specifically where the lens placode would normally develop (indicated by smaller white arrow).

B) Whole-mount *in situ* hybridization of a stage 27 embryo injected with *rax1* mRNA and probed with *foxe3*. The eye field indicated by the smaller white arrow shows over-expression of *foxe3*.

C-E) Whole-mount *in situ* hybridization of rescue experiments probing for *foxe3*. C) Unilateral co-injection of *pm298* mRNA and *rax1*-MO shows that the expression domain of *foxe3* is restored and slightly over-expressed as indicated by the white arrow. This, however, is not the case when *pax6* or *six3* are co-injected with *rax1*-MO (D,E). In both D and E, we see that *foxe3* expression is not rescued at all in the eye field region (smaller white arrow).

White arrows in images indicate the injected side of the embryo and are highlighting the expression pattern of the treated side. Black arrows indicate an un-injected side of the embryo, opposite of the dashed line, showing normal expression pattern (untreated) of *foxe3*. 
Rax1 has an effect on pitx3 and γ-crystallin expression patterns: six3, pax6 and rax1 are interacting to regulate downstream genes

Pitx3 is expressed in the presumptive lens ectoderm and is a major player in the lens induction process (Khosrowashahian et al., 2005). γ-crystallins are expressed in the developing lens vesicle and show high levels of expression throughout the differentiation of the lens fibre (Van Leen et al., 1987). Both genes show highest expression around stages 24-27 of development. When rax1 was knocked down, we found that the expression domains for both pitx3 and γ-crystallin were completely abrogated (Figure 2.4 A; Figure 2.5 A). When rax1 mRNA is injected, the result is slight over-expression of pitx3 and γ-crystallin in the injected side (Figure 2.4 B; Figure 2.5 B).

Furthermore, rescue experiments are imperative to clarify the interaction of rax1, six3, and pax6 on these downstream genes. Co-injecting pm298 mRNA with rax1-MO successfully rescues both pitx3 and γ-crystallin expression (Figure 2.4 C; Figure 2.5 C). Pax6 has been proven to be necessary for normal expression of both genes. Pax6 mRNA was able to restore pitx3 and γ-crystallin expression patterns almost completely (Figure 2.4 D; Figure 2.5 D). The same outcome was observed when six3 mRNA was co-injected with rax1-MO, showing almost completely rescued expression domains in the lens for both pitx3 and γ-crystallin (Figure 2.4 E; Figure 2.5 E).
**Figure 2.4: Rax1 has an affect on pitx3 expression pattern**

**A)** Whole-mount *in situ* hybridization of *rax1*-MO injected embryos probed with *pitx3* at stage 27 of development. The expression domain of *pitx3* is abolished in the injected side by *rax1* mediated knockdown, specifically in the lens region of the eye field (smaller white arrow).

**B)** Whole-mount *in situ* hybridization of a stage 27 embryo injected with *rax1* mRNA and probed with *pitx3*. The injected side shows slight over-expression of *pitx3* in the eye field region, as indicated by the smaller white arrow.

**C-E)** Whole-mount *in situ* hybridization of rescue experiments probing for *pitx3*. **C)** Unilateral co-injection of *pm298* mRNA and *rax1*-MO shows that the expression domain of *pitx3* is restored and slightly over-expressed in the entire eye field (smaller white arrow). *Pitx3* expression in the eye field is somewhat restored when *pax6* or *six3* are co-injected with *rax1*-MO (**D,E**). In both **D** and **E**, we see that *pitx3* expression is present in the eye field (white arrows) but not to normal amounts (black arrows).

White arrows in images indicate the injected side of the embryo and are highlighting the expression pattern of the treated side. Black arrows indicate an un-injected side of the embryo, opposite of the dashed line, showing normal expression pattern (untreated) of *pitx3*.
Figure 2.5: *Rax1* knockdown disrupts $\gamma$-crystallin expression

**A)** Whole-mount *in situ* hybridization of *rax1*-MO injected embryos probed with $\gamma$-crystallin at stage 27 of development. The expression domain of $\gamma$-crystallin is abolished in the injected side, specifically in the lens (smaller white arrow) by *rax1* mediated knockdown, confirming the absence of differentiating lens.

**B)** Whole-mount *in situ* hybridization of an embryo injected with *rax1* mRNA and probed with $\gamma$-crystallin. The smaller white arrow shows slight over-expression of $\gamma$-crystallin in lens compared to the control side (black arrow).

**C-E)** Whole-mount *in situ* hybridization of rescue experiments probing for $\gamma$-crystallin. **C)** Unilateral co-injection of *pm298* mRNA and *rax1*-MO shows that the expression domain of $\gamma$-crystallin is restored and slightly over-expressed in the eye (smaller white arrow). This is also the case for *six3* when co-injected with *rax1*-MO (white arrow) (**D**). In **E**, we see that $\gamma$-crystallin expression is completely rescued in lens by *pax6* mRNA (smaller white arrow).

White arrows in images indicate the injected side of the embryo and are highlighting the expression pattern of the treated side. Black arrows indicate an un-injected side of the embryo, opposite of the dashed line, showing normal expression pattern (untreated) of $\gamma$-crystallin.
Effects on notable eye marker genes after *rax1* knockdown

I looked at the effect of *rax1* on other eye marker genes, namely *otx2* and *mafA*, through *rax1* morpholino-mediated knockdown experiments. Following *rax1* translational knockdown, *otx2* expression is slightly reduced in the anterior neural plate and the eye field (Figure 2.6 B). Knocking down *rax1* shows the same effect on *mafA* by reducing its expression in the lens (Figure 2.6 A). *mafA* can directly induce the expression of γ-crystallin (Kataoka et. al., 2007) which is also affected by *rax1* knockdown. I also unilaterally injected a *rax1* control morpholino for a visual comparison of the effects on both eye marker genes. The mismatch control morpholino produced no affect on *otx2* or *mafA*. 
**Figure 2.6:** *Rax1* knockdown effects observed for *otx2* and *mafA* eye marker genes

*Rax1* morpholino was unilaterally injected in *Xenopus* embryos to study the effects that *rax1* knockdown has on two notable eye marker genes. A *rax1* control morpholino was also unilaterally injected to serve as another control, showing no effect on expression patterns in the injected side. Black arrows are used to show the injected side of the embryo and highlight any changes in expression levels. The expression of *mafA* in lens (A) is slightly reduced as indicated by the black arrow. The expression of *otx2* in the anterior-dorsal region and eye field is abolished (B) as highlighted by the black arrow.

A & A': *MafA*

B & B': *Otx2*

*rax1*-MO: *Rax1* morpholino

*rax1* CO MO: *Rax1* control morpholino
Discussion

Many studies have examined the roles of Eye Field Transcription Factors (EFTF's), specifically the relationship of *rax1* with upstream and downstream eye field markers (Zuber et. al., 2003; Muranishi et. al., 2012; Andreazzoli et. al., 2003; Giudetti et. al., 2014; Kamijyo et. al., 2015). Although extensive research has been done on these genes, their interaction with one another and hierarchal relationships are still unclear. I am proposing an alternative model based on results found by past and present work outlined in my thesis (Figure 3.1). Ultimately, this will help us understand the inductive patterns of the main transcription factors associated with eye development and put to bed debate concerning the hierarchal order of these genes.
Figure 3.1: A model summarizing the eye field genes and their functional roles in eye development, derived from studies in our lab and relevant literature. This model demonstrates the complexity of signalling cascades involved in eye development and the possible pathways in which these genes interact. Dashed lines signify a potential feedback role that is suggested by the data but not yet to be confirmed.
Rax1 expression is necessary for normal eye development

Understanding the molecular pathways controlled by rax1 and its interaction with other eye field transcription factors is necessary to unraveling the basic mechanisms of eye development as a whole. Previous studies have highlighted the importance of rax1 in different organisms. Loss of the Rax genes in mice resulted in reduced optic vesicles and also provided abnormal forebrain phenotypes (Muranishi et. al., 2012). In humans, RAX gene mutations are associated with microphthalmia and anophthalmia (Bailey et. al., 2004). Furthermore, in Xenopus and zebrafish, rax1 over-expression causes extensive proliferation of retinal cells (Terada et. al., 2006). Conversely, rax1 was suppressed via morpholino-mediated knockdown in zebrafish (Nelson et. al., 2009). Moreover, Terada et. al. (2006) found that rax1 morpholino-mediated knockdown experiments reveal a reduced eye size in Xenopus embryos; this is confirmed by work done in our lab.

Using antisense morpholino oligonucleotides directed against the rax1 translation start site, I determined that knocking down rax1 expression in Xenopus embryos reduces the size of the eye, characterized as small-eye phenotype. Lower concentrations of morpholino produce minor effects on eye development, with some dorsoventral abnormalities. Increasing morpholino concentrations, however, result in significant reduction of the eye field and at high enough concentrations can cause total loss of eye structures. My results are consistent with previous findings mentioned above.
and emphasize the significance of \textit{rax1} in eye development (Andreazzoli et. al., 2003; Terada et. al., 2006).

The specificity of the morpholinos was confirmed and can be viewed in Tables \textbf{2} and \textbf{3}. The two experimental morpholinos Rax1.1 and Rax1.2 were injected unilaterally in 2-cell embryos and examined at stage 37/38 of development. Both morpholinos produced the same phenotype consistently when injected at a range of concentrations. Rax1.1b mismatch control and Rax1.2 mismatch control were injected at two dosages, 10ng and 20ng. Both controls produced no effects on eye morphology, suggesting the phenotypic effects produced by Rax1.1 and Rax1.2 morpholinos are not because of off-target or toxic effects. To further confirm specificity of the morpholinos, a low dose synergistic injection was conducted. Rax1.1 and Rax1.2 were co-injected at lower doses along with a Fluorescent Standard Control morpholino that does not carry a sequence found in \textit{Xenopus}. The synergistic injection provided the same results as Rax1.1 and Rax1.2 morpholinos produced when injected at a higher dose individually. This suggests the two morpholinos are specific in blocking the translation of \textit{rax1} to produce the phenotypes observed when each morpholino is unilaterally injected.

Three different rescue experiments were carried out: 1) mRNA of an anti-\textit{rax1} morpholino plasmid named \textit{pm298} was co-injected with the \textit{rax1} morpholino; 2) \textit{pax6} mRNA was co-injected with \textit{rax1} morpholino; 3) \textit{six3} mRNA was co-injected with \textit{rax1} morpholino. Examining the phenotype of unilateral 2-cell injected embryos at stages 37/38 exhibited normal eye morphology (Table \textbf{2}). Therefore, the eye developed
normally which confirms the specificity of the morpholinos since the mRNA injected was able to translate properly.

**Rax1 is downstream of six3 and does not directly regulate six3 expression in early eye development**

Both *rax1* and *six3* are responsible for stimulating proliferation (Zuber et. al., 2003). Neural differentiation begins in the posterior neuro-ectoderm following gastrulation. Eye field progenitor cells continuously proliferate in order to reach high populations sufficient to produce an eye. Pro-neural differentiation genes are inhibited in the presumptive eye field by *rax1* expression which is dependent on *pax6* and *six3* activity (Gestri et. al., 2005; current study). *Six3* is necessary in early development for the proliferation of cells in the anterior neural plate by promoting *rax1* enhancement (Andreazzoli et. al., 2003). Previous work done in our lab has shown that *six3* knockdown results in loss of *rax1* expression in the anterior neural plate region (Sachani, 2011). This is most likely via an indirect route to *pax6* levels being reduced.

To confirm this hierarchy and further understand *rax1'*s effect on the other markers, we knocked down *rax1* and examined the outcome it has on *six3* expression. Three stages of development were observed: stage 14, 19, and 27. Our results show that *six3* expression is unaffected at all three stages when *rax1* is knocked down. This contradicts results found by Zuber et. al., (2003) who were working with cultured animal cap explants - ectopic cultures of ectodermal tissues removed during gastrulation and
analyzed at stages equivalent to neurula. *Rax1* enhances proliferation and when over-expressed in animal caps produces high numbers of optic progenitor cells, leading to greater expression of *six3* (Zuber et. al., 2003). However, there are more constraints in whole embryos and ventral factors are most likely playing a dominant role. Our data suggests that *pax6* plays an intermediary role between *rax1* and *six3* (Figure 3.2).

**Figure 3.2:** A summary model of the genes involved in eye development highlighting the placement of *rax1* and *six3* in the cascade (red). *Rax1* is downstream of *six3* based on my findings and those of Sachani (2011). Dashed lines signify a potential feedback role that is suggested by the data but not yet to be confirmed.
Rax1 regulates pax6 expression only at later stages of eye development

Pax6 mRNA over-expression has led to expansion of the expression domain for rax1 in the anterior neural plate, specifically in the eye field (Sachani, 2011). This finding implies that pax6 is working either directly or via other key factors to enhance rax1 expression (current study) and is confirmed by pax6 occupancy of rax1 promoter (Thakurela et. al., 2016). Co-injecting six3 morpholino with pax6 mRNA results in almost complete rescue of rax1 expression, telling us that six3 is upstream of pax6, and that pax6 is in turn likely upstream of rax1. Therefore, the real inhibition by six3 knockdown is likely due to the secondary effect of pax6 expression being reduced. It is very likely that pax6 has a role in activating rax1 early in development in order to promote proliferation (Blixt et. al., 2000; current study).

These findings unambiguously situate pax6 between six3 and rax1. Interestingly, rax1 has a regulatory role on pax6. When rax1 is knocked down at stages 14 and 19 there is no apparent affect, however at stage 27 of development, the expression domain of pax6 appears to be slightly reduced. It is possible that rax1 regulates pax6 in late embryogenesis directly or via intermediaries such as otx2 (Thakurela et. al., 2016). Alternatively, rax1 mis-regulation may affect the number of cells in presumptive organs indirectly, altering the domains of transcription factors such as pax6 (Current study). Since rax1, six3, and pax6 have overlapping expression domains, it is likely that pax6 is relying on rax1 activity during later stages in order for pax6 to continue regulating other downstream genes - namely, pitx3, foxe3, γ-crystallin, and mafA. Therefore, rax1, six3,
and *pax6* may refine and support each other’s expression domains differentially in a stage dependent manner. It is likely there are other players and this triad is but a part of a more complicated network where other genes cross-regulate each other (Figure 3.3).

**Figure 3.3**: A summary model of the genes involved in eye development highlighting the placement of *rax1* and *pax6* in the cascade (red). *Rax1* is downstream of *pax6* based on my findings and those of Sachani (2011). *Pax6* appears to be intermediary of *six3* and *rax1*. Dashed lines signify a potential feedback role that is suggested by the data but not yet to be confirmed.
Rax1 is necessary for Foxe3 activation

Foxe3 is normally expressed in the lens. Although Foxe3 is not involved in lens differentiation per se, it initiates a lens forming bias in the presumptive lens ectoderm (Kenyon et. al., 1999). Furthermore, Foxe3 induces proliferation of lens progenitor cells which go through differentiation in the posterior region of the lens (Kenyon et. al., 1999). Previous work in our lab has shown pax6 and six3 knockdown experiments result in complete loss of Foxe3 expression in the lens (Sachani, 2011). Conversely, pax6 may play a role in activating Foxe3 (Thakurela et. al., 2016). Our model situates rax1 and Foxe3 downstream of both six3 and pax6; therefore, Foxe3 expression is driven by pax6 and six3 either with rax1 as an intermediary, or directly via a combination of indirect routes. It is possible that one indirect route involves otx2 and pax6. Ogino et. al. (2008) suggests pax6 is controlling Foxe3 expression indirectly by regulation of otx2. Alternatively, pax6 could be involved in combinatorial activation with pitx3 which has been found to be a direct regulator of Foxe3 (Ahmad et. al., 2013).

When rax1 is knocked down via morpholino-mediated translation inhibition, a complete loss of Foxe3 expression is the outcome. Martinez-de Luna et. al., (2010) has found that the rax1 promoter/enhancer possesses binding sites for sox, its, and pou transcription factors. In isolation none of these is sufficient to recapitulate rax1 expression domain. A further element is required, namely forked binding element (FBE) which suggests that Foxe3, a forkhead box transcription factor, may be able to regulate rax1. When the forked binding element is removed, rax1 expression is lost (Martinez-de
Luna et al., 2010). This suggest that there must be some feedback mechanism between \textit{rax1} and \textit{foxe3}, where both regulate each others’ expression. A suite of rescue experiments were performed to confirm this relationship. When \textit{pm298} (an anti-\textit{rax1} morpholino plasmid) mRNA was co-injected with \textit{rax1} morpholino, the expression of \textit{foxe3} was restored in the injected side, and more so, was slightly over-expressed. Similarly, when \textit{rax1} is over-expressed via \textit{rax1} mRNA injections, the expression of \textit{foxe3} in lens is slightly enhanced. However, when either \textit{pax6} or \textit{six3} mRNA is co-injected with \textit{rax1} morpholino, \textit{foxe3} expression remains lost in both cases. These findings suggest that there is a direct regulatory role between \textit{rax1} and \textit{foxe3} since \textit{pax6} and \textit{six3} are not able to rescue the \textit{rax1}-MO affect. Although \textit{rax1} is appears to be driving \textit{foxe3} expression, the two genes may have a more complex relationship with parallel factors contributing to their regulatory roles (Figure 3.4); other players such as \textit{otx2}, \textit{pax6} and \textit{pitx3} are known regulators of \textit{foxe3}, either directly or indirectly (Ahmad et al., 2013; Ogino et al., 2008; Thakurela et al., 2016).
**Figure 3.4:** A summary model of the genes involved in eye development highlighting the interactions between *otx2*, *foxe3*, *pitx3*, *pax6* and *rax1* (red). *Rax1* has an affect on *foxe3* and appears to regulate *foxe3* based on my results. However, it is still unclear if *foxe3* regulates *rax1*. *Pax6*, *pitx3*, and *otx2* all have a role in regulating *foxe3* (Ahmad et. al., 2013; Ogino et. al., 2008; Thakurela et. al., 2016). Dashed lines signify a potential feedback role that is suggested by the data but not yet to be confirmed.
**Rax1 can regulate pitx3 expression**

Previous studies have highlighted the significance of *pitx3* in *Xenopus* eye development, especially its effect on *pax6*. When *pitx3* is over-expressed, the *pax6* expression domain is expanded implying a role for *pitx3* on *pax6* expression in the lens (Khosrowshahian et al., 2005). *Pitx3* knockdown experiments also revealed down-regulation of *pax6* expression. A similar outcome was observed for *rax1, foxe3*, and *otx2* expression patterns when *pitx3* was knocked down (Khosrowshahian et al., 2005). Conversely, *pax6* induces the expression of *pitx3* in animal cap explants, but *pitx3* is unable to activate *pax6* (Khosrowshahian et al., 2005). Our lab found that knocking down *pax6* resulted in *pitx3* expression being diminished in the lens ectoderm. This suggests that *pax6* has a direct regulatory role on *pitx3* (Sachani, 2011; *Figure 3.5*). However, it does not tell us about the regulatory relationship between *pitx3* and *rax1*.

*Rax1* knockdown experiments resulted in loss of *pitx3* expression in the lens placode. Rescue experiments give better insight on the interactive roles indicated between *rax1, pitx3, pax6*, and *six3*. When *pm298* mRNA and *rax1* morpholino are co-injected, *pitx3* expression is completely restored. Similarly, when *pax6* or *six3* mRNA are co-injected with *rax1* morpholino, *pitx3* expression is restored close to normal. These results suggest that *rax1* and *pitx3* are regulating each other’s expression since it *rax1* is able to inhibit and rescue *pitx3* expression and *pitx3* reduces *rax1* expression (Khosrowshahian et al., 2005; Current study). Furthermore, *pax6* and *six3* have a role in regulating *pitx3* expression as they are both able to rescue *pitx3* expression when *rax1*
is knocked down. My findings confirm that \textit{pitx3} is downstream of \textit{pax6} and \textit{six3} but has a direct regulatory relationship with \textit{pax6} and \textit{rax1} (Figure 3.5).

\textbf{Figure 3.5}: A summary model of the genes involved in eye development highlighting the interactions between \textit{pitx3}, \textit{pax6} and \textit{rax1} (red). \textit{Rax1} and \textit{pitx3} appear to have a regulatory relationship based on my results and previous findings in our lab (Khosrowshahian et al., 2005). \textit{Pax6} also has a direct relationship with \textit{pitx3} where both are able to regulate each other (Khosrowshahian et al., 2005; Sachani, 2011). Dashed lines signify a potential feedback role that is suggested by the data but not yet to be confirmed.
*Rax1* has an indirect affect on \( \gamma\text{-crystallin} \) and *mafA* in eye development

*MafA* and \( \gamma\text{-crystallin} \) are normally expressed in the lens of *Xenopus*. *Pax6* knockdown results in complete loss of the lens specific differentiation target \( \gamma\text{-crystallin} \) (Shimada et. al., 2003; Sachani, 2011). Furthermore, in *rax1* morphant *Xenopus*, \( \gamma\text{-crystallin} \) expression is down-regulated. However, there are likely parallel factors causing this effect. Due to *rax1’s* ability to regulate *pax6*, mis-expression of *pax6* caused by *rax1* knockdown could be the cause of *mafA* inhibition (Figure 3.6).

Activation of *mafA* by *pax6* is crucial for \( \gamma\text{-crystallin} \) expression (Takeuchi et. al., 2009). When *pax6* is knocked down, *mafA* is totally abolished in the lens which results in the same outcome for \( \gamma\text{-crystallin} \). Similarly, down-regulation of *rax1* reduces *mafA* expression significantly (current study). In parallel, *rax1* mediated activation of *foxe3* regulates \( \gamma\text{-crystallin} \) expression levels. When *foxe3* is over-expressed lens differentiation is inhibited which is reflected by a complete loss of \( \gamma\text{-crystallin} \) expression. This is most likely the case when *rax1* is over-expressed, as we see little \( \gamma\text{-crystallin} \) expression in the lens.

Clearly there are competing pathways in the regulation of at least some eye field transcription factors: impaired function of one player in the signal cascade can promote and repress downstream targets via different indirect routes. The end result of this combinatorial mix of activation/inhibition routes is summed and spatially delimited by the
intermediaries that are present, their concentrations, and the number of cells (size of field) available. For example, since rax1 has an affect on γ-crystallin and mafA, our results imply overlapping and competitive signalling cascades to the regulation of downstream genes. *Xenopus* embryos unilaterally injected with a cocktail of *pm298* mRNA and rax1 morpholino produced a close to normal expression pattern of γ-crystallin. Since rax1 is rescued, foxe3 is activated and in turn suppresses γ-crystallin. However, because pax6 is functioning normally too, it activates mafA which is critical for γ-crystallin expression. This is seen when pax6 mRNA and rax1 morpholino are co-injected, producing a normal expression domain for γ-crystallin. The same outcome is observed when six3 mRNA rescues γ-crystallin expression against rax1 morpholino. These findings agree with the assumed hierarchy placing six3 and pax6 upstream of rax1 and confirm their positioning with respect to direct and indirect action upon downstream genes (Figure 3.6).
Figure 3.6: A summary model of the genes involved in eye development highlighting the placement of rax1, mafA, and γ-crystallin (red). My results suggest that rax1 has an indirect effect on mafA and γ-crystallin which could be due to combinatorial effects with pax6 and pitx3. Dashed lines signify a potential feedback role that is suggested by the data but not yet to be confirmed.
**Rax1 and otx2 are required to activate one another**

*Rax1* and *otx2* are believed to have some interaction in eye development. Past studies using *in situ* hybridization experiments revealed that the two genes have different expression domains at early stages of eye development, suggesting any interplay between them must be occurring at later stages (Andreazzoli et al., 1999). It was also found that *rax1* over-expression inhibited *otx2* which suggests that *otx2* is not required for eye field specification at early stages of development, but is necessary for the specification of the anterior neural domains (Andreazzoli et al., 1999).

More recently, *rax1* has been identified as having an ultra-conserved element (UCE) that allows members of the *otx* family to bind to its regulatory regions (Martinez-de Luna et. al., 2010). Zuber et al., (2003) suggested that the expression domains of *otx2* and *rax1* are mutually exclusive and proposed an inhibition cycle in his model where *otx2* and *rax1* are exclusionary. However, Danno and colleagues (2008) showed that *otx2* and *sox2* are required to bind cooperatively to *rax1*’s ultra-conserved element for *rax1* activation. Furthermore, these studies reveal an increase in *rax1* expression when *otx2* is over-expressed, but found that *sox2* is required for *otx2*-induced *rax* expression *in vivo*. Therefore, *otx2* and *sox2* interact directly with one another and synergistically activate *rax1* expression (Danno et. al., 2008). Interestingly, more recent studies have revealed that *rax1* is essential for *otx2* activation in embryonic retina. *Otx2* has *cis*-regulatory region named *EELPOT* which is an enhancer that contains consensus sites for homeodomain binding (Muranishi et. al., 2011). Therefore, *rax1*
activates $otx2$ through the $EELPOT$ enhancer. I found that knocking down $rax1$ reduced $otx2$ expression and decreased the diameter of expression in the eye field. This result could be direct or indirect through reduction of eye field competent cells. However, $six3$ knockdown experiments proved to have a greater impact on $otx2$ by causing its expression to diminish completely at both early and later stages of development (Sachani, 2011). These findings agree with our proposed model which displays a co-dependant relationship for $otx2$ and $six3$, as well as a direct regulatory relationship between $otx2$ and $rax1$ (Figure 3.7).

**Figure 3.7:** A summary model of the genes involved in eye development highlighting the interaction between $rax1$, $otx2$, and $six3$ (red). $Rax1$ and $otx2$ activate each other based on my results along, with relevant literature discussing the relationship between $rax1$ and $otx2$ (Danno et. al., 2008; Muranishi et. al., 2011; Martinez-de Luna et. al., 2010). $Six3$ and $otx2$ also appear to have a co-dependant relationship (Sachani, 2011). Dashed lines signify a potential feedback role that is suggested by the data but not yet to be confirmed.
Summary: Eye field signalling model for *Xenopus* eye development

Zuber and colleague’s (2003) sophisticated model suggested *rax1* is upstream of *six3* and *pax6*. RT-PCR assays on animal caps (cultured ectodermal explants) has helped establish this model, but this technique does not distinguish eye from general neural effects. It is also inconsistent with the timing of *rax1*, *pax6*, and *six3* expression defined by Zuber using whole embryos as source material. However, my work along with previous work done in this lab on *six3* and *pax6*, proposes *six3* and *pax6* to be upstream of *rax1*. This was determined through morpholino mediated knockdown experiments that cause loss of function, as well as through rescue experiments which involve a *rax1* morpholino injected with a anti-*rax1* morpholino plasmid (*pm298*), *pax6* or *six3*. The main difference is that my work utilizes whole embryos which integrates proliferative and inductive effects, something that cannot be faithfully recapitulated using animal cap assays.

I have also carried out over-expression experiments which are crucial in characterizing genetic networks. However, parallel pathways are likely missed using this technology and what is going on *in vivo* may be misrepresented. If *rax1* over-expression enhances proliferation, there will be an increase in eye progenitor cells as a result. Therefore, an indirect effect might be comparable increase in expression of eye field markers. RT-PCR assays of animal caps serve as sensitive screens that exaggerate these relationships (Zuber et. al., 2003). Knockdown and rescue experiments provide new information: *rax1* inactivation has no affect on *six3* but slightly reduces *pax6*
expression only at later stages. Under these circumstances, \textit{pax6} and \textit{six3} cannot rescue \textit{foxe3} expression, but appear to rescue the eye development process by later stages. It is evident that \textit{rax1}, \textit{six3}, and \textit{pax6} are part of a more complicated network where genes cross-regulate one another, rather than a simple linear signalling cascade. This has allowed me to develop an alternative proposed model (\textbf{Figure 3.1}).
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CHAPTER 3

Conclusion and Future Directions

Eye field transcription factors and eye field markers are part of a complex, interconnected cascade that requires genes to turn on and off at the perfect time for eye development to occur flawlessly. *In situ* hybridization following knockdown, over-expression, and rescue experiments have shown us that the genes involved in eye development are operating in a delicate, combinatorial, and nuanced manner to cross-regulate and control eye morphogenesis.

My work and work already conducted in our lab has exhibited the ability of eye field markers to regulate one another both *in vivo* and *in vitro* (Khosrowshahian et al., 2005; Sachani, 2011; Current study). With respect to *rax1*, *pax6*, and *six3*, we see an overlapping expression domain amongst the eye field transcription factors that allows each one to regulate the other in a embryonic stage-specific manner. *Six3* sets the cascade for eye development and is required to activate *pax6*. *Pax6* activation helps maintain *six3* and *rax1* expression levels and promotes proliferation at later stages.

During later stages, *rax1* regulates the number of cells *pax6* is expressing. There is a refining feedback mechanism between *rax1* and *pax6*. *Rax1* controls proliferation of the retinal progenitor cells where *six3* expresses (Current study). This suggests that the three transcription factors are differentially regulating each other during specific stages of development. My findings along with those of Sachani (2011) confirm the overlapping
domains of *rax1*, *six3*, and *pax6* and their regulatory relationship occurring via a stage-specific manner.

Further downstream, *rax1* is required to activate *foxe3* so that it can promote proliferation of lens progenitor cells. However, over-expression of *foxe3* results in ectodermal cells remaining in a proliferative state, causing inhibition of differentiation which leads to loss or delayed *crystallin* expression (Kenyon et. al., 1999). A *foxe3* promoter analysis could lead to a better understanding of *foxe3* interactions with other notable eye field. Currently, there are no studies that have found a *rax1* site within the *foxe3* promoter region. A reporter assay would reveal real-time *in vivo* translational activity of *foxe3* promoter. By using *rax1* (or *otx2, pax6, pitx3*) as a reporter gene on *foxe3* promoter region, we can determine if the reporter gene signals for translation of endogenous proteins driven from the same promoter.

It is clear that *rax1* has a big role to play in eye development, observed by the *small-eye* phenotype produced when it is down-regulated, but also its effect on other eye field markers. Although *rax1* down-regulation impairs *pitx3* expression, *pax6* can rescue *pitx3* which is important for lens induction and retinal development (Khosrowshashian et. al., 2005). In spite of the fact that *mafA* expression is slightly reduced by *rax1* knockdown, *pax6* has a regulatory role which supports the *mafA* activation that is essential for *γ-crystallin* expression (Takeuchi et. al., 2009). However, there is a possibility that *mafA* and *rax1* share a direct role in regulation. Since *mafA* is important in *crystallin* activation and regulation, future work should consider knocking
down mafA and examining the effect it has on rax1 in eye development. Furthermore, ChIP sequencing could reveal more about the protein-DNA interactions between mafA and rax1, as well as determine whether pax6 and pitx3 are involved in regulation. This will provide a better understanding of the relationship between rax1 and mafA.

Understanding eye development and the hierarchy involved requires more work to identify the direct or indirect roles between each eye field transcription factor and eye field marker. We have identified regulatory relationships between many of the genes involved in eye development, as outlined in our proposed model (Figure 3.1), but some interactions and effects remain a mystery. It would benefit our model to perform morpholino mediated-knockdown experiments on other genes such as xoptx2, foxe3, pax2 and sox2 to determine the effect they have on rax1, pax6 and six3, as well as other eye field markers. Over-expression and rescue experiments involving these genes would also tell us more about their interactions with one another. With respect to all the eye field genes listed in Figure 3.1, performing alternative techniques such as RNA sequencing (RNA-seq) and chromatin immunoprecipitation sequencing (ChIP-seq) could potentially reveal more about their regulatory roles both at the RNA, DNA and protein level. RNA-seq would allow for discovering, quantifying and profiling RNAs and would be useful in monitoring changes of gene expression at a given time. This would be very beneficial since the genes involved in eye development are working in a stage-specific manner. ChIP-seq, on the other hand, would be beneficial for identifying protein interactions with DNA-binding sites since most of the the eye field markers are transcription factors. The relationship between rax1 and foxe3 or between foxe3 and
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*otx2* or *pax6* is a great example where potential binding sites may be present and unveiling them would tell us more about the relationship that exists between these eye field genes. Furthermore, ChIP-seq could help identify whether the transcription factors involved in eye development are working alone or are interacting with other outside factors that could form complexes to activate transcription of lineage specific genes. This may lead to new information on transcription factor binding sites that were not previously identified.

Further promoter analysis is possible by conducting a gel mobility shift assay. Electrophoretic Mobility Shift Assay (EMSA) is a useful technology that evaluates the degree of specificity of protein binding to DNA probes. This technique would provide better insight on the protein-DNA binding interactions that already exist for the eye field transcription factors, and can be used to reveal any alternative binding sites for the other eye field markers. It would be extremely beneficial to understanding some of the interactions we’ve examined in our proposed model. A DNA pull-down assay is another strategy that can be employed to further study protein-DNA complexes. Pull-down assays selectively extract protein-DNA complexes from a sample and requires the use of a DNA probe labeled with a high affinity tag to examine the protein-DNA interaction. Lastly, it would be wise to make use of transgenic *Xenopus laevis* embryos to gain a better understanding of the function of genes in eye development and to study of the regulation of these genes *in vivo*. Transgenesis in frog embryos can be used to express wild-type and mutant forms of genes at distinct periods of development and in a specific location. More specifically, using GFP-tagged reporter genes that are driven by a wild-
type or mutant promoter could prove very beneficial in transgenic frogs as it would provide information on their activity during specific development stages in the area of interest via fluorescence activation. This would provide a more comprehensive understanding of the stage-specific manner that the eye field markers are turning on/off.

Making use of new and readily available technologies that produce loss of function effects, allow for *in vivo* live imaging, and study protein-DNA interactions will assist in identifying the key roles carried out by genes involved in the development of the eye.
References


APPENDICES

APPENDIX A

Plasmid map

pCS2+ vector cloned with rax1 insert lacking Rax1.1 and Rax1.2 morpholino binding sites, named pm298.
APPENDIX B

In Situ Hybridization Protocol

Solutions:

**TTw for 100 ml**
- Tris pH 7.5 1M 5 ml
- NaCl 4 M 5 ml
- Tween 20 1 ml

**MEMPFA for 40 ml** (Stocks and fresh solution store in fridge)
- MgSO\(_4\) 1M 40 ul
- MOPS pH 7.5 1M 4 ml
- EDTA pH 8.0 20 mM 4 ml
- Paraformaldehyde 8M 20 ml

**RNA Hyb Mix for 100 ml**
- Formamide 50 ml
- SSC 20X 25 ml
- RNA (bulk yeast) 0.1g in 2 ml 50% formamide
- Denhardts 100X 1 ml
- Tween 20 10% 1 ml
- EDTA 5 mM 1 ml
- Heparin\(^1\) 0.01 g

\(^1\)Heparin Sigma grade II porcine Sigma H-7005 or equivalent

**Denhardts 100X for 100 ml**
- Ficoll 400 2g
- PVP 2g
- BSA (Fraction V) 2g

**SSC 20X for 1 l**
- NaCl 175.3 g
- Na Citrate 88.2 g
- pH to 7.0 with NaOH

**MAB for 100 ml**
- Maleic Acid 1.16 g
- NaCl 0.88 g
- pH to 7.5 with NaOH

**APB for 40 ml** (optional – depends upon whether BM purple or NBT/BCIP used)
- Tris pH 9.5 1M 4 ml
**MgCl\textsubscript{2} 1M** 2 ml  
**NaCl 4M** 1 ml  
**Tween 20 10%** 400 ul

**PBT** for 100 ml (for double in situ)  
**PBS 10x** 10 ml  
**Tween 20 10%** 1 ml

**PBS 10 x for 1l**  
**NaCl** 74.97 g  
**Na\textsubscript{2}HPO\textsubscript{4}** 12.46 g  
**NaH\textsubscript{2}PO\textsubscript{4}** 4.8 g  
ph to 7.0 and autoclave

**General Comments**

1. The protocol cannot be accelerated or steps left out (I've tried....)
2. The greatest source of stress for the embryos is getting suctioned out of the vial. It seems that keeping them *just* covered with fluid during changes works best.
3. You have to be meticulous about maintaining an RNase-free environment, fluids, gloves, desk etc., until after the formamide-containing hybridization steps have been completed.
4. Change gloves after using the RNase.
5. **NEVER** dispose of the used hybridization buffer/probe solution. Probe can be reused dozens of times, and in fact gets better with use for the first few deployments. It is expensive to make so cherish it and store it fully labeled on the back of the freezer door.
6. Gently mix the antibody tube before use – they tend to settle.
7. For the colour reaction, if you are using Roche’s BM purple, there is no need to go through the APB buffer. You can go straight from MAB to 0.5 ml BM Purple. This is one instance where it pays to remove all liquid between refills.
8. Bleaching is done under fluorescent lamps and removes the brown pigmentation over the back, and in the eye. It usually takes between 1 and 2 hours – monitor it. Magenta phos. pigmentation bleaches from dark purple to bright red, but the other substrates don’t change much at all over the span of time necessary to bleach melanocytes.
Day 1

MeOH  70%  2 min
MeOH  50%  2 min
MeOH  25%  2 min
TTw    2 min
TTw    2 min

Add 1 ul ProK/10ml TTw  5min

MEMPFA  20 min
TTw      5 min
TTw      5 min
TTw      5 min
RNA Hyb  10 min
RNA Hyb  1 hr 65°C
RNA Hyb and probe  o/n 65°C

Day 2

RNA Hyb  10 min 65°C
2 x SSC  20 min 37°C
2 x SSC  20 min 37°C
2 x SSC  20 min 37°C with 1 ul RNaseA / 5 ml
0.2 x SSC 0,1% Tween 20  1 hr 65°C
0.2 x SSC 0,1% Tween 20  1 hr 65°C
0.2 x SSC 0,1% Tween 20  1 hr 65°C
0.2 x SSC 0,1% Tween 20  10 min R.T.
MAB, 20%Lamb Serum, 2% Roche Blocking Buffer  1 hour R.T.
MAB,  “”  “”  1:4000 Ab  o/n 4°C

Day 3

MAB  30 min x 10 (fill vials each time)
BM Purple hours-o/n 4°C (0.5 ml)
or
APB    5 min
APB, 4.5ul NBT and 3.5 BCIP, or 5 ul Magenta Phos - 2 hours to o/n 4°C
Keep dark! This is one instance where it pays to remove all liquid between refills.
You have to monitor the reaction. If there is no colour reaction after 3 or 4 hours, you can probably get away with leaving it in the fridge overnight. If there is still no colour by the next morning, bring it out to room temperature, keep it dark, and check the reaction every couple of hours. You can stop the reaction with a rinse or two with MAB followed by 20 minutes MEMPFA. Magenta Phos can take several days.
Day 4

If double labeling:

PBT 5 min
PBT 5 min
PBT 4% paraformaldehyde 20 min (or MEMFA)
PBT 5 min X 4
PBT 30 min 65 °C

Or skip the fixation, and heat treat directly, or instead wash in

Glycine 100 mM pH 2.2, Tweens-20 0.1% 30 min
PBT 5 min X 4

Then repeat Day 3

Bleach

Formamide 5%, H2O2 1%, SSC 0.5 X

Finally, take to Methanol or Fix and then take to methanol
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