2011

Role of Six3 and Pax6 in regulating the gene networks involved in vertebrate eye development

Saqib Sachani
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Role of Six3 and Pax6 in regulating the gene networks involved in vertebrate eye development

by

Saqib S. Sachani

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 at the University of Windsor

Windsor, Ontario, Canada

2011

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Role of Six3 and Pax6 in regulating the gene networks involved in vertebrate eye development

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May 27, 2011
DECLARATION OF ORIGINALITY

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ABSTRACT

*Xenopus* eye field transcription factors display a dynamic and overlapping expression pattern but their signaling hierarchy is unclear. Current signaling models are inconsistent with regard to some eye phenotypes. The object of my study is to clarify the role of some of the early and major players in eye development: is *Rx1* really an upstream regulator of *Pax6* and *Six3*? Its mutant phenotype is very much milder that those of the latter two. Morpholino-mediated *Six3* knockdown caused severe phenotypes and absence of *Pax6* expression in the eye field. Conversely, *Pax6* knockdown produced a milder phenotype with reduced *Six3* expression. *Six3* phenotypes can be rescued by *Pax6*, and perturbation of either demolishes *Rx1* expression. This suggests a reversed order of dominance in signaling than previously described. I also examine the hierarchical relationships shared between *Six3, Pax6, Rx1* and other eye field candidates – *Otx2, Sox2, Pitx3, MafA, Lens1, Pax2* and *γ-crystallin*. 
DEDICATION

To my parents and family for supporting and encouraging me at all times.
ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Michael Crawford for introducing me to the field of Developmental Biology and for sharing with me his great interest and enthusiasm in it. I have been grateful for having worked on a project which was interesting and close to me, manage and develop it with great independence, along with his support, guidance and motivation. I am extremely thankful for all the support he has given to me over the last few years as well as the advice and comments on all matters.

I would also like to thank my committee members, Dr. John Hudson, Dr. Sirinart Ananvoranich and chair for the defense, Dr. Andrew Swan for their advise, help and support throughout my graduate and undergraduate career at Windsor.

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<thead>
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<th>Description</th>
</tr>
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<tbody>
<tr>
<td>µM</td>
<td>micromolar</td>
</tr>
<tr>
<td>4C</td>
<td>Chromosome conformation capture-on-chip</td>
</tr>
<tr>
<td>ANP</td>
<td>Anterior Neural Plate</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic Helix Loop Helix</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CDKI</td>
<td>Cyclin dependent kinase inhibitors</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>CMO</td>
<td>Control morpholino oligonucleotide</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>dUTP</td>
<td>Deoxyuridine Triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene-diamine-tetra-acetic acid</td>
</tr>
<tr>
<td>Ef1α</td>
<td>Elongation factor 1-alpha</td>
</tr>
<tr>
<td>EFTFs</td>
<td>Eye Field Transcription Factors</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>EnR</td>
<td>Engrailed repressor</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed sequence tag</td>
</tr>
<tr>
<td>Ey</td>
<td>Eye field</td>
</tr>
<tr>
<td>ey</td>
<td><em>Eyeless</em> gene</td>
</tr>
<tr>
<td>GCL</td>
<td>Ganglion cell layer</td>
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<tr>
<td>Gem</td>
<td>Geminin</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>HD</td>
<td>Homeodomain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>HMG</td>
<td>High mobility group</td>
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<tr>
<td>Hox genes</td>
<td>Homeotic genes</td>
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<tr>
<td>HPE</td>
<td>Holoprosencephaly</td>
</tr>
<tr>
<td>MAB</td>
<td>Maleic Acid Buffer</td>
</tr>
<tr>
<td>Maf</td>
<td>Musculoaponeurotic fibrosarcoma oncogene homolog</td>
</tr>
<tr>
<td>MBS</td>
<td>Modified Barth’s saline</td>
</tr>
<tr>
<td>Mitf</td>
<td>Microphthalmia-associated transcription factor</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MO</td>
<td>Morpholino oligonucleotide</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitro-blue tetrazolium</td>
</tr>
<tr>
<td>ng</td>
<td>Nanograms</td>
</tr>
<tr>
<td>nr</td>
<td>Neural retina</td>
</tr>
<tr>
<td>nt</td>
<td>Neural tube</td>
</tr>
<tr>
<td>Otx2</td>
<td>Othrodenticle homeobox homolog 2</td>
</tr>
<tr>
<td>Pax genes</td>
<td>Paired-box genes</td>
</tr>
<tr>
<td>pg</td>
<td>Picograms</td>
</tr>
<tr>
<td>PLE</td>
<td>Presumptive lens ectoderm</td>
</tr>
<tr>
<td>RPC</td>
<td>Retinal progenitor cells</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal pigmented epithelium</td>
</tr>
<tr>
<td>RPL</td>
<td>Retinal pigmented layer</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>Rx genes</td>
<td>Retinal homeobox genes</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>Six genes</td>
<td>Sine oculis homeobox genes</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------------------------------------------------------------------</td>
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<tr>
<td>so</td>
<td><em>sine oculis</em></td>
</tr>
<tr>
<td>Sox genes</td>
<td><em>SRY</em>-related HMG box genes</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>XCG</td>
<td>Xenopus cement gland specific gene</td>
</tr>
<tr>
<td>Xnr</td>
<td>Xenopus nodal related gene</td>
</tr>
<tr>
<td>Zic2</td>
<td>Zinc finger protein of cerebellum</td>
</tr>
</tbody>
</table>
Overview

Organ development is a very systematic and organized process which requires genes to turn on at precisely the right time, and in the right place. Co-ordination and interaction between genes and their products helps to assemble complex regulatory hierarchies that regulate tissues, and these in turn interact to direct organ differentiation. In recent years, *Xenopus* has served as a powerful system to study early embryonic events. With the help of this model system, where large numbers of eggs can be fertilized simultaneously and then observed to progress synchronously through developmental stages, embryologists laid the foundation for some one most important tenets of developmental biology including: determination, specification, body-axis formation, regulative development, and embryonic induction (reviewed in Zuber M.E., 2011).

Eye Development

One well studied model of such a process is embodied in the induction and development of the eye. Inductive interactions can result in the formation of germ layers or complex organ systems such as the central nervous system (Henry et. al., 2002). Experimental evidence has shown that vertebrate eye development requires inductive interactions between the presumptive head ectoderm and the underlying neural tissue which will eventually give rise to the lens and optic cup respectively.
Amphibian lens induction takes place in two phases, early and late. The early phase commences during stage 11 which is around gastrulation (Henry & Grainger, 1990), and this is when the ectoderm is rendered competent to respond to signals. The late phase starts at around stage 19 when the neurally derived optic vesicle comes into contact with the ectoderm. The late phase involves the specification and differentiation stages of the lens ectoderm (Henry et al., 2002).

Development of the neurally derived side of the vertebrate eye begins as the early specification of anterior neural plate forms immediately following gastrulation. This patch of cells comes to be bordered by a ridge that separates it from ectoderm, and will later develop into the eye and the brain. The first morphological sign of eye development occurs internally as a bilateral evagination of the late neurula forebrain upon the closure of the anterior neural tube. In mammals, an external marker of this is the appearance of the optic pit in the overlying ectoderm, whereas in amphibians, bulging of the optic primordia from the side of the head is observed (Henry and Grainger 1990; Chow and Lang, 2001; Zuber M, 2011). Continued evagination of the optic primordia from the diencephalon towards the non-neural ectoderm leads to the formation of optic vesicles (Figure 1.1). Mesenchymal tissue, which is located between the optic vesicle and the surface ectoderm, gets displaced and consequently the two tissues come into physical contact, whereupon inductive signals are exchanged between them. The distal end of the optic vesicle will finally induce the non-neural ectodermal surface to form the lens and cornea (Chow and Lang, 2001; Henry et. al., 2002; Grainger, 1992). This early induction signal to the non-neural ectoderm operates to induce lens placode, which in turn reciprocally induces the optic vesicle to invaginate and transform into optic cup. The
outer layer of the cup differentiates into the pigmented retina, and the inner layer becomes the neural retina (Grainger, 1992; Lang, 1999).

Following placode formation, the presumptive lens ectoderm displays its first sign of differentiation: the placode enlarges, forms a central pit, and vesiculates into the cavity that is forming in the enclosing optic cup as seen in Figure 1.1. Eventually, the developing lens vesicle separates from the head ectoderm and completes its differentiation process within the optic cup (McAvoy, 1980; Piatigorsky, 1981; Grainger, 1992; Chow and Lang, 2001). The formation of the lens placode coincides with crystallin synthesis and deposition. The crystallin family of proteins is required for lens generation and maintenance of its transparency (Piatigorsky, 1992). Finally, the mature lens forms as a polarized structure with its anterior surface covered by cuboidal epithelium and the posterior regions dominated by lens fibre cells in the interior (Lang, 1999; Chow and Lang, 2002).

The induction of lens and retina is reciprocally inductive; in amphibians, the absence of one causes the other to fail to form (Spemann, 1938; KhosrowShahian et. al., 2005). Physical manifestations of this close interaction are revealed by transmission electronic microscopy which, in rats, shows that the optic vesicle and the lens placode are tightly associated through a network of collagenous fibrils (McAvoy, 1980).
Figure 1.1: Summary of lens induction.

(A) Un-induced head ectoderm responds to lens inductive signals. (B) Stage 14 neural plate stage – planar inductive signals (dashed arrows) originate from the neural plate. Vertically transduced signals originate from the underlying endoderm and mesoderm towards the Presumptive Lens Ectoderm (PLE). (C) Neural tube closure and the contact of the optic vesicle with the lens ectoderm. (D) Thickening of the lens ectoderm to form the lens placode and the beginning of induced optic cup formation. (E) Lens vesicle detaches and resides in the cavity formed by invagination of the optic cup (adapted and modified from Henry et. al., 2002).

Lens Induction Model

Early experimental evidence and surgical manipulations were performed in amphibians as a tool to study and understand the inductive events which give rise to tissue levels and organs in embryonic development. The first induction studies were carried out by Spemann, Herbst and Lewis (Spemann, 1901; Herbst, 1901; Lewis, 1904; cited in - Grainger 1992; Chow and Lang, 2001). Spemann conducted ablation experiments in which he used fine glass needles to unilaterally remove the optic vesicle anlage at early neural stages: lens induction was inhibited (Figure 1.2). The contra-lateral
control side developed a normal eye with lens. He concluded that the optic vesicle plays a role in inducing lens structures in the overlying ectoderm.

Figure 1.2: Model of lens ablation experiment by Hans Spemann, 1901.

(A) Using fine glass needles, the lens forming ectoderm was surgically lifted, the optic vesicle cauterized, and the ectoderm replaced. (B) Destruction of the optic vesicle inhibited lens formation. (C). Lens and eye development was normal on the contra-lateral control side. (Adapted and Modified from Scott F. Gilbert – Developmental Biology 8th Ed)
Herbst (1901) proposed that the number of optic cups determined the number of lenses to be formed. The location of the optic vesicle specifies the location from which lens arises, and surgical manipulations by removal the mesenchyme that normally separates non-eye ectoderm from vesicles results in cyclopia: only one central eye is located above the nose rather than two distinct eyes (Herbst, 1901). This condition can also arise due to the failure of the optic field to split within the developing brain – which can be linked to present day identified holoprosencephaly (HPE) in humans, which arises as a consequence of the hemispheres of the telencephalon to separate thereby resulting in a single eye field due. This is due to mutant Six3 failing to activate Sonic hedgehog expression (shh) (Geng et. al., 2008).

Lewis surgically grafted optic vesicle rudiments and transplanted them under head ectodermal regions other than the normal eye location in Rana palustris and Rana sylvatica tailbud stage embryos. This resulted in induction of ectopic lenses suggesting that the optic vesicles potent to induce lens formation in any ectoderm competent to receive the signal, and that essentially all head ectoderm was competent to respond (Lewis, 1904; Lewis 1907).

In addition, Fessler (1920) observed in Salamander that when the optic vesicle was unable to contact the overlying ectoderm, lens formation was abrogated (reviewed in Grainger, 2002). More sophisticated experiments using fluoresceinated lineage markers and transplantation (Henry and Grainger, 1987), confirm Spemann and Lewis’ identification of the tissues involved, their derivates post-transplantation, and eliminated concerns regarding surgical graft cross-contamination.
The role of the optic vesicle as sole lens inducing tissue must be questioned as the Presumptive Lens Ectoderm (PLE) acquires the bias to form a lens at gastrulation, much earlier than formation of and contact with the optic vesicle. To some extent, the fate of the PLE is already determined prior to induction (Henry and Grainger, 1987). Therefore, lens induction takes place in two phases – an early phase which commences during gastrulation and ends around neural tube closure – and a late phase which is initiated upon contact between optic vesicle and overlying ectoderm. (Chow and Lang, 2001; Grainger, 2002). These can be further sub-divided: a period of lens forming competence in the late gastrula where the responding tissue develops a competence to receive specific inductive signals, and later, the acquisition of a lens forming bias comprise the early stage; specification of cells towards lens fate in the presumptive lens ectoderm and finally, differentiation of lens define the later phase (Figure 1.3; Grainger, 1992)
Figure 1.3: Early and Late phase of Lens Induction.

Summary of lens induction relative to the stages of development. (A) Defines the boundary of early and late phase of lens induction. (B) Summary of lens induction (described in Figure 1.1). (C) Specific stages, competence, bias, specification and differentiation relative to the lens induction model (adapted and modified from Henry et. al., 2002).

**Early phase of lens induction**

This acquisition of competence is cell-autonomous event since it is not dependent on the context: ectoderm may be cultured in isolation and still become competent (Servetnick and Grainger, 1991). The competence acquired in these cultured tissues can be confirmed and measured experimentally by transplanting specific ectodermal tissues of various developmental stages into different inductive environments (Servetnick and Grainger, 1991).
The presumptive lens ectoderm (PLE) enters the bias phase following receipt of planar signals from the adjacent anterior neural plate, which is a strong inducer of lens in the mid-neural embryos (Henry & Grainger, 1990). In *Xenopus*, during stages 14 to 19 i.e. between the neural plate and closed neural tube stages, the PLE receives the maximum amount of signal to become biased towards the development of the lens. Indeed, the competence of this biased ectoderm shows a stronger response when grafted beside the anterior neural plate staged embryo than when grafted over top a later staged optic vesicle (Henry and Grainger, 1990; Chow and Lang, 2001). Grafting experiments indicate that the optic vesicle can induce lens formation in any region of the head ectoderm outside of the presumptive lens area, however flank ectoderm remains incompetent (Spemann, 1901; Lewis, 1904; Grainger et. al., 1997). This confirms that strong signals from the anterior neural plate confer a lens forming bias over the entire head ectoderm region and indeed, the expression of key eye field transcription factors is restricted to this region (Zuber et. al., 2003). The late phase of induction subsequently involves domain-refining interactions between these genes and the tissue layers (Grainger et. al., 1996).

**Late phase of lens induction**

The moment that the optic vesicle makes direct contact with the PLE marks the beginning of lens specification and the late phase of lens induction (Figure 1.3; Grainger et. al., 1996). Following induction the PLE can be explanted and cultured separately resulting in autonomous differentiation and expression of lens specific markers. When the ectoderm is cultured in isolation at this stage it eventually results in formation of small
crystallin expressing structures called lentoids (lens like structures) (Henry and Grainger, 1990; Chow and Lang, 2001; Jin et. al., 2011). At this stage, the PLE thickens and starts to express lens markers resulting in formation of the lens placode such as Pitx3 (KhosrowShahian et al, 2005). Towards the end of specification, there is another cascade of inductive events between the PLE and the optic vesicle that marks the beginning of lens differentiation that is characterized by expression of crystallin in the placode (Figure 1.3; Grainger et. al., 1996). The lens placode invaginates to form the lens vesicle where progenitor cells proliferate and undergo terminal differentiation (Menko et. al., 1984; Chow and Lang, 2001). During this final step, proliferative cells are observed only in the anterior vesicle epithelium, while those situated posteriorly differentiate into lens fibres (Grainger et. al., 1996; Piatigorsky 1981). The terminal differentiation of epithelial lens progenitor cells coincides with expression and synthesis of crystallins that constitute the major structural and functional proteins of the lens (discussed later).

**Genes involved in eye development**

Eye development appears to be genetically and morphologically similar in all vertebrates. In *Xenopus*, the genetic processes that regulate eye development include genes that begin expression during gastrulation at around stage 10.5 (Barsacchi et. al., 2000).
Figure 1.4: Summary of expression of early eye field transcription factors involved invertebrate eye development

Expression of early eye field transcription factors is dynamic and domains are overlapping (Figure 1.4). *Six3* and *Otx2* expression dominate the eye field whereas *Pax6*, *Pax2* and *Rx1* domains are restricted to regions within those of *Six3* and *Otx2*. The expression of *Sox2* is observed along the neural tube and in the eye field domain where *Pax6*, *Six3* and *Rx1* overlap. Most of these particular genes commence expression during the early phase of eye induction (Figure 1.5) and have been characterized by their respective expression patterns and mutant phenotypes.

Among the best studied are the homeobox genes (*Six3*, *Pax6*, *Rx1*, *Otx2*, *Pitx3*), however other players include members of the High Mobility Group (*Sox1, 2, 3*), fork head box (*Lens1/FoxE3*), and leucine zipper (*MafA, MafB, c-Maf*) families of transcription factors. Figure 1.5 below describes the expression pattern of the eye field
transcription factors during the early and late phases of eye development and lens induction.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Competence/Bias</th>
<th>Specification</th>
<th>Differentiation</th>
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<tbody>
<tr>
<td>Six3</td>
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<td>Pax6</td>
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<td>Otx2</td>
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<td>Lens1</td>
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<td>MafA</td>
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<td>-</td>
</tr>
<tr>
<td>γ-crystallin</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Stage: 11.5  14  19  26  32  38+

[Images of embryos at different stages with labels: anp, nt, PLE, ef, pm, le]
Figure 1.5: Summary of expression of eye field markers during the early and late phase of lens induction.

- Indicates gene expression  - Indicates no expression

anp: anterior neural plate; nt: neural tube; PLE: presumptive lens ectoderm ef: eye field; rpe: retinal pigmented epithelium; le: lens
Homeobox Factors

The homeobox motif encodes a protein domain which is 60 amino acids long called the homeodomain. The homeodomain containing proteins recognize a TAAT consensus target sequence or motif (Beebe, 1994). The key homeobox genes that play an important role in eye development are outlined below:

Six3

The Six family of transcription factors *Xenopus* are homologous in nature to the *sine oculis* (so) gene in *Drosophila melanogaster*. Homologues for human, fish, avian, mouse and *Xenopus* have been cloned. (Gruss et. al., 2000; Brandli et. al., 2001). The *Six3* coding sequence contains the protein interacting *Six* domain and the DNA binding homeodomain (Gruss et. al., 2000).

In *Drosophila*, the *so* gene is important for the development of the visual system. Defects and irregularities in the expression of the *so* gene result in degeneration of the retina and aborted development of the optic lobe. (Serikaku & O'Tousa, 1994). In mice, *Six3* expression was first reported in the lens placode, followed by its subsequent expression in the lens epithelium during the progressive differentiation stages (Oliver et. al., 1995). Over-expression of *Six3* in medaka fish results in enlarged optic vesicles along with expansion of the presumptive midbrain. (Wittbrodt et. al., 1999). Loss of function experiments via a morpholino mediated knockdown of *Six3* in medaka fish results in craniofacial, forebrain, and eye anomalies (Wittbrodt et. al., 2002). Increasing concentrations of the morpholino results in small eyes, cyclopic eyes and finally, absence
of eyes (Wittbrodt et. al., 2002). *Six3* null mutant mice embryos lose telencephalic regions and exhibit craniofacial abnormalities (Oliver et. al., 2008). Typically, in both mouse and human mutants, holoprosencephaly occurs in varying degrees with the result that there is an absence in the brain of an interhemispheric fissure, reduced or absent olfactory bulbs, and microphthalmia or cyclopia (reviewed in Lacbawan et al., 2009).

In *Xenopus*, *Six3* expression is commences at stage 10.5, during gastrulation. At stage 14, the expression domain of *Six3* is located primarily in the anterior end of the neurula in prospective neuroectoderm. This expression is limited to a small group of cells only at this stage. At around stage 20, the middle region of the ventral diencephalon is dominated by *Six3* expression, which then decreases anteriorly towards the telencephalon. At around stage 32, the expression is restricted primarily to the eye region and the ventral diencephalon between the eyes (Ghanbari et. al., 1998).

**Pax6**

*Pax6* belongs to the *Pax* family of *paired*-related transcription factors. The gene encodes two DNA binding motifs: the *paired* box and a homeodomain (Stuart et al., 1994). *Pax6* is critical for lens and retina development: mutation of *Pax6* results in the aniridia syndrome in humans, *small eye* (sey) in mouse and the eyeless phenotype in *Drosophila* (Chow and Lang, 2001). *Pax6* retains a high level of structural and functional conservation between species, since murine *Pax6* ectopically expressed in *Drosophila* elicits formation of ectopic eyes, thus *Pax6* is acclaimed as the master regulator of eye development (Halder et. al., 1995; Chow and Lang, 2001). Moreover, *Pax6* homologues have also been found to express in the sensory organs of nematodes.
and the photosensitive ocellus of *ascidians* which clearly suggests a conserved role for the gene in light sensory organs broadly speaking (Johnson et al 2001; Zhang and Emmons, 1995; Glardon et. al., 1997).

*Pax6* heterozygous mutant mice exhibit the small eye (*sey*) phenotype which further leads to cataracts, hypoplasia of the iris, and microphthalmia (Hill et. al, 1991; Matsuo et. al., 1993). On the other hand, *Pax6* null mutant mice are observed to be anophthalmic and to die at birth (Grindley et. al., 1995). In *Xenopus*, ectopic *Pax6* expression results in formation of fully differentiated ectopic eyes along with enhanced expression of eye field markers which include *Otx2*, *Rx1* and *Six3* mainly (Chow et. al., 1999). Consistent with its predicted role, in animal cap assays (explants of uninduced ectoderm), *Pax6* can induce the expression of lens specific markers that include β*-crystallin* and γ*-crystallin*. However, in animal caps, *Pax6* is unable to induce the expression of neural and mesodermal markers which clearly suggests a direct role in lens formation by activation of *crystallins* (Zygar et. al., 1998; Cvekl et. al., 2004). Dominant negative *Pax6* targeted against wild type *Xenopus Pax6* results in proximal deformities and reduced eye formation (Chow et. al., 1999)

In *Xenopus*, *Pax6* expression is first expressed at stage 12.5 immediately subsequent to gastrulation. Later *Pax6* is expressed in the presumptive lens and retina. *Pax6* is also expressed in the developing brain and the neural tube. At around stage 14, *Pax6* expresses in neuroepithelial cells which are the prospective retinal epithelium cells and neural retina. A cross section of this region shows that the expression of *Pax6* is restricted to the neuroectoderm and is not seen in the mesoderm that underlies it. *Pax6* is expressed throughout stages 12.5 to 28 during the eye development process. After stage
28, the lens thickens and can be distinguished morphologically from the overlying epithelium. *Pax6* expression is not seen in the epithelium, however expression in the lens remains high during this period up to stage 33. By the end of stage 42, the expression of *Pax6* is limited to the ganglion cell layer and the inner nuclear layer which constitute the retinal laminae (Harris & Hirsch, 1997).

**Rx1**

The *Xenopus* *Rx* genes were the very first to be isolated from a cDNA library obtained from animal cap explants. *Rx* genes were identified as eye field transcription factors and were defined as key proliferative markers (Mathers et. al., 1997). The homeodomain sequence of the *Rx* family shares high homology with the *paired*-like genes (Mathers et. al., 1997). In mice, loss of *Rx* genes results in the loss of eye structures (Barsacchi et. al., 2000; Mathers et. al., 1997). Additionally, *Rx1* mutant mice also exhibit irregularities in forebrain development (Mathers et. al., 1997). Over-expression of *Rx1* in *Xenopus* embryos results in enlarged retinal pigment epithelia (Mathers et. al., 1997).

In *Xenopus*, expression for *Rx1* begins at stages after gastrulation. The gene first expresses at stage 13 and continues up to stage 45 (Barsacchi et. al., 1997). After stage 45, the expression declines (Barsacchi et. al., 1997). During development of the optic vesicle, and before it comes in contact with the overlying ectoderm (around stage 16 and 17), a strong field of expression for *Rx1* is observed. At later stages, *Rx1* is only expressed in regions of the eye that are of neural origin i.e.; the retinal structures (Barsacchi et. al., 1997).
Otx2

Otx2, a homeobox gene related to the orthodenticle family of genes which are expressed in *Drosophila melanogaster*, possesses the bicoid class of homeodomain (Simeone et al., 2003). In *Drosophila*, the orthodenticle family of genes are responsible for development of the head region and eye structures. (Boncinelli et. al., 1995). In humans and mice, Otx2 homologs have been identified and classified as critical genes that play an important role in the, the early specification of the neuroectoderm to become the fore-and mid-brain (Simeone, 1998). In mice, deletion of the Otx2 gene using homologous recombination results in phenotypes having embryonic lethal gastrulation defects. Mice also lack the pre-chordal mesoderm and notochord precursors which induce normal formation of the brain (Matsuo et. al., 1995). Otx2 null mutant mice display an absence of forebrain and midbrain structures that are likely linked to aberrant neural induction. As a result of this complex phenotype in mice, the precise role of Otx2 in the developing eye was difficult to isolate and understand (Pannese et. al., 1995).

In *Xenopus* embryos, Otx2 is detected as a maternal transcript right from the unfertilized egg to the late blastula. However, the expression levels at these stages are very low although still observable. After gastrulation, around stage 14, the expression pattern restricts primarily to the mesendoderm and anterior ectoderm regions (presumptive lens ectoderm) (Boncinelli et. al., 1995). In the eye field, Otx2 is activated well before many other eye field transcription factors and is detectable around stage 10.5 in the mid-gastrula embryo (Simeone et. al., 1993). This is coincident with expression of Six3 (Gestri et. al., 2005; and present study). Otx2 expression progressively restricts to
the anterior dorsal region of the embryo after gastrulation. At later stages, the expression is detectable only in the anterior neural plate region (Boncinelli et. al., 1995).

Double in situ hybridization studies to localize the expression of Otx2 with Rx1 showed that their expression domains were distinct at the very early determinative stages of the eye field (Andreazzoli et. al., 1999). Over-expression of Rx1 results in significant inhibition of Otx2, suggesting that Otx2 is not required for eye field specification in the early stages, even though it is required to specify anterior neural domains (Andreazzoli et. al., 1999). At later stages, Otx2 expression expresses in the optic vesicle and RPE (Bovolenta et. al., 1997). Morpholino mediated Otx2 knockdown in Xenopus yields abnormal anterior development and malformed eyes (Caron et. al., 2005). Over-expression of Otx2 by mRNA microinjection results in the induction of ectopic cement glands and abnormal eyes which are enlarged in size (Gammil and Sive, 1997; Pannese et. al.,1995). Otx2 is a direct activator for cement gland marker genes which include XCG, and at the same time Otx2 appears to inhibit more posteriorly expressing genes such as xCad3 and Xbra suggesting that it acts as both a transcriptional activator and a repressor in a context-specific manner (Gammil and Sive 2001; Isaacs et. al., 1999). In the context of eye development, Otx2 acts as an activator since inhibitory constructs, such as Otx2-EnR mRNA, produce eyeless tadpoles that can be phenotypically rescued with a functional transcript of Otx2 (Isaacs et. al., 1999). This suggests that Otx2 is required to specify the anterior structures (Zuber et. al., 2003).
The *Pitx* family of paired-like homeodomain genes consist of the homologs, *Pitx1*, *Pitx2*, and *Pitx3* (Pommereit, Pieler, & Hollemann, 2001; Gage et. al., 1998) the latter of which is involved in eye development (Semina et. al., 2000; Khosrowshahian et. al., 2005). In mice, deletion of *Pitx3* results in aphakia, which is a recessive mutation that is characterized by small eyes without lenses (Semina et. al., 2000). In humans, *PITX3* mutations result in anterior segment mesenchymal dysgenesis and development of congenital cataracts (Semina et. al. , 1998).

In *Xenopus*, *Pitx3* expresses in the pituitary, brachial arches, presumptive lens ectoderm, otic vesicle, somites, heart and the gut (KhosrowShahian et. al., 2005). *Pitx3* expresses in the mid-blastula and early gastrulation stages which include stages 9 – 11.5 respectively. After the mid-neurula stages, the expression is up-regulated through stages 12 to 19 into the late phase of induction (Khosrowshahian et. al., 2005). Expression of *Pitx3* is reported prior to the thickening of the lens placode, suggestive of a role in lens induction. Expression is maintained in the lens placode, lens pit and the lens vesicle (Khosrowshahian et. al., 2005). From stage 19 onwards expression remains strong in the lens and continues up to stage 34. Most prominent expression of *Pitx3* is reported at around stage 24 when the optic vesicle is in contact with competent ectoderm (Khosrowshahian et. al., 2005). Expression at this stage is marked at the PLE. At the later stages when the PLE develops into the lens placode, the expression is strong in the lens placode. At later stages i.e. stage 38, expression of *Pitx3* is restricted only to the lens epithelial layer and no expression is reported in lens fibres (Pommereit, Pieler, & Hollemann, 2001). Inhibition of *Pitx3* expression in *Xenopus* embryos using morpholino
mediated knockdown impairs eye development leading to reduced eyes. With higher doses of morpholino no eye develops (Khosrowshahian et. al., 2005). Over-expression of Pitx3 in Xenopus results in expansion of the Pax6 domain indicating that Pax6 may be under the control of Pitx3 in lens. Pax6 expression continues to be enhanced at later stages resulting in expansion of its domain in whole embryos. By contrast, in animal cap assays, Pax6 activates the expression of Pitx3 (Khosrowshahian et. al., 2005). Nevertheless, in whole embryo Pitx3 knockdown experiments, Pax6 expression is observed to be slightly down regulated. Pitx3 is also reported to regulate Lens1, Rx1 and Otx2. (Khosrowshahian et. al., 2005)

Pax2

Pax2 shares features in common with Pax6. It is defined by the presence of a paired-box which encodes a paired domain – a highly conserved 128 amino acid DNA binding domain which resembles the Drosophila prd gene (Schneitz et. al., 1993; Pichaud and Desplan, 2002). Along with the paired domain, Pax proteins also contain the homeodomain and the octapeptide domain (except Pax4 and Pax6) and are classified as multi-functional transcription factors (Callaerts et. al., 1997; Eccles and Schimmenti, 1999; Eccless et. al., 2002). In the paired class group of genes, Pax6 claimed most of the attention due to its evolutionary conserved role in eye development (Halder et. al., 1995). However Pax2 also plays a significant role: it is first detected in the ventral half of the optic cup, and after invagination expression restricts to the glial cells that extend to form the optic stalk (Torres et. al., 1996; Macdonald and Wilson, 1998). In Pax2 null mutant mice no glial cells develop and the optic stalk collapses: the optic nerve fails to run
between the optic cup and the brain (Pichaud and Desplan, 2002). *Pax2* is also expressed in the otic vesicle primordium which comes to play an auditory role (Hill et al., 1991; Quinn et al., 1996). Along with its expression in the eyes and the otic regions, *Pax2* also specifies regions in the central nervous system (CNS) and the kidney (Tavassoli et al., 1997).

**High Mobility Group Factors**

The High Mobility Group (HMG) proteins are transcription factors which contain the HMG box domain (75 amino acids) which is a DNA-binding domain. HMG box domains have also been found in many chromatin remodeling complex-associated proteins (Stros et al., 2007).

**Sox2**

The *SRY* (sex determining region Y chromosome) related high mobility group (HMG) box (and *Sox*) transcription factors play an important role in cell fate and differentiation in variety of cellular lineages (Lefebvre et al., 2007). Members of the *Sox* family of transcription factors have been shown to bind to the minor groove of cognate sequence and to initiate alterations of chromatin structure: they demonstrate a unique characteristic as transcriptional enhanceseosomes (Penvy et al., 1997; Lefebvre et al., 2007). This interaction results in the widening of the minor groove at the expense of compression of the major groove, in some instances resulting in higher protein accessibility and the formation of functionally active complexes of transcription factors on the gene enhancer sequences (Lefebvre et al., 2007).
A variety of roles for the Sox family of transcription factors have been reported. These include: sex determination (Polanco and Koopman, 2007); eye development – lens induction, activation of crystallins, lens fibre differentiation (Uchikawa and Kamachi, 2004; Kamachi et. al., 1998; Kondoh et. al. 2004); embryonic stem cell pluripotency maintenance (Avillion et. al., 2003); maintenance of neural stem cell identity (Wegner and Stolt, 2005); and anterior pituitary development (Kelberman et. al., 2006). These roles have been identified across a variety of species and involve Sox family members working individually or in tandem with other Sox proteins.

In Xenopus, Sox2 plays an important role in the early steps of neural differentiation. In combination with basic Fibroblast Growth Factor (bFGF), Sox2 can induce neural fate in animal caps, probably by competing with ventralizing signals (Mizuseki et. al., 1998). Consistent with this interpretation, impairment of Sox2 activity by a dominant negative mRNA results in inhibition of neural differentiation in animal caps due to enhanced (ventralizing) Bone Morphogenetic Protein 4 (BMP4) levels concomitant with loss of neural markers such as N-CAM and Krox20 (Kishi et. al., 2000).

In chicken, Sox2 binds cooperatively with Pax6 and together they bind to the δ-crystallin enhancer (DC5) (Kamachi et. al., 2001). In chick embryos, Sox2 alone cannot induce lens tissue differentiation, however, when co-expressed with Pax6, lens tissue is induced in ectoderm (Kamachi et. al., 2001). However, in medaka fish, Sox2 alone can induce ectopic lens formation (Koster et. al., 2000). The expression of Sox1, 2 and 3 overlap in the PLE region, and this may indicate redundant functionalities.

During lens development in Xenopus, chick and mice, Sox1 first expresses in the lens placode region and later restricts to the lens fibre cells. In Xenopus, Sox2 expression
is first detected in the anterior neural plate region at stage 14, then along the forming neural tube, and finally, in the anterior dorsal head region at stage 19 when neural tube folding is complete. During the late phase of eye development, the expression of Sox2 is also detected in the optic cup and the PLE region. Sox2 expression is observed to be increased during the thickening of the lens placode region during these stages (Kamachi et. al., 1998). Sox3 also expresses in the lens placode region during induction suggesting the coordinated role of these genes to activate crystallins (Kamachi et. al., 1998). De novo mutations of Sox2 in mammals can result in the absence of eyes (Ragge et al, 2005) and graded diminution of Sox2 activity appears to impair neurally derived retina in particular (Taranova et al., 2006)

**Leucine Zipper Factors**

The leucine zipper factors are transcriptional factors that contain a basic-leucine zipper DNA binding motif as well as a distinct acidic domain which functions as a transactivation domain. (Moens et. al., 1998).

**MafA**

The Maf family of transcription factors are basic-leucine zipper transcription factors that play a major role in lens induction, placode thickening and differentiation. Key members of the Maf family identified are MafA (also known as Lens specific Maf or L-Maf), MafB and c-Maf (Ishibashi and Yasuda, 2001). MafB is expressed in the optic vesicle anlagen, whereas the MafA is primarily expressed in the lens ectoderm (Ishibashi and Yasuda, 2001). MafA expression occurs in the lens placode around stage 24 when the
PLE induction has taken place and differentiation marks its onset, and it is shown to up-regulate expression of the *crystallin* genes. In *Xenopus* and chick embryos, over-expression of *MafA* results in ectopic induction of *crystallin* in the PLE region. On the other hand, expression of *MafB* is reported around stage 20, after the closing of the neural tube and during induction by the optic vesicle. *MafB* is expressed early in development and is thought to induce expression of *MafA* in the PLE. Morpholino mediated *MafB* knockdown results in less *MafA* being induced. The genes can functionally substitute for each other: in *MafB* knockdown embryos, *MafA* mRNA expression can rescue the activation of crystallins (Ishibashi and Yasuda, 2001). In animal cap ectoderm explants in *Xenopus, MafB* can enhance the expression of *Pax6, Lens1, Sox3, Six3* and *MafA* along with other *crystallin* genes – suggesting a role in induction and lens epithelium maintenance. Thus *MafA* is the important connecting link between *MafB* and the *crystallins*. Also, *Pax6* over-expression enhances both *MafA* and *MafB* expression early in development (Ishibashi and Yasuda, 2001; Chow and Lang, 2001; Reza et. al, 2002).

In contrast to *Xenopus* and chicks, mice require neither *MafA* or *MafB* for lens development. The expression of crystallin remains completely normal in *MafA* and *MafB* double mutants, and moreover, *c-Maf* appears to be the key lens regulator in mice (Takeuchi et. al., 2009). Finally, in *Xenopus* and chick, *MafA* in combination with *Sox2* can induce and expand the expression domain for both *γ-crystallin* and *δ-crystallin* by binding to their enhancers thus positively regulating their expression (Reza et. al., 2002; Shimada et. al., 2003).
Fork head factors

The fork head transcription factors are not one of the largest families of transcription factors but they do display a remarkable functional diversity in a wide variety of biological processes which include cell growth, lens progenitor cell proliferation, cell-cycle regulation and other cellular processes (reviewed in Carlsson and Mahlapuu, 2002). The fork head transcriptional factors in contrast to helix-turn-helix proteins bind to DNA sequences as monomers via the fork head box (80 to 100 amino acids) (Kaestner et. al., 2000).

Lens1

Lens1 (or Foxe3), a member of the sub-family of the fork head family of transcription factors that resembles the helix-turn-helix and are commonly identified as a winged-helix motif (Kaufmann and Knochel, 1996). Lens1 has an expression pattern restricted to the lens lineage (Blixt et. al., 2000. Brownell et. al., 2000). Lens1 expression in Xenopus is up-regulated by ectopic Pax6 expression, however, Lens1 cannot in return enhance the expression of Pax6 (Kenyon et. al., 1999). The role of Lens1 is to promote proliferation and to maintain an undifferentiated state followed by lens specification. Once lens specification is completed, the expression of Lens1 progressively restricts from the PLE, to lens placode, and finally to the epithelium of differentiating lens (as a border). Mis-expression of Lens1 in Xenopus results in complete suppression of lens differentiation reflected by loss of γ-crystallin expression. Higher levels of Lens1 appear to sustain ectoderm in a specified but undifferentiated state. Expression of Six3 and Pax6 are not affected in the PLE under these conditions (Kenyon et. al., 1999). During
differentiation of the lens, *Lens1* is down-regulated in a mosaic pattern as Sox2 and Sox3 are upregulated in the PLE and terminal differentiation commences.

**Differentiation Markers**

Expression of differentiation markers marks the final fate of the cell. These are late expressed during organogenesis.

**Crystallins**

The expression of *crystallins*, which are structural proteins of the lens, marks terminal differentiation of the lens fibre cells (Wistow and Piatigorsky, 1998). They are members of the heat shock protein superfamily and play a role in stress response and cellular protection (Ghosh et. al., 2005). In the lens, crystallins are specialized proteins which comprise over 90% of lens protein and they confer structural characteristics that play an important role in transparency and refraction of light (Clark, 2004; Jaffe and Horwitz, 1992). Several categorizes of crystallins have been identified based on their separation by size exclusion chromatography – but the most commonly discussed are are – *α*-crystallins, *β*-gamma crystallins and *γ*-crystallins (Wistow and Piatigorsky, 1998).

*α*-crystallins – comprises *α*-A-crystallin and *α*-B-crystallin present in a 3:1 ratio in the lens (about 40% of the *crystallins* in the lens). *β* and *γ* crystallins have originated from a common ancestor, and contain two types of Greek key motif (anti-parallel β-sheets that are fused) (Blundell et al., 1981; Bax et al., 1990). Despite their high structural similarity both *β* and *γ* crystallins expression levels varies between different species.
The β-crystallin family of genes contain seven members – βA1, βA2, βA3, βA4, βB1, βB2 and βB3 crystallin. The β-crystallin genes are well conserved between a variety of species which include mammals, amphibians and fish. (Wistow and Piatigorsky, 1998). On the other hand, γ-crystallin family of genes contain eight members - γA, γB, γC, γD, γE, γF, γN and γS crystallin (Wistow et. al., 2005). By contrast to β-crystallins, the γ-crystallins exist only as monomers due to the unique compact complex formed between its domains. Due to this condensed structure, γ-crystallin packs tightly and provide transparency by folding in regulated manner (Lubsen et. al., 1998).

In Xenopus, the α and γ crystallins both express in the developing lens vesicle and their expression remains high throughout lens fibre differentiation (Van Leen et. al., 1997). A little later, the expression for β-crystallin is observed in the lens fibre cells. Finally, the crystallins remain active from primary lens fibre differentiation to secondary fibre formation (Treton et. al., 1991).

The special conformational packing of the fibre with extracellular spaces smaller than the wavelength of light alters light scattering characteristics to optimize for transparency and minimal diffraction. Specific lens membrane proteins such as aquaporin play an important role in adhesion between differentiated lens cells, electric coupling, circulation of water, and ions to maintain a homeostasis (Chepelinsky, 2009). In the lens, expression of filamen proteins CP49 and filensin are observed. These two unique intermediate filament proteins form a beaded filament network. They are important to maintain the transparency and refraction of light. Mutation in the CP49 in humans has been linked to cataracts (Alizadeh et. al., 2003).
The various genes listed above with their functions are summarized below in Table 1.1. The eye field transcription factors display an overlapping pattern of expression – however their expression in terms of function can be divided into subunits of the eye namely: extension from the diencephalon (optic stalk); pigmented retina; neural retina; and lens as seen in Figure 1.6
Table 1.1: Summary of genes involved in eye and lens development with their functions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Important Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Six3</td>
<td>- BMP4 inhibition&lt;br&gt;- Pax6 activation&lt;br&gt;- Proliferation (Sequestering Geminin)&lt;br&gt;- Sox2 activation&lt;br&gt;- Lens differentiation</td>
</tr>
<tr>
<td>Pax6</td>
<td>- Establishing lens bias&lt;br&gt;- Lens epithelium maintenance&lt;br&gt;- Crystallin expression&lt;br&gt;- Eye field specification&lt;br&gt;- Proliferation</td>
</tr>
<tr>
<td>Otx2</td>
<td>- Neural Bias&lt;br&gt;- Inhibiting BMP4&lt;br&gt;- Lens competence</td>
</tr>
<tr>
<td>Rx1</td>
<td>- Proliferation&lt;br&gt;- Inhibitor of differentiation markers/cell cycle exit</td>
</tr>
<tr>
<td>Sox2</td>
<td>- Proliferation&lt;br&gt;- Regulation of crystallin expression&lt;br&gt;- Lens fibre differentiation</td>
</tr>
<tr>
<td>Pax2</td>
<td>- RPC proliferation</td>
</tr>
<tr>
<td>Pitx3</td>
<td>- Lens and Retina Induction</td>
</tr>
<tr>
<td>Lens1</td>
<td>- Proliferation</td>
</tr>
<tr>
<td>MafA</td>
<td>- PLE Induction&lt;br&gt;- Lens differentiation&lt;br&gt;- Crystallin activation</td>
</tr>
<tr>
<td>γ-crystallin</td>
<td>- Lens differentiation and development</td>
</tr>
<tr>
<td>BMP4</td>
<td>- Dorsal-ventral eye patterning&lt;br&gt;- Promotes ventral fate</td>
</tr>
</tbody>
</table>
Figure 1.6: Diagrammatic representation of genes expressed at the different tissue levels of the eye.

Proximal (Left – Diencephalon, early expressed markers); Distal (Right – Lens, late expressed markers); NR – Neural Retina.
Role of **BMP4** in dorso-ventral eye patterning

**BMP4** plays an important role in dorso-ventral patterning of the eye (Schmidt et. al., 1995). Lens and retina are derivatives of the dorsal ectoderm and neuro-ectoderm respectively, and **BMP4** is a ventralizing agent that acts as an antagonist. In *Xenopus*, over-expression of **BMP4** results in ventralization of embryos (Gestri et. al., 2005), and neuro-ectoderm is converted to an epidermal fate, reducing the competency of the tissue to respond to neuralizing cues from the underlying mesoderm (Nakayama et. al., 1998). In mouse, **BMP4** null mutants fail to survive past the E10.5 suggesting the importance of this gene in maintaining the dorsal ventral axis (Furuta and Hogan, 1998). In mouse explant studies, **BMP4** over-expression in the presumptive lens ectoderm can abrogate lens development (Furuta and Hogan, 1998). The PLE of **BMP4** null mutants recovers its ability to form normal lens and to induce retina if transplanted back on to wild type optic vesicles, however, in presence of **BMP4**, the isolated ectoderm, fails to form lens (Furuta and Hogan, 1998). Therefore **BMP4** inhibition is an important requirement for neural plate induction and consequently for eye field development. Furthermore, when BMP4 coated beads are surgically implanted to the anterior neural plate region during mid-neurula stages, the expression of key neural markers *Otx2*, *Rx1*, and *Pax6* are significantly repressed (Hartley et. al., 2001). Ectopic expression of **BMP4** via a *Pax6* promoter results in repression of *Otx2* and *Rx1*. Additionally, eye formation was abrogated in greater than 90% of the derived transgenic tadpoles (Hartley et. al., 2001). **BMP4** represses a dorsalizing pathway necessary to the lens and optic cup formation.
Six3 plays an important defensive role in repressing the expression of \textit{BMP4} in the anterior neural plate region. Six3 binds directly to the \textit{BMP4} promoter and represses its expression (Wittbrod et.al., 2002) In medakafish a Six3 factor/activator fusion construct, \textit{Six3-VP16} causes \textit{BMP4} expression domain to be expanded and dorsal regions to diminish (Gestri et. al., 2005).

\textbf{Balancing proliferation and differentiation}

As for any other developmental field, early eye development requires a population of undifferentiated and proliferative precursors before the organ can differentiate. In \textit{Xenopus}, neural differentiation starts right after gastrulation in the posterior region, however, in the anterior region at this time only the anterior neural plate is specified. The Retinal Progenitor Cells (RPCs) of the neuro-ectoderm in the optic vesicular region must undergo multiple rounds of proliferation to produce the quantity of cells which can then differentiate into the diverse populations of cells within the retina: differentiation events in the RPCs are closely linked with proliferation controls (Nelson et. al., 2009).

Cells in the anterior neural plate proliferate until they reach a minimal threshold required for normal eye morphogenesis (Ando et. al., 2005; Nelson et. al., 2009). Normally, \textit{Rx1} positively regulates the expression of \textit{Zic2} and \textit{Hairy2} which act as anti-neurogenic transcription factors (Ando et. al., 2005). \textit{Rx1} also represses the cell cycle inhibitor \textit{p27Xic1} thus promoting proliferation (Andreazzoli et. al., 2003) To forstall differentiation of competent cells, the expression of pro-neural genes, which includes \textit{xNgnr-1} and \textit{xDelta-1}, are repressed by \textit{Rx1} (Andreazzoli et. al., 2003). Loss of \textit{Rx1} activity by introduction of an \textit{engrailed} repressor chimera, \textit{Rx-EnR}, results in expansion
of \textit{Ngnr-1} and \textit{Delta-1} in the anterior neural plate thereby reducing proliferation, encouraging neuralization, and consequently reducing size of the eye field.

\textit{Six3} also plays a regulatory role in proliferation of the retinal progenitor cells. First, \textit{Six3} positively regulates the expression of \textit{Zic2} and \textit{Hairy2} which are proliferative markers (Gestri et. al., 2005). Additionally, \textit{Six3} influences the expression of cell cycle modulators – \textit{cyclinD1} and \textit{p27Xic1} (Bernier et. al., 2000; Gestri et. al., 2005). In medaka fish a screen to identify the direct interacting partners of \textit{Six3} yielded \textit{geminin} (Gem) – an inhibitor of DNA replication (Del Bene et. al., 2004). The \textit{Six3} and \textit{Gem} proteins form a complex that inhibits \textit{Gem}’s ability to inhibit DNA replication by sequestering \textit{Cdt1} (Del Bene et. al., 2004). By partnering with \textit{Gem}, \textit{Six3} promotes cellular proliferation thereby increasing size of the eye field.

The decision to terminally differentiate or continue to proliferate is made during the G1 phase of the cell cycle by the RPCs (Ohnuma et. al., 1999). Usually, if the decision is to differentiate, then the cell enters G0 phase and is prohibited from re-entering the cell cycle. An exception to this behaviour is represented in the Müller glial cells of the retina which enter the G0 phase but still have the capacity to re-enter the cell cycle (Welcker and Clurman, 2005). Certain Cyclin/Cyclin-dependent kinase (CDK) enzyme complexes are active during the G1 to arbitrate between differentiation or proliferation. These complexes are: CyclinD:CDK4/6 or CyclinE:CDK1/2 (Welcker and Clurman, 2005; Duparc et. al., 2007). On the other hand, certain CDK inhibitors also influence decisions by altering the activity of cyclin/CDK complexes. Out of the two major families of CDK inhibitors in mammals (INK and Cip/Kip family), in \textit{Xenopus} only the Cip/Kip family has been identified (Su et. al., 1995).
Over-expression of Rx1 by injection of Rx1-mRNA significantly increases the levels of CyclinD1 (which is one of the major expressed cyclins in the RPCs), and simultaneously inhibits the expression of p27Xic1 (Casarosa et. al., 2003). Six3 and Six6, in combination bind to the promoter of p27Kip1 and repress its activity, thereby promoting proliferation (Li et. al., 2002). Six3 also regulates the expression of cyclinD1 and p27 (Gestri et. al., 2005). Collectively, this suggests that the various eye field transcription factors have the ability to modulate cell cycle parameters during eye development. Cyclin D1 null mutant mice display hypocellular retinas which can be attributed to reduced proliferation (Sicinski et. al., 1995). On the other hand, over-expression of p27Xic1 results in increased number of ganglion cells, due to early cell-cycle exit, whereas over-expression of p27 keeps the RPCs in proliferation mode and produces later born cell types as well. (Ohnuma et. al., 1999).

**Project Outline**

Previous study involving the eye field transcription factors undertaken by Zuber and colleagues (2003) reported a model for eye field specification and lens induction (). They proposed a model of progressive tissue specification in which neural patterning is Otx2 driven - without it there is no anterior neural plate for eye field specification. Lastly they proposed a permissive feedback loop that exists between subsidiary eye field transcription factors such as Pax6 and Six3 that determine the eye field domain. Their model suggests that Otx2 is one of very early genes expressed followed by Rx1, Pax6 and Six3. The model was derived by injection of transcription factor RNA (alone or in combination) into embryos, and the subsequent analysis of ectoderm explants by RT-
PCR. Two of the interesting conclusions were derived in the model were firstly, _Rx1_ is be upstream of _Six3_ and _Pax6_; secondly, _Rx1_ inhibits the expression of _Otx2_.

**Figure 1.7:** Summary model of eye field induction in the anterior neural plate proposed by Zuber and colleagues.

Arrows indicate regions of discrepancies observed between proposed model and our study (Adapted and modified from Zuber et. al., 2003)

There are several weakness of this model. First, _Otx2_ and _Six3_ are the very first expressed markers in the late gastrula and their expression precedes _Rx1_ even before the anterior neural plate is defined (Ghanbari et. al., 2003; Chow and Lang, 2001; Zuber et. al., 2003). It is hard, therefore to understand how _Rx1_ could enjoy hierarchical prominence given that it expresses too late to be a candidate. Second, the null mutant phenotypes for _Six3, Pax6_, and _Rx1_ respectively define progressively diminished spheres of influence: if _Rx1_ was the primary instigator of optic patterning, then it would have been logical for it, not _Six3_ to cause the more globally deleterious phenotype of holoprosencephaly. Instead, _Rx1_ mutants display partial eye phenotypes.
As a result we decided to further investigate the relationship shared between Six3, Otx2, Pax6, Rx1 and other eye field genes – in a whole embryo system, employing whole-mount in situ hybridizations and RT-PCRs.

The main objective of my project was to understand the role of Six3 and Pax6 in early inductive events associated with eye development. To characterize and understand the functional role of Six3 and Pax6, loss of function analysis was carried out by microinjection of morpholino oligonucleotides directed against Six3 and Pax6. Translational knockdown of Six3 and Pax6 creates phenotypes that have not been observed previously in Xenopus. The relationship between Six3 and Pax6 is intriguing, and not many studies have examine it closely – to what extent do they operate in parallel, or does Six3 regulate Pax6 during retina specification as it does for lens (Zuber et. al., 2003; Loosli et. al., 1999; reviewed in Zuber M, 2011). Another important question was to what extent is the relationship between the two transcription factors identical in optic and brain regions? How do they exert their effects upon each other and upon Rx1 during eye development? The final step was to look at the putative downstream genes, including Sox2, Otx2, BMP4, MafA, Lens1, Pitx3, γ-crystallin and Krox20.
References


Ref Type: Serial (Book,Monograph)


Chepelinsky, A. B. Structural function of mip/aquaporin 0 in the eye lens; Genetic defects lead to congenital inherited cataracts. 190, 265 -297. 2009. Ref Type: Serial (Book,Monograph)


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the bicoid class and demarcates anterior neuroectoderm in the gastrulating mouse embryo. EMBO Journal 12:2735-2747.


Zuber, M. E. Eye field specification in Xenopus laevis. 93[C], 29-60. 2010. Ref Type: Serial (Book,Monograph)

Chapter 2:
Six3 activation of Pax6 is essential for normal eye morphogenesis

Summary

Amphibians have provided an accessible model to study eye development for almost a century. Despite the amphibian markers that have been developed, the surgical manipulations performed, and the surplus of information derived from mammalian and other genomes, just how the transcription factors involved in patterning of the brain and eye interact in a network has remained remarkably opaque to understanding. The various transcription factors expressed in the Xenopus eye region have a dynamic and overlapping pattern of expression in the anterior neural plate and presumptive lens ectoderm. We have used inactivation of Six3 and Pax6, by morpholino mediated knockdown, to study craniofacial abnormalities that involve, holoprosencephaly, reduced forebrain and eyeless phenotypes. We here that Six3 is required for early Pax6 activation and maintenance. Using mRNA over-expression studies – we report here that Pax6 can rescue the expression of Rx1 and Sox2 upon Six3 knockdown. Lastly, we also examine the effects of Six3 and Pax6 knockdown on a subset of eye field markers – Otx2, MafA, Pitx3, Lens1, Pax2, and γ-crystallin. Each of them plays an important role specifying a neural bias, controlling proliferation in the anterior neural plate, and inducing lens and retina. Lastly we refine the eye field induction model which situates, Six3 and Pax6 in the eye development process.
Introduction

Amphibians have provided an accessible model to study eye development and the genetics that underlies induction for almost a century. However the orchestration and interaction of transcription factors involved in patterning the brain and eye have remained remarkably opaque to understanding. Experimental evidence has shown that vertebrate eye development, specifically formation of the lens, requires a well coordinated process with interactions between the neural retina and the non-neural surface ectoderm (Spemann 1938 and Grainger 1996). These interactions give rise, respectively, to the optic stalk, pigmented and neural retina on the one hand, and the lens and cornea on the other (Chow et. al., 1999).

The genetic processes that regulate eye induction and development include genes that begin their expression during gastrulation, and continue to express in the regions of presumptive eye field in the anterior dorsal region, and at later stages are restricted to the eye domain. Several of these genes have been identified to play an important role in eye development, and a group of them that have been classified as the eye field transcription factors include, but are not restricted to: Six3, Pax6, Otx2, Rx1, Sox2, Pitx3, MafA, Pax2 and Lens1 (Zuber et. al., 2003; KhosrowShahian et. al., 2005).

The two most prominent players appear to be the paired box gene Pax6, and a sine oculis (so) homolog, Six3. With the evolutionary evidence available it is clear that the common factor associated with eye development across species is Pax6, hence accredited as the eye master gene (Quiring et. al., 1994; Gehring et. al., 1996; Chow et. al., 1999).
al., 1999) Both, *Pax6* and *Six3* encode homeodomain transcription factors, but their hierarchical relationship is not clear.

*Six3*, a SINE class homeobox gene is expressed in the anterior neural plate in *Xenopus*. At later stages, *Six3* expression restricts to the eye and the base of the diencephalon (Ghanbari et. al. 2001). In *Drosophila*, the *so* gene is important for specification of the eye primordium (Serikaku and O’Tousa, 1994, Wawersik and Maas, 2000). *Six3* null mice mutant embryos display both loss of telencephelon and abnormalities of craniofacial regions (Oliver et. al. 2008). In humans *SIX3* mutation results in holoprosencephaly and cyclopia (Wallis et. al., 1999).

Over-expression of *Six3* in medaka fish results in formation of ectopic retinal primordial and enhanced expression of *Pax6* and *Rx2* in the brain (Loosli et. al., 1999). In zebrafish *Six3* overexpression resulted in expansion of rostral brain structures and enhanced expression of *Pax2* in the optic stalk (Kobayashi et. al, 1998). *Six3* has also been shown to indirectly regulate proliferation in the retinal precursor cells by binding to *Geminin* (an inhibitor of DNA replication), allowing *Cdt1* to assemble to the pre-replication complex (Del Bene et. al., 2004). During the early stages of development, *Six3* promotes proliferation and inhibits pre-mature neurogenesis by negatively regulating cell cycle exit markers such as *cyclinD1* and *p27Xic1* and positively regulating proliferation markers which include, *Xic2, X hairy2, Xbf1* and *Rx1* (Gestri et. al. 2005). Moreover, acting as a direct inhibitor of *BMP4*, *Six3* supports dorso-anterior patterning thereby creating a neural bias in formation of the neural plate (Gestri et. al. 2005; Ando et. al., 2005).
Along with BMP4 repression, Six3 also directly represses Wnt1 resulting in a proper anterior-posterior patterning of the diencephalon (Gestri et. al., 2005; Lavado et. al., 2008). As a result, Six3 plays a dual role both in dorso-ventral, as well as anterior-posterior neural and eye patterning.

Pax6, encodes a paired class homeodomain transcription factor that is critical for lens and retina development: its mutation results in aniridia in humans, small eye (sey) in mouse, and the eyeless phenotype in Drosophila (Gehring et. al., 2002). In Xenopus embryos, Pax6 expression is observed in neuroepithelial cells which lay the foundation for the prospective retinal epithelium and the neural retina. Ectopic expression of mouse Pax6 in Drosophila imaginal discs results in formation of ectopic eyes, suggesting that both its function as well as the context of its genetic interactions are evolutionarily conserved (Halder et. al., 1995). On the other hand, Pax6 over-expression results in the induction only of ectopic lens and not retina in Xenopus: Pax6 is potent and capable to induce lens formation factors in ectoderm and in the absence of retinal factors (Altmann et. al. 1997).

Eye development and lens induction involves discrete steps. Early expression of Six3 and Otx2 in the dorsal anterior neural plate region at the completion of gastrulation defines the eye field and sets a road map for eye development. Six3 expresses as an autoregulating planar signal from the anterior neural plate which forms the optic vesicle and eventually induces factors along for a coordinated development of the eye resulting in induction of the lens in the neuro-ectoderm (Chow and Lang, 2001).
Previous studies have employed gain- and loss-of-function analysis, as well as whole embryo or animal cap RT-PCR assays to elucidate the hierarchical relationships of these two genes during eye development. These strategies have delivered fruitful insights, but have not permitted a direct analysis of gene effects at the level of discrete tissues. We further refine the models proposed by others by suggesting that role of \( \text{Six3} \) is important to the early activation and maintenance of \( \text{Pax6} \) expression specifically in eye primorida.

Morpholino mediated knockdown of \( \text{Six3} \) in \textit{Xenopus} confirms expectations resulting in the loss of eye and brain structures and its role in early eye and brain patterning and results in impaired \( \text{Pax6} \) activity. On the other hand \( \text{Pax6} \) knockdown results in absence of lens, distorted retinal pigmentation development and a small eye phenotype suggesting that \( \text{Pax6} \) plays an important but relatively subsidiary role in early eye field domain specification. Consonant with this hierarchy of effect, \( \text{Six3} \) morphants can be rescued by \( \text{Pax6} \) ectopic expression. We also report here that in absence of \( \text{Six3} \), \( \text{Pax6} \) fails to orchestrate and co-ordinate the expression of \( \text{Rx1} \) and \( \text{Sox2} \), two key players in proliferation and differentiation. Finally, we also report that \( \text{Six3} \) and \( \text{Pax6} \) perturbation have hierarchically consistent effects on \( \text{Otx2}, \text{Lens1}, \text{Pax2}, \text{Pitx3}, \text{MafA} \) and \( \gamma \)-crystallin expression during early eye morphogenesis.
Materials and Methods

Embryos

*Xenopus laevis* were obtained from Xenopus I, Inc., (Michigan, USA). Animals were reared in accordance to University, Federal and Provincial regulations. Ovulation was induced in adult female frogs by injecting 0.6 – 0.8 cc of Chorionic Gonadotrophin (HCG) hormone (Intervet Canada Corp., Ontario, Canada). Dejellying and fertilization of eggs were done as previous described (Drysdale and Elinson, 1991). Embryos were staged as per Nieuwkoop and Faber (1967), fixed with MEMPFA, and stored in 70% methanol.

Morpholino Design

Morpholino oligonucleotide (MO) directed against *Six3* and *Pax6* were designed and ordered from Gene Tools, LLC (Orlando, USA). *Six3-MO* sequence targeting the 5’ UTR *Six3* region was GGGACAGCACGAGCCGCACACAAAA. An alternate *Six3-MO* sequence was designed to confirm the specificity of morpholino effect. *Six3-MO-ALT* sequence was: GAAGCAGCAAAAACATAGCGACAGCGA. *Pax6-MO* sequence targeting the transcriptional start site was: CAAGGGACTGTGTAATTCCCAACAT. *Pax6-MO-ALT* sequence designed to confirm phenotype effects was: GATCAACGCCTAGTGATTTTCCCCCT. Sequences for Control-MO were as follows: *Six3-Control-MO*: GGcACAcCAGAcCCcCACAgaAAA *Pax6-Control-MO*: CAtGaGACTcTGTAaTaTgCCAAACAT. Morpholinos directed against the gene were labeled with 3’-Carboxyfluoroscein and Control Morpholinos was labeled with 3’-
Lissamine. A generic 3’-Carboxyfluoroscein morpholino was injected - controlled for fluoroscein effects.

**Generation of Rescue RNA and RNA for over-expression**

*Six3* full length cDNA minus the 5’UTR (thereby losing the *Six3-MO* target site) was amplified using Phusion High Fidelity DNA Polymerase (NEB), and cloned to pCS2- vector at the EcoRI and XhoI sites. Initial denaturation 98°C (30 seconds), denaturation 98°C (10 seconds), annealing temperature 67°C (30 seconds), extension 72°C (30 seconds) for 35 cycles, and final extension 72°C (10 minutes). The forward primer was (EcoRI): CC(GGAATTCC)ATCCCATGCTGTCG and reverse primer (XhoI): CCG(CTCGAG)TGGCTAAATAGGGGCTCG. Clones for *Six3Δ5UTR* were confirmed by sequencing (Robarts Research Institute, London, Ontario, Canada).

*Pax6* full length cDNA was amplified and site directed mutagenesis was used to alter the morpholino binding site without affecting the sequence of translated protein. The following primers were used to amplify and clone into the PCS2- vector at the BamHI and XhoI sites:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>CGC(GGATCC)GCAGATTTATGcATcACtCAaTCCCTGGGAGGAGAAGC</td>
</tr>
<tr>
<td>Reverse</td>
<td>CCG(CTCGAG)GTCCTTTTCCAGTTTGTCAGTC</td>
</tr>
</tbody>
</table>

Using Phusion High Fidelity DNA Polymerase (NEB), initial denaturation 98°C (30 seconds), denaturation 98°C (10 seconds), annealing temperature 68°C (30 seconds), extension 72°C (30 seconds) for 35 cycles, and final extension 72°C (10 minutes). An internal primer (GGTCGGCCGTTGACAAACACTC) downstream of the mutation was designed to
confirm the mutation for Pax6-MorphALT was verified by sequencing (Robarts Research Institute, London, Ontario. Canada).

Synthetic capped mRNA was transcribed using mMessage machine (Ambion, Inc.) for Six3 (SP6/KpnI, Zuber et.al., 2003), Six3Δ5UTR (SP6/KpnI), Pax6 (SP6/NotI, Zuber et. al., 2003), Pax6-MorphAlt (SP6/NotI) and GFP (T3/NotI, Khosrowshahian et. al., 2005). Capped RNA was aliquoted and stored in RNAse free water at -80°C until use. 150pg of RNA was injected into embryos, unless otherwise specified.

**Microinjection**

Both control as well as targeting morpholino and mRNA injections were made into the eggs using a Drummond nano-injector. Injection volume was maintained at 4.6nL and injections were made into: the animal pole of the embryos either at 1-cell; or unilaterally into one of the blastomeres at the 2-cell stage. Injected embryos were permitted to heal in 0.3x MBS with 2% Ficoll-400 (Sigma) at 12°C for 60 to 90 minutes and then later transferred to 0.1x MBS. Each plate was labeled with the number of viable embryos and detailed record was kept of the number of surviving embryos every few hours and the day after injection. Dose response curves were derived for all treatments. The amount of morpholino injected was 20ng for most injections and 150pg of RNA. GFP RNA was co-injected as lineage tracer when transcription factor mRNA injections were performed.
**Wholemount *in situ* hybridization**

Digoxigenin labeled probes were synthesized (as described in Table 2.1) and *in situ* hybridization was performed essentially as per Smith and Harland, 1991. Post *in situ* hybridization, embryos were bleached and cleared through a treatment of benzyl alcohol/benzyl benzoate. A minimum of three biological replicates of treatment cohorts were assayed for each probe. Images were captured using Northern Eclipse software (Empix, Canada).
Table 2.1: List of plasmids with their respective linearizing restriction enzyme and RNA polymerase used to synthesize riboprobe.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Restriction Enzyme</th>
<th>Polymerase</th>
<th>Source/Reference</th>
</tr>
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<tbody>
<tr>
<td>Six3</td>
<td>HindIII</td>
<td>T3</td>
<td>Zuber et. al., 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dr. M. Zuber</td>
</tr>
<tr>
<td>Pax6</td>
<td>Xba</td>
<td>T7</td>
<td></td>
</tr>
<tr>
<td>Rx1</td>
<td>HindIII</td>
<td>T3</td>
<td></td>
</tr>
<tr>
<td>Sox2</td>
<td>EcoRI</td>
<td>T7</td>
<td>Accession: AF022928</td>
</tr>
<tr>
<td>Ptx3</td>
<td>EcoRI</td>
<td>T7</td>
<td>Khosrowshahian et. al., 2005</td>
</tr>
<tr>
<td></td>
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<tr>
<td>MafA</td>
<td>BamHI</td>
<td>T7</td>
<td>Kataoka et. al., 2004</td>
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<tr>
<td>Otx2</td>
<td>SacI</td>
<td>T7</td>
<td>Blitz and Cho, 1995</td>
</tr>
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<td></td>
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<td>Dr. Ira Blitz</td>
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<tr>
<td>Lens1</td>
<td>SacI</td>
<td>T7</td>
<td>Accession: AF186464</td>
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<tr>
<td>Pax2</td>
<td>EcoRI</td>
<td>T3</td>
<td>Heller and Brändli, 1997</td>
</tr>
<tr>
<td>γ-crystallin</td>
<td>SacI</td>
<td>T3</td>
<td>Dr. Jonathan Henry</td>
</tr>
<tr>
<td>Krox20</td>
<td>EcoRI</td>
<td>T7</td>
<td>Dr. Marc Amoyel</td>
</tr>
</tbody>
</table>
Protein Isolation and Western Blots

Protein was isolated from a batch of 20 embryos using a lysis buffer which comprised 20mM Tris (pH8.0), 100mM NaCl, 1mM EDTA, 0.5% TritonX-100, 0.5% SDS, 10% glycerol (protocol kindly provided by Dr. Kristen Kroll) and protease inhibitor cocktail tablets (Roche). Embryos were lysed, sonicated, centrifuged, and the lysate was stored at -20°C. Protein concentration was determined using the Bradford Assay. 30µg of protein was loaded per well onto mini-protein 12% SDS-PAGE gels. Transfer to PVDF membrane (Roche) was performed using standard protocols (Hoefer Scientific, Semi-Phor Blotter). Membranes were blocked in 5% milk in Tris Buffer Saline-Tween (TBST) and incubated over-night with 1:1000 of primary antibody (mouse) for Six3 (kind gift of Dr. Paola Bovolenta) in 5% TBST and Pax6, 1:10,000 in 3% TBST (Developmental Studies Hybridoma Bank, University of Iowa, USA) respectively. Anti-mouse secondary antibody (Chemicon, AP308PMI) was used at 1:10,000 in 5% milk in TBST for 2 hours. For actin, 1:10,000 primary antibody raised in rabbit (A2066, Sigma) used was diluted in 2% milk/ TBST. Secondary antibody was goat anti-rabbit, 1:10,000 (Chemicon, AP132P) in 5% milk in TBST. Membranes were then washed 5x with TBST for 10 minutes each and exposed using chemiluminescence reagents (Super Signal West Pico, Thermo Scientific). Three biological replicates were assayed by Western blot for each experiment.

RNA Isolation, cDNA synthesis and RT-PCR analysis

RNA was isolated using TRIzol (Invitrogen) from batches of 20 embryos and stored at -80°C for each stage of interest. cDNA was synthesized using OminiScript RT
(Qiagen) and oligodT primer (Sigma). RT-PCR analysis was performed as previously described (Khosrowshahian et. al. 2005). cDNA used was equivalent to RNA pooled from 2 embryos. Dream Taq Polymerase (Fermentas) was used to determine the linear amplification range and the midpoint number of cycles was employed in probe-specific manner to perform semi-quantitative RT-PCR. Band density was quantified using Gene-Tools imaging software (Syngene). Lists of primers used for RT-PCR described in Table 2.2. Experiments were replicated a minimum of three times.

**Histological Sectioning**

Embryos were dehydrated through methanol to xylene and embedded in paraplast media (Sigma). Sectioning was carried out using microtome (American Optical Company, 820 Spencer). Images were acquired using Northern Eclipse software (Empix, Canada).
Table 2.2: List of primers used for semi-quantitative RT-PCR analysis (supplementary table)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Annealing Temperature (°C)</th>
<th>Cycle Number</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Pax6 | FP: GCAACCTGGCGAGCGATAAGC  
      | RP: CCTGCCGTCTCTGGTTCCGTTAGTT | 57           | 28              | 450               | Zuber et. al., 2003 |
| Rx1  | FP: CCCCAACAGGAGCATTTAGAAGAC  
      | RP: AGGGCACTCATGGCAGAAGGTT   | 67           | 27              | 416               | Zuber et al., 2003  |
| Sox2 | FP: GAGGATGGACACTTATGCCAC    
      | RP: GGACATGCTGTAGGTAGGCGA     | 68           | 27              | 214               | Nitta et. al., 2006 |
| BMP4 | FP: GAGATTGTCCATTCTCTGTCGC   
      | RP: TCAGTGGAAGAAGTCAGCGC      | 62           | 26              | 262               | Malarte et. al., 2006 |
| Otx2 | FP: GGATGGATTTGTACATCCGTC    
      | RP: CACTCTCGAGCTCAGCTCCC     | 57           | 27              | 315               | Zuber et. al., 2003  |
| Six3 | FP: TTGTCTGTCTGCTTTGTT       
      | RP: TTGTGTGTTGTGTTATATC       | 57           | 28              | 369               | Zuber et. al., 2003  |
| MafA | FP: CTTGCTCTCCCTCAATCTCTGG   
      | RP: CCGACAGAGGCGAAGGCTGGT     | 57           | 30              | 331               | Ishibashi et. al., 2001 |
|----------|---------------------------|-------------------|-----|-----|------|-----|-----|------|-----------------------------|
| Pitx3    | FP: AAGTCCGTTGTCACTCAG    | RP: CTTCTGGAAAGTGGAGC | 57  | 32  | 560  |     |     |      | Khosrowshahian et al., 2005  |
| γ-crystallin | FP: CAAGGGCAGATGAGGAGTT | RP: GAGGCTCCCCAGTCAGTGA | 57  | 30  | 185  |     |     |      | U48901 (Unigene)             |
| Krox20   | FP: AACCGCAGCCAAGACC     | RP: GTGTCAGCCTGTCTGTAG | 57  | 28  | 448  |     |     |      | Xenopus Resource Centre (Xenbase) |
| EF1α     | FP: CAGATTGGTGGATATGG    | RP: ACTGCCTTGATGACTCTTA | 57  | 24  | 268  |     |     |      | Khosrowshahian et al., 2005  |
Results

Morpholino mediated *Six3* knockdown confirms its role in eye and brain development

To understand the role of *Six3*, we impaired translation of *Six3* mRNA by means of a morpholino oligonucleotide. *Six3* inactivation was dose dependant and correlated with increasingly severe phenotypes (Table 2.3). Western blots confirmed that 20 ng of the morpholino resulted in complete translational block (Figure 2.1A, lane 5). By contrast, when 150 pg of *Six3* mRNAΔ5’UTR (lacking the morpholino target site) was co-injected with 20 ng of *Six3* morpholino, Six3 translation could be restored and phenotypes rescued (Figure 2.1A, lane 6).

Phenotypes were classified into three categories characterized as: severe – improper closure of the neural fold and absence of anterior structures (Figure 2.1 B,C); moderate – complete or severe loss of eye structures with head nevertheless identifiable (Figure 2.1 F, G); mild - slightly smaller eyes (Figure 2.1 D, E). Table 2.1 summarizes *Six3* knockdown phenotypes. An alternate *Six3* morpholino was used to confirm the phenotype (data not shown), and a control morpholino with positional substitutions produced few effects nonspecifically, and only at the highest doses.

*Six3* knockdown also confirmed a role for the gene in modulating dorsal – ventral characteristics. Lower morpholino concentrations left 75% of the pigmented retinal domains circular, however when the morpholino concentration was increased, only dorsal retinal regions formed (Figure 2.1 H, I. J). This, in conjunction with RT-PCR data of
BMP4 confirms a role for Six3 role in dorso-ventral patterning of the eye by inhibiting activity of BMP4 (current study and Gestri et. al., 2005). Conversely, over-expression of Six3 results in reduced BMP4 expression (Figure 2.1K).
**Figure 2.1: Six3 expression is essential for normal eye and brain development**

**A:** Efficacy of Six3 morpholino was determined using Western blots. Embryos injected with control Six3-MO did not show any reduction in Six3 protein levels (Lane 2). Control-MO in combination with Six3 resulted in enhanced Six3 expression (Lane 3). Down regulation of Six3 using morpholino at 20ng resulted in complete translational inhibition (Lane 5). Six3 morpholino co-injected with Six3 mRNAΔ5’UTR (lacking the morpholino site) restored translation of Six3 (Lane 6). Actin was used a loading control.

**B,C:** Six3 morpholino injections resulted in severe phenotypes which exhibit improper closing of the neural tube and the loss of anterior structures. 

**D, E:** Moderate phenotype – lacking eye structures or displaying reduced eye structures. Early (D) and late (E) stage embryos displaying mild phenotype as characterized by reduced retina and eye structures.

**F,G:** Tadpole injected on the left side with Six3 morpholino completely lacks eye structures (D). Histological Section of early stage embryo injected with Six3 morpholino suggesting absence of retina and eye structures in comparison to the uninjected side. (E).

**H-K:** Six3 is important for dorsal-ventral eye patterning. Lateral view of tadpoles injected with Six3-MO with increasing concentrations. 5ng of Six3 morpholino inhibits only 25% of retinas to develop (H), 10ng inhibits roughly close to 50% of the retinas but most especially dorsal structures to form (I), 20ng of morpholino results in aberrant retinal development retina to develop (J) due to enhanced BMP4 expression as confirmed by RT-PCR (K, lane 5). Six3 over-expression results in depressed BMP4 mRNA levels (K, lane 7).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Six3-MO</th>
</tr>
</thead>
<tbody>
<tr>
<td>5ng</td>
<td>Wild type</td>
</tr>
<tr>
<td>10ng</td>
<td>Six3-CMO</td>
</tr>
<tr>
<td>20ng</td>
<td>Six3-CMO, 10ng</td>
</tr>
<tr>
<td>20ng</td>
<td>Six3-CMO, 20ng</td>
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<tr>
<td>20ng</td>
<td>Six3-MO, 10ng</td>
</tr>
<tr>
<td>20ng</td>
<td>Six3-MO, 20ng</td>
</tr>
</tbody>
</table>

**Western Blot**

- **A**
  - Six3
  - Actin
  - Six3-MO, 10ng
  - Six3-MO, 20ng
  - Six3-MO, 20ng + Six3 mRNA-5'UTR

**Immunohistochemistry**

- **B**
  - Six3-MO
  - Six3-MO

- **C**
  - Six3-MO
  - Six3-MO

- **D**
  - Six3-MO
  - Six3-MO

- **E**
  - Six3-MO
  - Six3-MO

- **F**
  - Six3-MO
  - Six3-MO

**Quantitative RT-PCR**

- **G**
  - Six3-MO
  - Six3 mRNA-5'UTR

**Gene Expression**

- **H**
  - 5ng Six3-MO
  - 10ng Six3-MO
  - 20ng Six3-MO

- **I**
  - BMP4
  - Ef1a

- **J**
  - Six3 Morpholino
  - Six3 mRNA (150pg)

**Control**

- Control
- 5ng
- 10ng
- 20ng
**Table 2.3:** Effect of *Six3* morpholino mediated knockdown with percentages of phenotypes observed at different concentrations of the Morpholino injected.

<table>
<thead>
<tr>
<th>Morpholino Concentration (Six3)</th>
<th>5ng</th>
<th>10ng</th>
<th>20ng</th>
<th>30ng</th>
<th>20ng Control MO</th>
</tr>
</thead>
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<tr>
<td></td>
<td>n</td>
<td></td>
<td></td>
<td></td>
<td>n</td>
</tr>
<tr>
<td>Severe Phenotype</td>
<td>0%</td>
<td>2%</td>
<td>34%</td>
<td>36%</td>
<td>1.3%</td>
</tr>
<tr>
<td>Moderate Phenotpye</td>
<td>0%</td>
<td>3.5%</td>
<td>29%</td>
<td>4%</td>
<td>1%</td>
</tr>
<tr>
<td>Mild Phenotype</td>
<td>11%</td>
<td>54%</td>
<td>17%</td>
<td>8.5%</td>
<td>5.2%</td>
</tr>
<tr>
<td>Normal eye</td>
<td>77%</td>
<td>22.5%</td>
<td>7%</td>
<td>3.5%</td>
<td>79%</td>
</tr>
<tr>
<td>Dead</td>
<td>12%</td>
<td>18%</td>
<td>13%</td>
<td>48%</td>
<td>13.5%</td>
</tr>
</tbody>
</table>
**Six3 expression is required to maintain Pax6 levels during early eye development.**

Unilateral morpholino-mediated knockdown of *Six3* - the un-injected side serves as a control - inhibited *Pax6* expression at both early and late stages (Figure 2.2B-D’). Western blot analysis for Pax6 protein shows it is reduced by *Six3* morpholino injection (Figure 2.2 A, lane 8). Conversely, and as demonstrated by others at the RNA level (Zuber et. al., 2003), we here confirm *Six3* over-expression enhances Pax6 protein levels (Figure 2.2A, lane 9). Although low concentrations of *Six3* morpholino do not appear to produce any change in *Pax6* mRNA levels as assessed by RT-PCR, higher levels of 20ng and 25ng morpholino reduced *Pax6* expression levels to half that of wild type controls (Figure 2.2 E, F). Conversely, ectopic expression of *Six3* mRNA results in *Pax6* up-regulation by 1.5 fold (Figure 2.2 E, F).

The next step was to see if *Six3* knockdown has any effect on activation of *Pax6* at the early neurula stage. Activation of *Pax6* in *Six3* morphants is delayed compared to wildtype. As seen in Figure 2.2G, in wild-type the expression of *Pax6* is first recorded at stage 12, however in *Six3* knockdown embryos – *Pax6* expression is delayed to start at stage 14. This suggests that *Six3* expression is required to activate and maintain *Pax6* levels during early eye development.
Figure 2.2: Six3 is essential for early Pax6 activation and maintenance

A: Western blot confirming the effect of Six3 knockdown on Pax6 protein levels. As seen, Six3 perturbation inhibits Pax6 (lane 4). Six3 over-expression enhances Pax6 (lane 5).

B-D’: Whole mount in situ hybridization showing the effects of Six3 morpholino mediated knockdown on Pax6 expression. Embryos were injected on the right side with Six3 morpholino. Pax6 expression is inhibited on the injected side with no change in the contralateral uninjected control side. Arrows show the effect of Six3 perturbation on Pax6 expression, stage 14 (B), stage 19 (C), stage 27 (D,D’). There is no change observed in Pax6 expression along the neural tube, only eye field expression is diminished (D’).

E,F: RT-PCR analysis to validate and complement the in situ results from B-D’ suggest that Pax6 mRNA expression level is inhibited by Six3-morpholino and that the reverse trend is produced by Six3 over-expression (E, lane 9). Pax6 levels are normalized with respect to Ef1a expression (F).

G: Six3 morpholino mediated knockdown results in delay in Pax6 activation. Wild type level of Pax6 can be detected stage 12 onwards, however in Six3 knockdown embryos, Pax6 is activated late in stage 14 (lane 4).
Wild type
Six3 - C-MO, 20 ng
Six3 - MO, 10 ng
Six3 - MO, 20 ng
Six3 mRNA, 150 pg
Pax6 C-MO, 20 ng
Pax6 MO, 10 ng
Pax6 MO, 20 ng
Pax6 mRNA, 150 pg

Pax6 mRNA levels

Relative Pax6 mRNA levels

stage 10.5 12 13 14 15 16 17
wt
Six3-MO

wt
Six3-MO
Pax6 morpholino mediated knockdown results in eye deformities including aberrant RPE development and lens induction

In Xenopus, Pax6 morphants display concentration dependent effects (Table 2.4). An alternate Pax6 Morpholino was used to confirm the phenotype (data not shown), and a control morpholino with positional substitutions produced rare non-specific effects, and only at the highest doses. Western blots confirmed that 20ng of Pax6 morpholino results in complete translational block (Figure 2.3A). Deformities in the RPE and absence of lens were clearly observed on the Pax6 morpholino injected side of the embryo as compared to the contra-lateral uninjected side. In contrast to the Six3 knockdown experiments, severe deficiencies of craniofacial patterning and neural fold were seldom if ever seen. Pax6 over-expression has been reported to induce ectopic eyes in flies but only lens in vertebrates, where it also expands the existing eye domain (Chow et. al., 1999; Zuber et. al. 2003).

Phenotypes obtained upon Pax6 knockdown ranged from: abnormalities in the retinal development (Figure 2.3B, C, D); absent lens (Figure 2.3 C, E); and no eyes (Figure 2.3 E). The most frequently recurring phenotype exhibited retinal deformities, indicating the role of Pax6 in the early inductive events involved with lens and retina (Figure 2.3 B, C, D, E, E’).

Upon Pax6 knockdown, the expression domain for Six3 was not affected at early stages, however at late stages the eye field domain for Six3 was reduced (Figure 2.3J). Compared to the uninjected side, ectopic Pax6 expression later expanded the Six3
expression domain at early to mid neurula stages – this suggests Pax6 feedbacks upon Six3 very early in development (Figure 2.3 H,I)
**Figure 2.3:** *Pax6* is important for retina and lens induction, development and maintenance.

**A:** *Pax6* morpholino mediated translational blocking efficacy was confirmed using Western blots. Control MO did not have any effect on *Pax6* protein levels, however, *Pax6* morpholino at 15ng (lane 5) and 20ng (lane 6) inhibited protein translation as compared to control (lane 2). *Pax6* mRNA with mutated morpholino sites co-injected with the morpholino was able to rescue the effect of the knockdown (lane 7).

**B-E:** Phenotype severity observed in *Pax6* morphants. Phenotypes showing retinal deformities (B,C). Loss of ventral retina (eye) structures and aberrant RPE development (D). Abrogated RPL and absence of eye primordium, lens, neural retina, corneal epithelium (E, E’). Uninjected side of embryo displaying completely normal lens development (E’’). cns: central nervous system; rpl: retinal pigmented layer; nr: neural retina; le: lens; ce: corneal epithelium.

**H-J’:** *Pax6* mRNA enhances *Six3* levels. Whole mount in situ hybridization displaying early neural plate development stage effect of ectopic *Pax6* levels results in enhanced *Six3* levels, stage 14, left (H), stage 19, left (I). *Pax6* feeds back upon *Six3* by defining the eye field domain and limiting *Six3* expression. Later stages show that *Pax6* morpholino reduces *Six3* expression, stage 26, injected (J), un-injected (J’).
Table 2.4: Effect of *Pax6* morpholino mediated knockdown with percentages of phenotypes observed at different concentrations of the Morpholino injected.

<table>
<thead>
<tr>
<th>Morpholino Concentration (Pax6)</th>
<th>5ng</th>
<th>10ng</th>
<th>20ng</th>
<th>30ng</th>
<th>20ng Control MO</th>
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<tr>
<td><strong>n</strong></td>
<td>133</td>
<td>101</td>
<td>156</td>
<td>84</td>
<td>136</td>
</tr>
<tr>
<td>Eyes absent</td>
<td>2%</td>
<td>3%</td>
<td>10%</td>
<td>6%</td>
<td>1%</td>
</tr>
<tr>
<td>RPE deformities</td>
<td>5%</td>
<td>8%</td>
<td>40%</td>
<td>30%</td>
<td>2%</td>
</tr>
<tr>
<td>Lens absent or reduced</td>
<td>2%</td>
<td>33%</td>
<td>38%</td>
<td>32%</td>
<td>3%</td>
</tr>
<tr>
<td>Normal eye</td>
<td>83%</td>
<td>44%</td>
<td>12%</td>
<td>5%</td>
<td>86%</td>
</tr>
<tr>
<td>Dead</td>
<td>8%</td>
<td>12%</td>
<td>10%</td>
<td>27%</td>
<td>8%</td>
</tr>
</tbody>
</table>
Pax6 is downstream of Six3: Pax6 alone augments Rx1 and Sox2 expression and can rescue Sox2 and Rx1 expression in Six3 knockdown embryos.

During the early stages of eye development, Rx1 and Sox2 are key markers expressed throughout the anterior neural plate, and at later stages, predominantly in the optic vesicles, and finally in the neural and pigmented retina (Kamachi et. al., 1998; Andreazzoli et. al., 1999; Gestri et. al. 2005). Rx1 plays a significant role in initial specification followed by successive proliferation of the retinal progenitor cells but is not essential for lens induction (Andreazzoli et. al., 1999). On the other hand, Sox2, concomitant with its expression in the optic vesicles, is also activated in the head ectoderm during lens placode formation. In co-operation with Sox3, it can induce \(\gamma\)-crystallin synthesis confirming a role in the differentiation of lens placode by the optic vesicle (Kamachi et. al., 1998).

Six3 inhibition results in near abolition of Rx1 at stage 19 (Figure 2.4B,C), when Rx1 normally expresses in the anterior neural plate, as well as later when Rx1 expression might otherwise express in the optic vesicle. Similarly, Pax6 knockdown phenocopies the same effect with regard to Rx1 expression in the anterior neural plate and presumptive eye field (Figure 2.4 D,E). Furthermore, in Six3 morphants Sox2 expression is reduced in the optic regions, however there is no effect outside the usual Six3 domains in the head ectoderm or in the neural tube at either early or late stages(Figure 2.5 B,C). Similar results are observed in following Pax6 knockdown (Figure 2.5 D, E). This confirms that Rx1 and Sox2 are downstream of Six3 and Pax6 in the optic field.
Over-expression of Pax6 results in expansion of Rx1 and Sox2 expression domains (Figure 2.4 F; Figure 2.5 F).

The next step was to see if ectopic Pax6 expression could phenotypically rescue Six3 morphants as reflected by restoration of Rx1 and Sox2. Rx1 and Sox2 expression are rescued and even expand slightly indicating that Pax6 likely operates upon these targets downstream of Six3(Figure 2.4G; Figure 2.5G). This is also consistent with our finding that in absence of Six3, Pax6 is ablated, and Rx1 and Sox2 are down-regulated. The results were confirmed by RT-PCR assays which confirmed the whole-mount in situ data (Figure 2.4 A,H; Figure 2.5A, H).
Figure 2.4: Pax6 can rescue Rx1 expression upon Six3 knockdown

A, H: RT-PCR analysis to confirm whole mount in situ hybridization results. Rx1 levels are reduced upon Six3 knockdown (lane 2) and Pax6 knockdown (lane 4). At the same time Pax6 over-expression enhances Rx1 levels (lane 6).

B-G: Whole mount in situ hybridization displaying Rx1 expression. Six3 morpholino injection results in abrogation of Rx1 expression at both early and late stages on the injected sides of the embryos (B,C). Pax6 morpholino treatment phenocopies Six3 knockdown with regard to Rx1 expression levels (D,E). Pax6 can enhance the expression domain of Rx1 (F). Co-injection of Six3 morpholino and Pax6 mRNA can rescue Rx1 expression (G). Arrows indicate change in expression levels.
A

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>Six3 - MO</th>
<th>Six3 - C - MO</th>
<th>Pax6 - MO</th>
<th>Pax6 - C - MO</th>
<th>Pax6 - mRNA</th>
<th>Six3-CMO + Pax6-mRNA</th>
<th>Six3-MO + Pax6-mRNA</th>
</tr>
</thead>
</table>

Rx1

Ef1a

B

C

D

E

F

G

H

Relative Rx1 mRNA levels

Wild type
Six3-MO
Six3-C-MO
Pax6-MO
Pax6-C-MO
Pax6-mRNA
Six3-CMO + Pax6-mRNA
Six3-MO + Pax6-mRNA
**Figure 2.5: Pax6 can rescue Sox2 expression upon Six3 knockdown**

**A,H:** RT-PCR analysis to confirm whole mount in situ hybridization results. Sox2 levels are reduced upon Six3 knockdown (lane 2) and Pax6 knockdown (lane 4). At the same time Pax6 over-expression enhances Sox2 levels (lane 6).

**B-G:** Whole mount *in situ* hybridization displaying Sox2 expression. Six3 knockdown inhibits Sox2 expression to be down regulated only in the eye field (PLE) region at both early and late stages (B, C). Pax6 knockdown inhibits Sox2 levels to be downregulated in the optic cup region at both early and late stages (D, E). Pax6 mRNA enhances Sox2 levels in the eye field (PLE) region (F). Six3 morpholino co-injected with Pax6 mRNA rescues Sox2 expression (G). Arrows indicate change in expression levels.
A

<table>
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<tr>
<th></th>
<th>Sox2</th>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>Six3 - C-MO</td>
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<td></td>
</tr>
<tr>
<td>Pax6 - C-MO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pax6 - mRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pax6 - C-MO + Pax6-mRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Six3-MO + Pax6-mRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Six3-MO</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B C D E F G H

**H**

Relative Sox2 mRNA levels

- Wild type
- Six3 - MO
- Pax6-C-MO
- Pax6-mRNA
- Six3-C-MO + Pax6-mRNA
- Six3-MO + Pax6-mRNA
Effect on eye marker genes following *Six3* and *Pax6* knockdown

The effects of *Six3* and *Pax6* knockdown upon other marker genes were also studied including: *Otx2*, *MafA*, *Pitx3*, *Lens1*, *Pax2*, *γ-crystallin* and *Krox20*. *Six3* knockdown has deleterious effects on *Otx2* expression at both early and late stages resulting in diminished *Otx2* levels (Figure 2.6A, B, C). Expression in the anterior neural plate and the eye field is diffuse. *Pax6* knockdown on the other hand does not show equally severe effects upon *Otx2*, however, it decreases the eye field diameter slightly when compared to un-injected contralateral controls (Figure 2.6D, D’). Both *Pitx3* and *MafA* are expressed in the presumptive lens ectoderm and play an important role in the lens induction process (Khosrowshahian et. al., 2005; Ishibashi et.al., 2002). *Six3* expresses in the pre-lens ectoderm (Gehring et. al., 1998) and when we knock it down, *MafA* expression is completely abolished from ectodermal regions (Figure 2.6.2 A., A’). *Pitx3* expression is also observed to be abrogated in *Six3* morphants (Figure 2.6.3 A, A’). Similarly, when *Pax6* is perturbed, *MafA* expression is completely abolished (Figure 2.6.2 B. B’) and *Pitx3* expression is reduced (Figure 2.6.3 B,B’). This is consistent with reports that *Pax6* controls the eye field size (Zuber et. al., 2003; Andreazzoli et. al., 2002). However, *MafA* expression remains relatively normal, and although it still reduces in 25% of cases (Figure 2.6.2 C,C’) there must be other factors playing a role in lens signaling in the absence of *Pax6*. *Lens1* and *γ-crystallin* expression was completely abolished in *Six3* and *Pax6* morphant embryos, confirming the absence of differentiating lens (Figure 2.6.3 C, D; 2.6.4 C, D). In *Six3* morphants, *Pax2* expression at the early stages was completely abrogated (Figure 2.6.4 A), however, at later stages expression in the presumptive ventral lens ectoderm was inhibited whereas expression in the hindbrain
and midbrain was close to normal (Figure 2.6.4 B). In *Pax6* morphants, *Pax2* expression was reduced at both early and late stages, however not completely abolished (Figure 2.6.4 C,D). For *Krox20*, a hindbrain marker, no significant change was observed in either *Six3* nor *Pax6* morphants (Figure 2.6.5 A-D’).
Figure 2.6: Whole mount in situ hybridization to study effect on eye marker genes upon Six3 and Pax6 knockdowns.

Six3 and Pax6 morpholinos were injected on the left side of the embryos. Arrows show changes in expression levels and absence of expression. The right side of the embryo was un-injected and used as contralateral control.

2.6.1: A-D – Otx2;
2.6.2: A-C’ – MafA;
2.6.3: A-B – Pitx3; C-D - Lens1
2.6.4: A-D – Pax2; E-F - γ-crystallin
2.6.5: A-D’ – Krox20

Six3-MO: Six3 Morpholino; Pax6-MO: Pax6 Morpholino
Discussion

*Six3 plays a primary role in eye and brain development*

*Six3* is expressed at the anterior end of the early gastrula where the future neural plate will eventually form (Eagleson and Theisen, 2008; confirmed by us - data not shown). At late neurula stages, *Six3* expression is detected in the presumptive telencephalic, ventral diencephalon and retinal tissues (Eagleson and Theisen, 2008). Not surprisingly, given its role in brain patterning, *Six3* perturbation also results in abnormal craniofacial development. Lower concentrations of morpholino do not generate significant effects, however, increasing concentrations yield phenotypes with craniofacial abnormalities ranging from improper neural tube closure, to reduced or lost forebrain, and complete loss of eye structures. Our knockdown results clearly confirm the early role of *Six3* in specifying the anterior neural field necessary for the co-ordinated development of the brain and the eye. *Six3* null mutant mice fail to develop anterior structures, including the rostral diencephalon and telencephalon (Lavado et. al., 2008). In medaka fish, *Six3* morphants yielded an absence of forebrain and eyes (Carl et. al., 2002).

*Six3* overexpression in *Xenopus* and zebrafish has been shown to expand anterior neural plate at the cost of the non-neural ectoderm by repression of *BMP4* in the ectoderm adjacent to neural plate (Gestri et. al., 2005). Our results demonstrate that inactivation of *Six3* results in progressive increases in the ventralizing agent *BMP4*. As a consequence pigmented retina is only induced in dorsal regions of the eye and not in ventral regions, and this confirms the antagonistic relationship of *Six3* towards *BMP4*. 
\textbf{Six3 activation of Pax6 is essential for eye morphogenesis and lens development}

The relationship between Six3 and Pax6 is intriguing; however there is no clear definition of their hierarchical relationship other than in lens (Carl et. al., 2002; Zuber et. al., 2003; Gestri et.al., 2005). Six3 is expressed earlier than Pax6 (Ghanbari et. al., 1998; Zuber et. al., 2003). Here we show that in our Six3 morphant embryos, Pax6 expression is abolished in the anterior neural plate region. At later stages, once the eye field is determined, Pax6 expression is diffuse suggesting that Six3 is required to support the optic expression of Pax6. By contrast, Pax6 expression is not affected in the posterior neural tube where Six3 is not normally expressed. These results are consistent with the observation that Six3 mutant mice experience down-regulation of Pax6 in the lens placode (Liu et. al., 2006) and that in medaka fish, absence of Six3 reduces Pax6 expression in the retina (Carl et. al., 2002). Six3 also plays an important role during development of the optic vesicle, and later in optic vesicle involution as demonstrated in medaka fish (Carl et. al., 2002). Due to the importance of Six3 in the development of these proximal structures (diencephalon and optic vesicle), it is not surprising that more latter distal structures are impaired (RPE, NR and lens).

\textbf{Pax6 morphants only partially phenocopy Six3 misregulation}

Pax6 knockdown results in retinal deformities and absence of lens. In addition, Pax6 knockdown produced no effect upon early Six3 expression, however at later stages the expression domain for Six3 was reduced. Possibly, in this latter context, smaller optic field size or impaired retinal patterning that were due the absence of lens rudiments diminished the number of cells competent to respond and thereby express Six3. As shown
before in Pax6/- mutant mice, Six3 expression is completely unaffected (Kroll et. al., 2005). This suggests, early activation of Six3 is independent of Pax6, however, Pax6 appears to later play a role in the maintenance of Six3 when Pax6 over expression can enhance Six3 levels. Whether or not this role is direct or indirect remains to be elucidated.

**Ectopic Pax6 expression can rescue the expression of Rx1 and Sox2 in Six3 knockdown embryos.**

The relationship between Rx1 and Pax6 has been examined in various studies (Chow et. al., 1999; Harris et. al., 2002; Zuber et. al., 2003). In animal cap assays, Rx1 over-expression elicits increases in Pax6 levels. On the other hand, in animal caps ectopic Pax6 expression does not enhance Rx1 (Zuber et. al., 2003). However in whole embryos Pax6 over-expression results in expansion of the expression domain of Rx1 (Chow et. al., 1999). The latter result is consistent with what is reported here. This difference in experimental results can be explained by relating to the function of each of the transcription factors. One of the main functions of Rx1 is to enhance proliferation, therefore when it is over-expressed in animal caps, greater numbers of optic progenitor cells result with the consequence of higher expression of transcription factors including Six3. By contrast, there are constraints in whole embryos - ventral factors which possibly play a dominant role.

Six3 knockdown results in abrogation of Rx1 expression. Similar observations were faithfully phenocopied in Pax6 knockdown embryos. This suggests that Six3 and Pax6 are acting upstream of Rx1. We then undertook a complementation study which involved injecting Six3 morpholino along with Pax6 mRNA: expression of Rx1 in the
combination injection was restored to approximately normal suggesting *Pax6* to be intermediary and downstream of *Six3*.

*Sox2* a SRY-box 2 transcription factor is expressed in the *Xenopus* presumptive lens ectoderm during the neural tube closure and before the lens placode is induced. Once the lens placode is induced, *Sox2* is up-regulated in the lateral ectoderm overlying the optic vesicle. *Sox2* is also expressed in head ectoderm and along the neural tube. (Zygar et. al., 1998; Schlosser and Ahrens, 2004; Donner et. al., 2006). *Six3* or *Pax6* knockdown diminishes expression of *Sox2* significantly in the ectoderm overlying the optic vesicle. This is consistent with a role for *Pax6* in the induction in *Xenopus* of presumptive lens ectoderm. In mice, *Six3* directly activates *Sox2* in the presumptive lens ectoderm during the early stages of lens induction (Liu et. al., 2006).

**Six3 and Pax6 perturbation abrogate the expression of early and late eye field genes.**

The expression domains for *Six3* and *Pax6* have a distinct and overlapping patterns with both the early and late eye field genes. *Six3* perturbation results in a diffuse and well reduced expression of *Otx2*, and the complete absence of *Pax2, Pitx3, Lens1, MafA*, and *γ-crystallin*. *Otx2* is first expressed at about the same time as *Six3* in late gastrula embryos. In *Xenopus*, *Otx2* repression mediated by fusion of an engrailed repressor results in disorganized anterior development along with loss of eye structures (Isaacs et. al. 1999). *Otx2<sup>-/-</sup>* mice display absence of forebrain and mid-brain structures (Acampora et. al. 1995). *Six3* alone cannot induce a neural fate in *Xenopus* animal caps and requires *Otx2* (Gestri et. al., 2005) therefore it is likely that *Six3* partners with *Otx2* from the very early stages to define and specify anterior neural plate preliminary to laying
the foundation for eye development and morphogenesis. A similar permissive interaction between *BMP4* and *Otx2* is observed in early development of the cement gland (Gammill and Sive, 1999). Diminished *Six3* activity further relaxes constraints upon the influence of ventral genes like *BMP4*, resulting in ventralization of the embryo in the anterior dorsal regions and resulting in a forced non-neural. Possibly, expansion of the *BMP4* expression domain presents an insurmountable obstacle for remaining *Otx2* to sustain neural fates, resulting in ablation of neural structures. By contrast, *Pax6* knockdown does not yield severe effects on *Otx2* possibly reflecting the attribute that *Pax6* expresses later. Moreover, in *Xenopus* *Pax6* predominantly influences distal eye structures while *Otx2* influences more proximal ones. Interestingly, *Pax6* does specify the eye field in its entirety, but is important to the size of neurally derived optic structures (Loosli et. al. 1999) – presumably, *Pax6* reduces organ size by diminishing expression of *Six3* and *Otx2*. Consonant with this interpretation, *Otx2* conditional knockout mice exhibit deficiencies in differentiation of photoreceptor cells (Nishida et. al., 2003).

*MafA, Lens1, Pitx3 and γ-crystallin* are all affected by *Six3* and *Pax6* perturbation. These genes are later expressing and this places *Six3* and *Pax6* temporally upstream. *MafA, Lens1* and *γ-crystallin* are normally expressed in the lens of eye. In case of *Six3* or *Pax6* knockdown, the expression of these genes is completely abolished. It has been shown that *MafA* can directly induce the expression of *crystallins* (Kataoka et. al., 2007; Ishibashi et. al., 2002). *Lens1* establishes a lens forming bias in the presumptive lens ectoderm, but does not play a role during lens differentiation itself (Kenyon et. al., 1999) Therefore, the absence of *crystallins* is likely due to downregulation of *MafA*. Induction assays have shown *Pax6* to directly induce *Lens1* which is required to thicken
and maintain the undifferentiated characteristic of the presumptive lens ectoderm (KhosrowShahian et. al., 2005; Ishibashi et.al., 2002). The early lack of Pax6 in Six3 or morphants produces a predictable effect upon Lens1.

An eye field signaling model proposed by Zuber and colleagues (2003, 2011) situates Rx1 upstream of Pax6 and Six3. This model was based predominantly upon RT-PCR assays of animal cap where it is impossible to distinguish eye from general neural effects. There is no doubt and question that additional interactions may exist and additional genes yet to functionally identified may be playing parallel roles or compensating for the absence of one. However our proposed model (Figure 2.7) suggests a hierarchical functional role for Six3 to set the agenda for a neural bias and eye development by partnering with Otx2 and inhibiting BMP4 thereby regulating factors needed for that cascade. Six3 dependent activation of Pax6, results in lens and retinal induction at later stages by another set of factors activated by Pax6, presumably including Rx1. Pax6 maintains a well regulated balance between proliferation and differentiation. By activating Rx1, Pax6 promotes proliferation on one hand and at the same time, via the Pitx3 activation of MafA, Pax6 promotes differentiation of the lens. At the same time Pax6 mediated activation of Sox2 promotes lens differentiation along with RPE development by Pax2 activated upon by Sox2 (Figure 2.7).
Figure 2.7: Summary model of eye field induction derived from the current study on how the various eye field transcription factors collaboratively express and cross-regulate each other to give rise to the eye.
References


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Zuber, M. E. Eye field specification in Xenopus laevis. 93[C], 29-60. 2010. RefType: Serial (Book,Monograph)

CHAPTER 3 : DISCUSSION

Overview

Many research studies have individually looked at the roles of Six3 and Pax6 with respect other eye field markers (Harris and Hirsch, 1997; Kobayashi et. al., 1998; Kenyon et. al., 1999; Zuber et. al., 2003; Gehring et. al., 2005; Gestri et. al., 2005), however the relationship between these two eye gene markers is still not clear. I propose a revised model interpreted from the results discussed here which will help in understanding the hierarchical pattern of molecular inductive events associated with the development of the eye.

Six3 expression is essential for eye and brain development

Six3 maintains neural identity in the forebrain by repressing the expression of Wnt1 by directly binding to the Wnt1 promoter in vivo (Zhu et. al., 2002; Lagutin et. al., 2003). In Six3 null mutant mice, Wnt1 expression is up-regulated and mutant mice demonstrate aberrant craniofacial development (Laugtin et. al., 2003; Lavado et. al., 2008). Due to expansion of the Wnt1 expression domain, normal forebrain development is abrogated, and Wnt signaling is critical to defining the anterior-posterior axis in the brain (Niehrs, 1999; Heisenberg et.al., 2001). The forebrain region is kept Wnt-free by Wnt-antagonists such as Otx1, Otx2 and Six3, and the absence of these markers results in caudalization of anterior structures (Kiecker and Niehrs, 2001). In Otx1 and Otx2 null mutant mice, Wnt1 expression expands into the anterior region. The remarkable similarities in phenotype of the Six3 and Otx2 null mutants clearly confirm their role in
anterior brain development and suggest a commonality of mechanism (Lagutin et. al., 2003). Similarly, animal cap studies in *Xenopus* show that both *Six3* and *Otx2* together are required to induce a neural fate (Gestri et. al., 2005), therefore it seems that amphibians share with mammals the same essential requirement for early brain development.

In medaka fish, morpholino mediated *Six3* knockdown results in absence of brain and eyes and expression of the forebrain marker *Vax1*, which plays an important role in early cell differentiation in the basal forebrain and optic stalk was completely lost in *Six3* knockdown medakafish embryos. Also, *Rx2* (functional homolog for *Rx1* in Xenopus), a marker for neural retina in medaka fish was absent (Carl et. al., 2002). This is consistent with what we report here for *Xenopus*: loss of *Six3* results in complete loss of eye structures and aberrant brain development. Considerably reduced expression levels for *Xenopus* brain and eye markers confirm the early and important role of *Six3* in anterior neural specification, brain regionalization, and eye induction. Levels for *Otx2* in the anterior neural plate region were severely affected upon *Six3* perturbation, possibly due to enhanced *Wnt* and *BMP4* (discussed later) activity in dorso-anterior regions. Due to enhanced levels of the above mentioned dorsal-posterior (*Wnt1*) and ventral marker (*BMP4*) – the dorso-anterior ectoderm (which is biased to become neural), instead transforms to an epidermal fate (Aybar and Mayor, 2002; Gestri et. al., 2005). The aberrant closing of the neural tube observed in *Six3* knockdown phenotypes might reflect ambiguity of dorso-ventral, anterio-posterior, and midline signaling. Proper folding and closing of the neural tube is essential in development of a normal telencephalon (Wallis et. al., 1999). Human fetuses carrying a maternally inherited mutation of the *SIX3* gene,
display a failure of neural tube closure at Carnegie stage 14 leading to holoprosencephaly (HPE): the two telencephalic hemispheres fail to separate (Pasquier et. al., 2005; Wallis et. al., 1999). As a consequence the telencephalic hemispheres fuse and other midline deficiencies intrude to create phenotypes such as cyclopia (Wallis et. al., 1999). The phenotype displaying defective neural tube closure in *Xenopus* provides future insights that could identify markers associated with HPE and cyclopia and to understand their functional roles in these disorders.

**Six3 inhibits BMP4 expression in the anterior neural plate and promotes a neural bias**

Once the anterior neural plate is defined, *Six3* expression up-regulates around stage 14 and restricts to that region. During the early stages, before gastrulation, *BMP4* is expressed throughout the ectoderm – however, after gastrulation, and once the anterior neural plate is defined – the expression of *BMP4* is excluded from the neural plate region (Kuroda et. al., 2004; Wilson and Edlund, 2001). Elsewhere, high levels of *BMP4* transform the unspecified ectodermal cells towards an epidermal fate rather than a neural fate. Thus, *Six3* expression in the anterior neural plate antagonizes *BMP4* and represses its expression in this region thereby promoting a neural bias from very early in development (Gestri et. al., 2005).

In *Xenopus*, *Six3* morphants confirmed that when the dorsal neural cue is absent, *BMP4* up-regulates as assayed by RT-PCR. Co-injection of *Six3* morpholino with *Six3Δ5’UTR*-mRNA rescued phenotypes and restored *BMP4* levels to close to normal. On the other hand, *Six3*-mRNA mis-expression resulted in *BMP4* repression. This clearly
confirms that *Six3* and *BMP4* share an antagonistic relationship. Gestri and colleagues (2005), report that *Six3* over-expression in zebrafish results in reduces *BMP4* levels and conversely, that *BMP4* over-expression results in *Six3* depression. Electrophoretic mobility shift assays (EMSA) revealed that *Six3* directly binds to the *BMP4* promoter in a concentration dependent manner thus confirming the antagonistic relationship shared between *Six3* and *BMP4* is direct (Gestri et. al., 2005).

Graded *Six3* knockdown in our studies revealed an interesting pattern for eye development. Lower doses of knockdown resulted in 75% of the RPE to form. With increasing morpholino concentrations, the RPE progressively restricted to the dorsal region of the embryo and the eye size was reduced. This observation supports the antagonistic dorso-ventral relation between *BMP4* and *Six3*. Since *Six3* knockdown permits *BMP4* levels to go up, resulting in conversion of more ventral ectoderm to a non-neural epidermal fate (Summarized in Figure 3.1). Ventralization of a portion of the eye field ensues as a consequence of the optic vesicle developing a reduced capacity to induce the outer ectoderm nor itself to internally pattern appropriately. A dose-dependent effect on eye development was not reported in zebrafish (Gestri et. al., 2005).
Figure 3.1: Antagonistic relationship shared between Six3 and BMP4.

*Six3* knockdown results in expansion of *BMP4* expression domain into the dorsal regions resulting in transforming the neural fate ectoderm to a non-neural epidermal fate.
**Six3 and Otx2 are both required to maintain a neural bias in the anterior neuro-ectoderm**

The expression for *Otx2* is detected in the dorsal marginal zone prior to gastrulation (Gammill and Sive, 1997; Pannese et. al., 1995). During gastrulation the expression for *Otx2* is first detected in the involuting mesoderm and later in the ectoderm that overlies it. This ectodermal region where *Otx2* is expressed will eventually form rostral brain and the eyes (Pannese et. al., 1995). The expression of *Six3* overlaps with *Otx2* in the ectoderm. Both *Six3* and *Otx2* transcripts are detected right from the egg stage to gastrulation by RT-PCR, however by *in situ*, the expression for *Otx2* precedes that of *Six3*. Upon *Six3* knockdown, *Otx2* expression is downregulated in the early neurula and at later stages, eye field expression is completely diffused (current study). In *Xenopus* animal cap explants, *Six3* mRNA alone cannot induce neurulation, however when coinjected with *Otx2* mRNA, neurulation is induced (Gestri et. al., 2005; Zuber et. al., 2003). This suggests that *Six3* partners with *Otx2* to define and specify the anterior neural plate which will eventually result in systematic eye and brain development. In maintaining a neural bias in the dorso-anterior structures, *Six3* holds BMP4 at bay (Gestri et. al., 2005; current study).

A similar antagonistic interaction is observed between *Otx2* and *BMP4* for normal development of the cement gland structures, thus contributing in development of anterior structures (Gammill et. al., 2000). Although cement gland is eventually situated ventrally, it is specified at the very anterior margin of what becomes neural plate – in other words at the dorso-ventral border. It is also an unusual site because it arises where endoderm touches ectoderm directly, and the cues appear to derive for planar diffusion from the
dorso-anterior neural plate. In *Six3* morphants *Otx2* is unable on its own to repress *BMP4* thus resulting in collapse of signaling to sustain neural fates. Not surprisingly, morpholino mediated knockdown of *Otx2* in *Xenopus* also results in abnormal development of anterior structures and the eye (Carron et. al., 2005). When the repression construct *Otx2-EnR* is deployed, eyeless tadpoles result, although this observation must be tempered by two caveats: first the injected mRNA is ubiquitously expressed; and second, it is present much earlier than normal – there is no assurance the phenotype is due to direct mechanisms (Isaacs et. al., 1999).

An early requirement for *Otx2* can be deduced from the Chuang and Raymond study (2002) in which over-expression of *Pax6, Rx1* and *Six3* resulted in ectopic eye formation, but only in the head where *Otx2* expresses. This clearly suggests that like *Six3*, *Otx2* is essential to eye morphogenesis and its expression early in development is obligatory to maintain a neural bias.

*Six3* plays an important role in promoting proliferation of cells in the early anterior neural plate

Maintaining a neural bias by inhibition of *BMP4* is one of the most important roles for *Six3* in the anterior neural plate (Gestri et. al., 2005). However, in *Xenopus, Six3* knockdown also abrogated the expression pattern for *Rx1* in the anterior neural plate region (present study). *Rx1* controls proliferation as well as neurogenesis in the anterior neural plate region (Andreazzoli et. al., 2003). Interfering with *Rx1* function by injection of *Rx1-EnR*-mRNA results in reduced eyes to complete loss tadpoles (Andreazzoli et. al., 2003). Neural differentiation marks its beginning in the posterior neuro-ectoderm
subsequent to gastrulation, however, the eye field progenitor cells continue to proliferate to reach numbers which will be sufficient to produce an eye. Pro-neural differentiation genes - Ngnr1 and Delta1 are repressed in the presumptive eye field by the expression of Rx1 which is driven by Six3 in a Pax6 dependent manner (Gestri et. al., 2005; Andreazzoli et. al., 2003; current study).

Down-regulation of Rx1 activity in Six3 or Pax6 morphants or by Rx1-EnR mediated target repression results in expansion of Ngnr1 into the anterior neural plate region, thereby reducing the size of the eye field: precocious differentiation robs the field of sufficient starting material (Andreazzoli et. al., 2003; Zuber et. al., 2003; current study). Rx1 has been implicated to positively regulate the expression of xZic2 and xHairy2 which are anti-neurogenic and repress the expression of cell cycle inhibitor p27Xic1 (Andreazzoli et. al., 2003). Therefore, by regulating the expression of Rx1 in a Pax6 dependent or independent manner, one role of Six3 is to indirectly promote cellular proliferation in the anterior neural plate prior to eye development.

Moreover, during the early stages of development, higher Six3 levels promote proliferation and inhibit pre-mature neurogenesis by negatively regulating cell cycle exit markers such as cyclinD1 (Appolloni et. al., 2008). Simultaneously, Six3 positively regulates proliferation markers which include, Xic2, Xhairy2, Xbf1 and Rx1 (Gestri et. al. 2005). Along with this, Six3 directly binds to Geminin causing Cdt1 to assemble the pre-replication complex thereby promoting proliferation. Geminin inhibits cell cycle progression by sequestering Cdt1 (Del Bene et. al., 2004; Truong et. al., 2011). In medaka fish, blocking Geminin function results in enhanced proliferation and expansion of the eye field (Del Bene et. al., 2004). Over-expression of Six3 in zebrafish results in
expansion of the rostral region of the brain whereas in medaka fish it results in enlarged retinal structures with enhanced $Rx1$ and $Rx2$ expression (Kobayashi et. al., 1998; Loosli et. al., 1999). These results support the role of $Six3$ as a positive regulator of proliferation by liberating Cdt1 as it directly affects cell-cycle players. Therefore $Six3$ is required early in development for the proliferation of cells in the anterior neural plate – first by promoting the activation of $Rx1$ and secondly by inhibiting early neurogenesis.

$Six3$ activation of $Pax6$ is essential to eye development

The expression of $Six3$ precedes that of $Pax6$ during early specification of the anterior neural plate. $Pax6$ is first recorded at around stage 12.5-13 (Ghanbari et. al., 1998; Zuber et. al., 2003). In the current study, $Six3$ knockdown in $Xenopus$ results in $Pax6$ expression to be perturbed in the anterior neural plate region. During later stages $Pax6$ expression was considerably reduced in the region around the closing of the neural tube (anteriorly) which forms the future eye. Finally at later stages, $Pax6$ expression in the PLE appears to be diffuse whereas the expression of $Pax6$ along the neural tube (posteriorly) is unaffected. Western blot analysis confirmed Pax6 protein levels to be downregulated upon $Six3$ perturbation in a dose-dependent manner. However, complete $Six3$ translational block reduces Pax6 levels to be 25% of normal and controls. Similar results were seen at the RNA level, where $Six3$ knockdown resulted in $Pax6$ mRNA to be reduced. Conversely, $Six3$ up-regulation by injection of mRNA results in up-regulation of $Pax6$ mRNA levels as well as protein levels (by a factor of 1.5). These results are consistent with the over-expression studies reported in medakafish, zebrafish and
*Xenopus* animal cap explants (Kobayashi et al., 1998; Carl et al., 2002; Loosli et al., 1999; Zuber et al., 2003).

In wild type embryos, *Pax6* expression is detected by RT-PCR at stage 12.5-13 and increases substantially by stage 17. Following *Six3* knockdown, *Pax6* activation was delayed and faint expression could first be detected at stage 15. In animal cap studies, *Six3* over-expression results in enhancement of *Pax6* expression (Zuber et al., 2003). This suggests that *Six3* is required to activate the expression of *Pax6* very early during neural plate specification and to maintain it throughout the eye developmental stages, but that a second phase of *Pax6* activation is later possible by other means or *Pax6* itself auto-regulating its expression. Our results reported are consistent with data in *Six3* lens-promoter mutant mice, where *Pax6* expression down-regulates in the lens placode region (Ashery-Padan et al., 2000), *Six3* morphants in medaka fish where *Pax6* downregulates in the retina (Carl et al., 2002). ChIP, EMSA and luciferase assays confirm that *Six3* directly activates *Pax6* (Liu et al., 2006; Singh and Tsonis, 2010). *Six3* also has also been attributed to play an important role in lens regeneration in newts. Over-expression of *Six3* in the ventral iris in newts results in transdifferentiation from that iris cells finally resulting in lens regeneration in the dorsal iris through by activation of *Pax6* (Grogg et al., 2005).

Another possible explanation for enhanced *Pax6* levels, other than direct activation (Liu et al., 2006), could be due to the role of *Six3* to enhance proliferation in the anterior neural plate. With more cells present in the anterior neural plate there is more capacity for the domain of *Pax6* to expand. Interestingly, in medaka fish, enhanced *Pax6* levels due to murine *Six3* mis-expression are detected outside the eye field region, where
Six3 and Otx2 are normally expressed but not Pax6. This suggests that Six3 alone or in combination with Otx2 activates the expression of Pax6 outside the eye field to induce ectopic lenses (Loosli et. al., 1999). Therefore, Six3 is a major player in the lens development and essential for Pax6 activation and maintenance.

Pax6 is essential to lens induction and specification

Pax6 down-regulation in morphants produces retinal deformities and absence of lens. During early stages of eye development, Pax6 knockdown does not have any effect on Six3. However during the later stages, once the optic primordium is determined, Pax6 down-regulation reduces the Six3 expression domain (current study).

Pax6 has been shown to play an important role in the lens induction process (Altmann et. al., 1997). In Xenopus, over-expression of Pax6 results in ectopic lens induction giving rise to supernumerary lenses (Altmann et. al., 1997; Zuber et. al., 2003). Also, in Xenopus animal cap assays Pax6 over-expression induces expression of β-crystallin and γ-crystallin. However, Pax6 does not induce the expression of mesodermal markers which clearly tells us that its role in lens induction is direct (Altmann et. al, 1997; Khosrowshahian et. al., 2005; Zuber M., 2011).

Our study confirms prior work (Shimada et. al., 2003) that Pax6 knockdown results in complete abrogation of the lens inducing gene MafA and its lens specific differentiation target γ-crystallin. The lens proliferative marker, Lens1 is also completely abolished upon Pax6 knockdown. This is consistent with the predicted role of Pax6 when over-expressed where it results in Lens1 up-regulation (Kenyon et. al., 1999). Also,
shown in zebrafish and medaka fish, Pax6 activates the expression of Prox1 which activates inhibitors of cell cycle – Cdkn1b (p27Kip1) and Cdkn1c (p57Kip2) which may be possibly responsible for the cell cycle exit resulting in terminal differentiation (Wigle et. al., 1999; Blixt et. al., 2000). Therefore functionally Pax6 likely acts a pro-differentiation gene and anti-proliferation candidate.

Pax6 knockdown also impairs expression of Pitx3 in the lens ectoderm (current study). In animal caps, Pax6 induces the expression of Pitx3 whereas Pitx3 is unable to activate the expression of Pax6 (Khosrowshahian et. al., 2005). This suggests Pax6 lies upstream of Pitx3. However, in Pax6 morphants, Pitx3 is not completely abolished, thus remnant Pitx3 expression in the pre-placodal ectoderm must be able to retain inducing abilities sufficient to stimulate retinal development, likely in cooperation with signaling by Six3 (current study). To completely abolish retinal development, Pitx3 expression in the pre-placodal ectoderm must be lost completely (Khosrowshahian et. al., 2005). This is observed in Six3 knockdown embryos, where Pitx3 expression is abolished thereby no retinal structures are observed. Therefore, the presence of key eye field transcription factors in the PLE can induce retina formation; nevertheless Pax6 is essential for lens induction, specification and differentiation (summarized in Figure 2.7)

**Pax6 plays an intermediate role in rescuing the expression of Rx1 and Sox2**

Rx1 must be downstream of both Six3 and Pax6 (current study). Six3 and Rx1 share the role of stimulating proliferation (Chow et. al., 1999; Harris et. al., 2002; Zuber et. al., 2003). On the other hand in our studies, Pax6 mRNA ectopic over-expression results in expansion of the expression domain for Rx1. This is consistent with earlier
work (Chow et. al., 1999), however in my study $Rx1$ expression is enhanced only in the anterior neural plate (ANP) and specifically in the eye field and not in any other area of the ANP. This suggests that $Pax6$ partners with other key factors co-expressed to induce $Rx1$ expression. Co-injection of $Six3$ morpholino and $Pax6$-mRNA results in restoration of $Rx1$ expression close to normal. This tells us that the secondary set of factors with which $Pax6$ interacts do not require $Six3$. Therefore loss of $Rx1$ in $Six3$ knockdown embryos is likely a secondary effect observed due to reduced $Pax6$ levels. Interestingly, $Pax6$ rescues the loss of $Rx1$ which is a proliferative marker – however, at later stages $Pax6$ itself indirectly activates cell-cycle inhibitors (Wigle et. al., 1999; Blixt et. al., 2000; current study). This suggests a dual role for $Pax6$ that is context and time-specific: early in development it activates $Rx1$ to promote proliferation, but, at later stages it activates $Prox1$ to inhibit proliferation (current study; Wigle et. al., 1999; Blixt et. al., 2000). $Pax6$ promotes survival of the eye primordium and prohibits early exit from cell cycle that would otherwise reduce chances for the later born cell types to develop (Ohnuma et. al., 1999). Later, $Pax6$ activation of $Prox1$ is more prominently observed during the lens placode thickening stages where differentiation is supported from that point onwards (Blixt et. al., 2000). Therefore, $Pax6$ along with other factors expressed in the placodal ectoderm ($MafA$, $MafB$, $Pitx3$) could be responsible for $Prox1$ mediated cell-cycle inhibition (Blixt et. al., 2000; Chow et. al., 1999).

$Sox2$ was one of the very first set of transcription factors implicated in lens differentiation (Kamachi et. al., 1995). $Six3$ and $Pax6$ morphants both down-regulate $Sox2$ in the optic vesicle, but elsewhere along the neural tube and anterior head ectoderm $Sox2$ remains normal. Furthermore, $Pax6$ over-expression can rescue the expression of
Sox2 in Six3 knockdown embryos, specifically in the optic vesicular region. Sox2 in combination with Pax6 can induce crystallin synthesis (Kamachi et. al., 2002). However it is still not clear how Pax6 regulates Sox2, but it has been shown that Six3 directly activates Sox2 expression in the presumptive lens ectoderm (Liu et. al., 2006).

Six3 and Pax6 perturbations display no effect on posterior and proximal genes

Krox20 and Pax2

Krox20 is expressed in rhombomeres 3 and 5 and serves as a marker for hindbrain development (Seitanidou et. al, 1997). Its expression does not overlap with anteriorly expressed Six3 and Pax6. Krox20 has been implicated in its role for hindbrain development and caudalization (Nieto et. al., 1991). Not surprisingly, since their respective expression patterns do not overlap, the effects of Six3 and Pax6 misregulation in anterior regions leave Krox20 is unaffected.

Similarly, Pax2 is expressed in the ventral optic vesicle, hind brain (presumptive ear vesicle) and the kidney (Heller et. al., 1997). Six3 and Pax6 perturbation affects Pax2 expression in the ventral optic vesicular region only, whereas the expression remains unaffected elsewhere (Heller et. al., 1997).

Proposed eye field signaling model

Currently, a signaling model by Zuber and colleagues (2003, 2011) proposes Rx1 to be upstream of Pax6 and Six3. This model is based predominantly upon RT-PCR assays of animal caps where it is impossible to distinguish eye from general neural effects. By contrast my study suggests Rx1 to act downstream of Six3 and Pax6 as
evident by loss of function experiments and rescue of \textit{Rx1} by \textit{Pax6} mRNA injection. One of the differences between the two studies could be that mine utilizes whole embryos with a spatial acuity which is not possible in the assays utilized by Zuber and colleagues (2003), and the latter study conflates proliferative with induction effects.

Over-expression studies are critical to the characterization of genetic networks, however, there are possibilities of parallel pathways and intermediates that can be missed, as well as misrepresentations of what occurs \textit{in vivo}. For example, if over-expression of \textit{Rx1} results in increased proliferation, there will be more eye progenitor cells. Consequently, there might also be a proportional increase in expression of eye field markers. Homogenate-based assays by sensitive screens such as RT-PCRs (Zuber et. al., 2003) will tend to exaggerate these relationships. However, with our present study, knockdown adds new information: \textit{Six3} inactivation impairs \textit{Rx1} expression, and \textit{Pax6} can rescue it – but does not rescue the eye development process. This clearly suggests that \textit{Six3}, \textit{Pax6} and \textit{Rx1} are acting in a more complex network than just a linear signal cascade – one where genes cross-regulate each other (Figure 3.3).
Figure 3.2: Summary model of eye field expressed genes complied from multiple studies illustrating the genetic interactions between them and the functional roles they play in coordinated eye development.
Conclusion

No single gene in the group of eye field transcription factors is exclusive and required for a particular segment of eye development, for example retina development. As a matter of fact, with the help of \textit{in situ}, semi-quantitative RT-PCR, over-expression and down-regulation studies, it becomes evident that the eye field transcription factors operate in an exquisitely cross-regulated network to control eye morphogenesis.

Many of the eye field markers regulate each other both \textit{in vitro} and \textit{in vivo}. \textit{Six3} expression is required to activate the expression of \textit{Pax6} (Liu et. al., 2006; current study). \textit{Six3} but it also plays two additional roles – first it maintains a neural bias in the anterior neural plate by inhibiting expression of \textit{BMP4} and \textit{Wnt1} (Gestri et. al., 2005; current study), and second, it promotes proliferation and inhibits differentiation by sequestering Geminin which releases Cdt1 to license replication complex assembly (Del Bene et. al., 2007). Lastly, by activating \textit{Pax6}, and helping to sustain its level, its promotes differentiation at later stages (Figure 3.2).

At later stages of eye development, \textit{Pax6} limits the expression of \textit{Six3} in the eye field (Liu et. al., 2006; current study), possibly by regulating the expression of \textit{Rx1} which controls proliferation of the retinal progenitor cells in which \textit{Six3} expresses (Harris et. al., 2003; current study; Figure 3.2). Over-expression of \textit{Pax6} results in large eyes and multiple lens induction due to more retinal progenitor cells (Altmann et. al., 1997; Zuber et. al., 2003; current study).

\textit{Pax6} mediated activation of \textit{MafA} is critical for crystallin expression (Takeuchi et. al., 2009). \textit{Pitx3} regulation by \textit{Pax6} is again important to lens induction and retinal
development (Khosrowshahian et. al., 2005). Lens1 – can act as a lens antagonist as well, upon overexpression, Lens1 can keep the ectodermal cells in a proliferative state thereby inhibiting differentiation resulting in loss of crystallin expression. However, at the same time, Lens1 promotes proliferation of lens progenitor cells which undergo differentiation in the posterior region of the lens (Kenyon et. al., 1999). At the same time, Pax6 activation of Prox1 results in cell cycle exit thereby promoting differentiation (Blixt et. al., 2000; Figure 3.2).

In the future, studies need to look at understanding the direct role between each of the eye field transcription factors and their targets and to identify the epigenetic behavior. Also, the transcription factors involved in eye development work alone or partner with other factors forming complexes to turn on transcription of lineage specific genes. An approach to identify transcription factor partner and complexes could involve following the dynamics of the transcriptome by exploiting chromatin immunoprecipitations (4C: chromosome conformation capture-on-chip; Mitchell and Fraser, 2008). This might result in identification of new transcription factor binding sites. It would be interesting to identify what lies upstream of the eye field transcription factors. Lastly, a final challenge will be understand the epigenetic program retinal progenitor cells undergo that permits multiple outcomes from common progenitor cells (reviewed in Cvekl and Duncan, 2007).

With the advancement in genome sequencing and the Xenopus White paper for 2011 (Khokha et. al., 2011) focusing on new technologies such as those required to produce loss of function, in vivo live imaging, etc., will facilitate identification of genes that play a key role in the developing eye.


Zuber, M. E. Eye field specification in Xenopus laevis. 93[C], 29-60. 2010. Ref Type: Serial (Book,Monograph)

APPENDICES

APPENDIX A

Normalized Pax6 protein levels with respect to Actin levels

Figure A1: Normalized Pax6 protein levels.

Upon Six3 morpholino knockdown Pax6 protein levels were reduced to 50% of control and wildtype levels. However, Six3 mRNA overexpression results in enhanced Pax6 levels suggesting Six3 positively regulates Pax6
APPENDIX B

Semiquantitative RT-PCR analysis for eye field genes upon *Six3* and *Pax6* knockdown

![Relative Six3 mRNA levels upon Pax6 knockdown.](image)

**Figure B1: Relative *Six3* mRNA levels upon *Pax6* knockdown.**

Upon *Pax6* knockdown, *Six3* expression levels were not reduced considerably. As confirmed by in situ data, *Pax6* knockdown results in smaller eye field. Upon *Pax6* over-expression *Six3* expression enhances as seen above.
Figure B2: Relative $Otx2$ mRNA levels upon $Six3$ and $Pax6$ knockdown

Upon $Six3$ and $Pax6$ knockdown, $Otx2$ levels are observed to decline. The severity is more in $Six3$ knockdown as compared to $Pax6$ knockdown.
Figure B3: Relative Pax2 mRNA levels upon Six3 and Pax6 knockdown

Pax2 expression level is abrogated upon Six3 knockdown in the anterior neural plate.

Upon Pax6 knockdown expression is slightly diminished.
Figure B4: Relative Pitx3 mRNA levels upon Six3 and Pax6 knockdown

Upon Six3 knockdown, the expression of Pitx3 is completely abolished in the PLE as confirmed by in situ hybridization. However, Pitx3 expression in the other regions (somites, heart and brachial arches) are unaffected upon Six3 knockdown.
Figure B5: Relative *Lens1* mRNA levels upon *Six3* and *Pax6* knockdown

*Lens1* levels are completely abolished upon *Six3* and *Pax6* knockdowns.
Figure B6: Relative *MafA* mRNA levels upon Six3 and Pax6 knockdown

*MafA* levels are completely downregulated in the eye field upon *Six3* and *Pax6* knockdown as confirmed by in situ hybridization.
Figure B7: Relative $\gamma$-crystallin mRNA levels upon Six3 and Pax6 knockdown

$\gamma$-crystallin expression – a lens differentiation marker is almost deleted upon Six3 and Pax6 knockdown
Dose-dependent Six3 knockdown resulted in BMP4 levels to be effected. At higher levels of Six3 knockdown, BMP4 levels increase by 1.5 fold whereas upon Six3 mRNA overexpression, BMP4 levels reduced to 50% of wildtype and control morpholino injections.
APPENDIX C

Six3 and Pax6 Control morpholino treatments

Figure C1: Effect of Six3 and Pax6 control morpholino on expression of eye marker genes.

*Otx2*: A, (Six3 MO), B (Pax6 MO)

*MafA*: C, C’ (Six3 MO); D, D’ (Pax6 MO)

*Pitx3*: E, E’ (Six3 MO); F, F’ (Pax6 MO)

*Lens1*: G, G’ (Six3 MO); H, H’ (Pax6 MO)

*γ-crystallin*: I, I’ (Six3 MO); J, J’ (Pax6 MO)
Six3 Control-MO

Pax6 Control-MO

Otx2

MafA

Pitx3

Lens1

γ-crystallin

* : control morpholino injected side

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APPENDIX D

TUNEL ASSAY PROTOCOL MODIFICATIONS

Protocol Adapted and Modified from Nancy May Hoo, Harland Lab (Dr. Richard Harland, University of California – Berkeley, California, USA)

Modifications:

1. Day 3: Alkaline Phosphatase Buffer washes at room temperatures: 10 minute - 3 times
2. Day 3: Staining in NBT/BCIP done in cold room in dark with reaction constantly monitored
3. Day 3: Stopping Chromogenic Reaction with MAB washes with 0.5M EDTA – done in cold room
Figure D1: TUNEL Assay to detect apoptotic positive cells upon Six3 and Pax6 knockdown. Left side injected. Six3 MO (A), Six3 CMO (B), Pax6 MO early stages (C), Pax6 CMO early stages (D), Pax6 MO - at late lens differentiation stages (E) and uninjected side (E’). No significant difference in number of TUNEL positive cells.
APPENDIX E

Plasmid Maps

_Six3Δ5'UTR:_

[Diagram of plasmid map showing restriction sites and features such as SP6 primer, Rep Origin 1, Six3Δ5'UTR, XSix3 (ORF), AMP^R, Misc Feature 2, Xho I (1168), Xma I (313), Kpn I (1447), Pst I (1188), Pst I (1456), Bst I (1188), Bam HI (640), Bam HI (80), Eco RI (95), Hind III (29), 5183 bp, and several other annotations.]
Pax6AltMO:

Pax6Alt-MO
5806 bp
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