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

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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.

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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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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

μM	micromolar
4C	Chromosome conformation capture-on-chip
ANP	Anterior Neural Plate
BCIP	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
bHLH	basic Helix Loop Helix
BMP	Bone morphogenetic protein
CDK	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitors
ChIP	Chromatin Immunoprecipitation
CMO	Control morpholino oligonucleotide
CNS	Central nervous system
dUTP	Deoxyuridine Triphosphate
EDTA	Ethylene-diamine-tetra-acetic acid
Ef1α	Elongation factor 1-alpha
EFTFs	Eye Field Transcription Factors
EMSA	Electrophoretic mobility shift assay
EnR	Engrailed repressor
EST	Expressed sequence tag
Ey	Eye field
ey	*Eyeless* gene
GCL	Ganglion cell layer
Gem	Geminin
GFP	Green fluorescent protein
HD	Homeodomain

HMG	High mobility group
Hox genes	Homeotic genes
HPE	Holosprosencephaly
MAB	Maleic Acid Buffer
Maf	Musculoaponeurotic fibrosarcoma oncogene homolog
MBS	Modified Barth's saline
Mitf	Micropthalmia-associated transcription factor
mM	millimolar
MO	Morpholino oligonucleotide
NBT	Nitro-blue tetrazolium
ng	Nanograms
nr	Neural retina
nt	Neural tube
Otx2	Othrodenticle homeobox homolog 2
Pax genes	*paired-box* genes
pg	Picograms
PLE	Presumptive lens ectoderm
RPC	Retinal progenitor cells
RPE	Retinal pigmented epithelium
RPL	Retinal pigmented layer
RT-PCR	Reverse transcriptase polymerase chain reaction
Rx genes	Retinal *homeobox* genes
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
Shh	Sonic hedgehog
Six genes	*Sine oculis* homeobox genes

so	*sine oculis*
Sox genes	*SRY –related HMG* box genes
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UTR	Untranslated region
XCG	Xenopus cement gland specific gene
Xnr	Xenopus nodal related gene
Zic2	Zinc finger protein of cerebellum

CHAPTER 1 :
INTRODUCTION

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 sychronously 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 verterbate eye development requires inductive interactions between the presumtive 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 primorida 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; C how 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 collageneous fibrils (McAvoy, 1980).

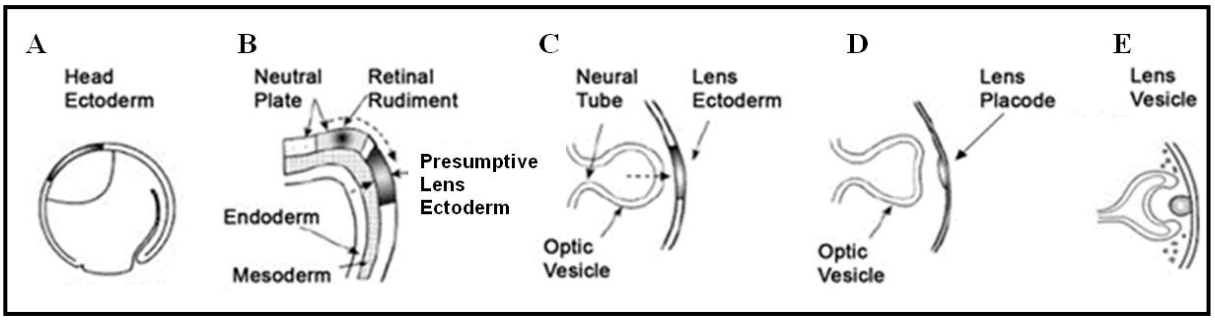


Figure 1.1: Summary of lens induction.

(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.

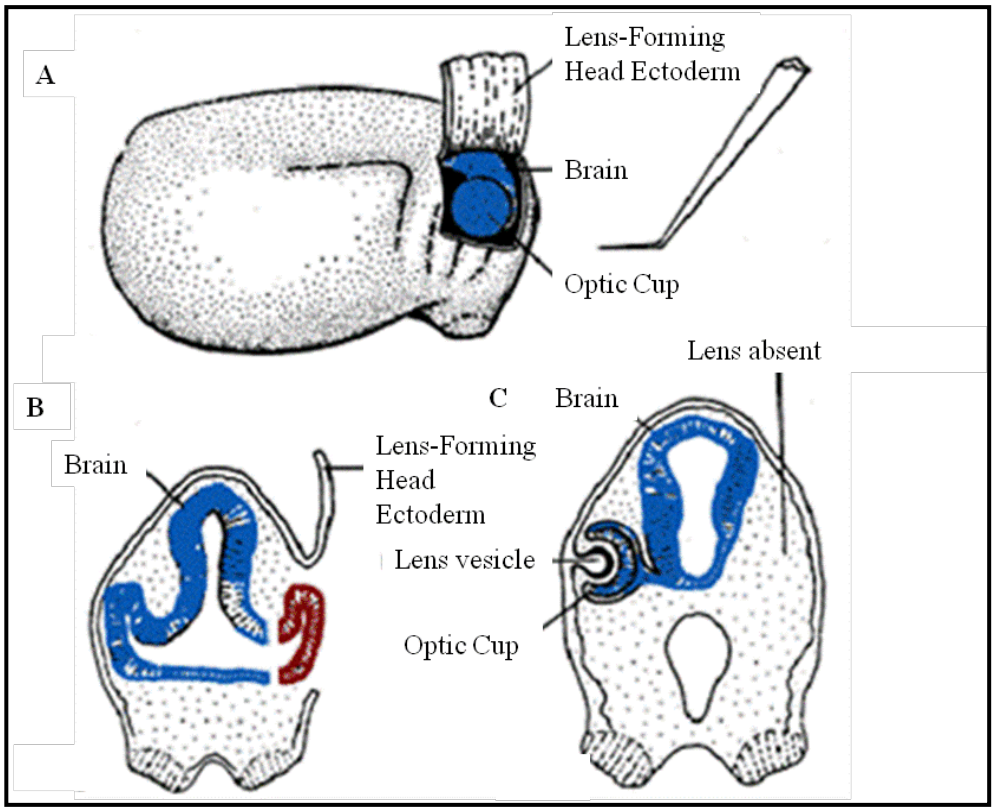


Figure 1.2: Model of lens ablation experiment by Hans Spemann, 1901.

(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 fluorsceinated 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)

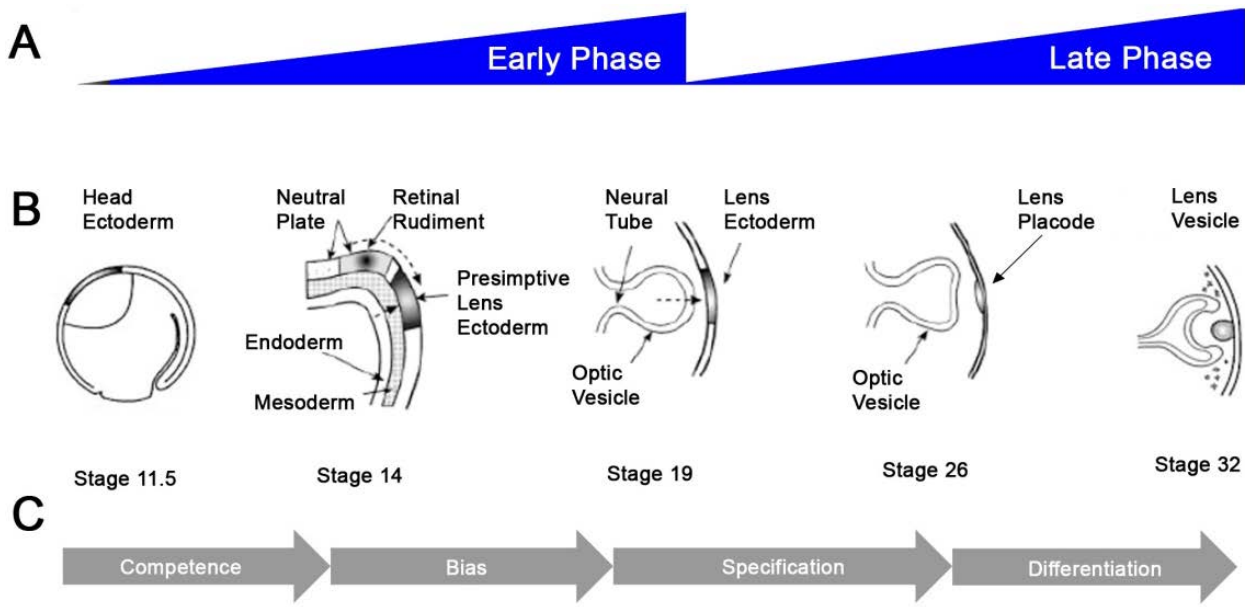


Figure 1.3: Early and Late phase of Lens Induction.

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-neurla 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).

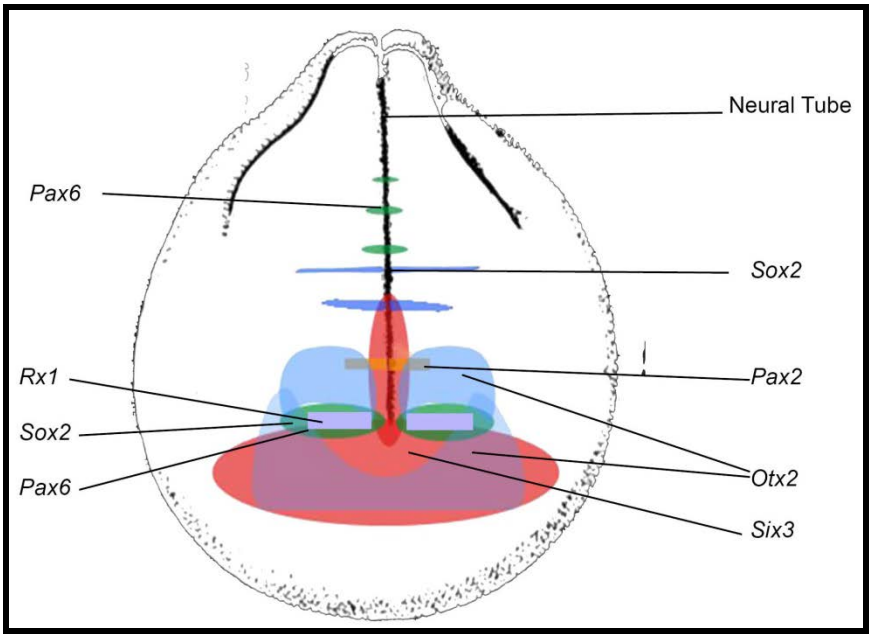


Figure 1.4: Summary of expression of early eye field transcription factors involved invertebrate eye development

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.

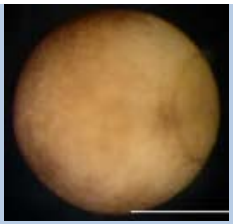
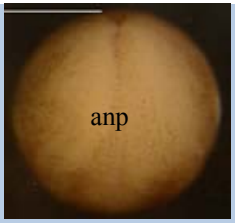
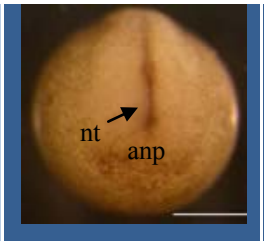
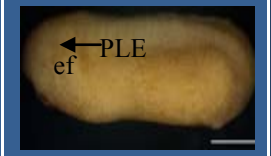
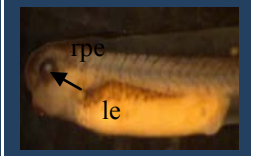
Stage	11.5	14	19	26	32	38+
						
Gene	***Competence/Bias***		***Specification***		***Differentiation***	
Six3	•	•	•	•	•	•
Pax6	-	•	•	•	•	•
Otx2	•	•	•	•	•	•
Rx1	-	•	•	•	•	•
Sox2	-	•	•	•	•	•
Pax2	-	•	•	•	•	•
Pitx3	-	•	•	•	•	•
Lens1	-	-	•	•	•	•
MafA	-	-	-	•	•	•
γ-crystallin	-	-	-	•	•	•

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 a mino 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 micropthalmia 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 (Barasachhi et. al., 1997). After stage 45, the expression declines (Barasacchi 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 (Barasacchi 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). O ver-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 co ntext-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).

Pitx3

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 dom ain (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 t heir 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 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

Gene	Important Functions
Six3	• BMP4 inhibition • Pax6 activation • Proliferation (Sequestering Geminin) • Sox2 activation • Lens differentiation
Pax6	• Establishing lens bias • Lens epithelium maintenance • Crystallin expression • Eye field specification • Proliferation
Otx2	• Neural Bias • Inhibiting BMP4 • Lens competence
Rx1	• Proliferation • Inhibitor of differentiation markers/cell cycle exit
Sox2	• Proliferation • Regulation of crystallin expression • Lens fibre differentiation
Pax2	• RPC proliferation
Pitx3	• Lens and Retina Induction
Lens1	• Proliferation
MafA	• PLE Induction • Lens differentiation • Crystallin activation
γ-crystallin	• Lens differentiation and development
BMP4	• Dorsal-ventral eye patterning • Promotes ventral fate

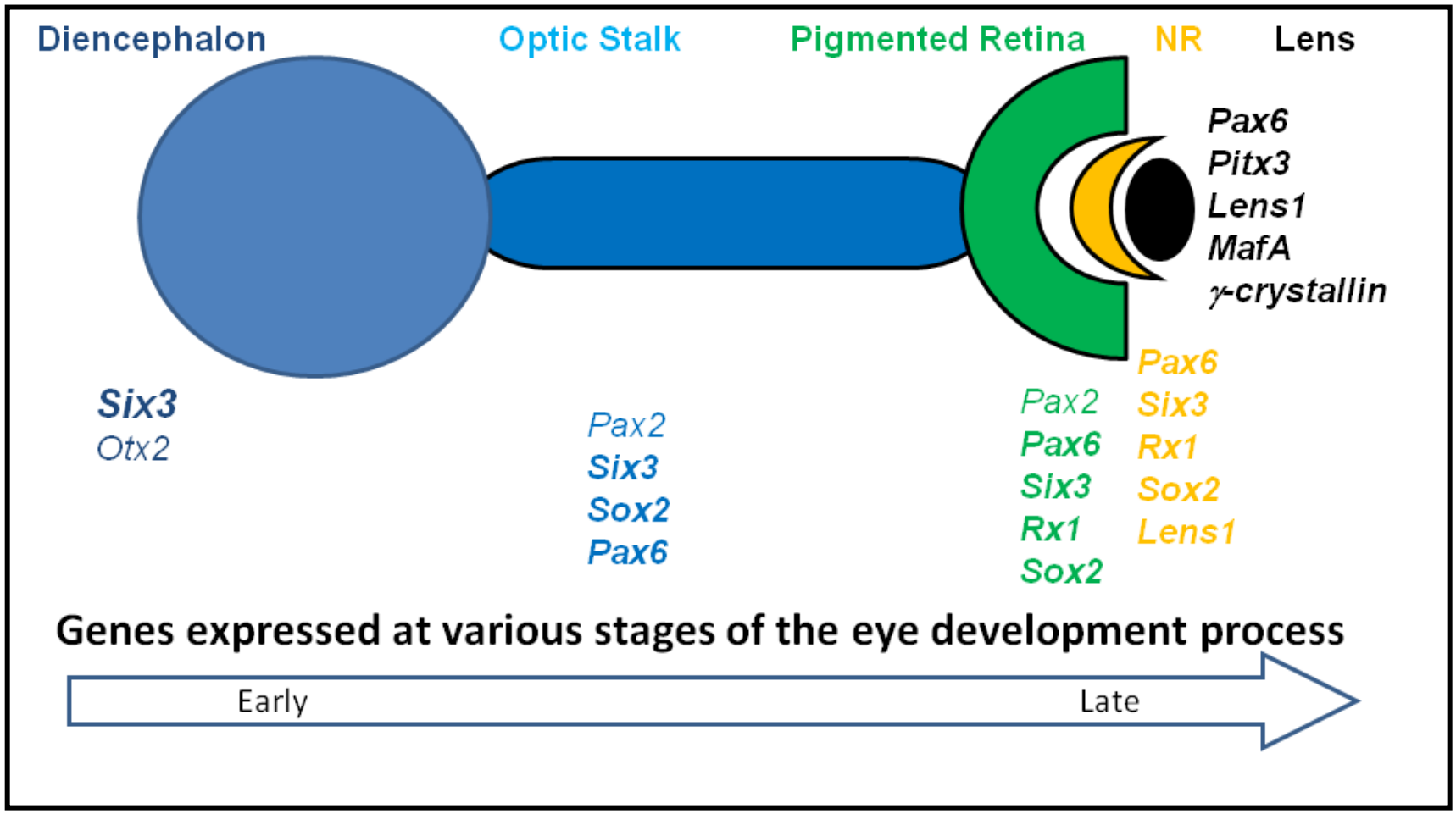


Figure 1.6: Diagrammatic representation of genes expressed at the different tissue levels of the eye.

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 *BMP4* in the anterior neural plate region. Six3 binds directly to the *BMP4* promoter and represses its expression (Wittbroddt et.al., 2002) In medakafish a Six3 factor/activator fusion construct, *Six3-VP16* causes *BMP4* expression domain to be expanded and dorsal regions to diminish (Gestri et. al., 2005).

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 *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, *Rx1* positively regulates the expression of *Zic2* and *Hairy2* which act as anti-neurogenic transcription factors (Ando et. al., 2005). *Rx1* also represses the cell cycle inhibitor *p27Xic1* thus promoting proliferation (Andreazzoli et. al., 2003) To forstall diffentiation of competent cells, the expression of pro-neural genes, which includes *xNgnr-1* and *xDelta-1,* are repressed by *Rx1* (Andreazzoli et. al., 2003). Loss of *Rx1* activity by introduction of an *engrailed* repressor chimera, *Rx-EnR,* results in expansion

of *Ngnr-1* and *Delta-1* in the anterior neural plate thereby reducing proliferation, encouraging neuralization, and consequently reducing size of the eye field.

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

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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) 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 el. 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*, *Xhairy2*, *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 *Six3* is important to the early activation and maintenance of *Pax6* expression specifically in eye primorida.

Morpholino mediated knockdown of *Six3* in *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 *Pax6* activity. On the other hand *Pax6* knockdown results in absence of lens, distorted retinal pigmentation development and a small eye phenotype suggesting that *Pax6* plays an important but relatively subsidiary role in early eye field domain specification. Consonant with this hierarchy of effect, *Six3* morphants can be rescued by *Pax6* ectopic expression. We also report here that in absence of *Six3*, *Pax6* fails to orchestrate and co-ordinate the expression of *Rx1* and *Sox2,* two key players in proliferation and differentiation. Finally, we also report that *Six3* and *Pax6* perturbation have hierarchically consistent effects on *Otx2*, *Lens1*, *Pax2*, *Pitx3*, *MafA* and *γ-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: GAAGCAGCAAAACTAGCGACAGCGA. *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*: GGcACAcCACGAcCCcCACAgAAAA *Pax6-Control-MO*: CAtGcGACTcTGTAAaTgCCAACAT. 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)ATCCCATGGTGTTCAGGTCC and reverse primer (XhoI): CCG(CTCGAG)TGGCTCAAATAGGGGGTCG. 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: forward- CGC(GGATCC)GCAGATGTTaGGcATcACtCAaTCCCTGGGAGGAGAAGC; and reverse- CCG(CTCGAG)GTCCTTTCCCCAGTTTGTCAGTC 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.

Plasmid	Restriction Enzyme	Polymerase	Source/Reference
Six3	HindIII	T3	Zuber et. al., 2003 Dr. M. Zuber
Pax6	Xba	T7	
Rx1	HindIII	T3	
Sox2	EcoRI	T7	Accession: AF022928
Pitx3	EcoRI	T7	Khosrowshahian et. al., 2005 Dr. M. Crawford
MafA	BamHI	T7	Kataoka et. al., 2004 Dr. K. Yasuda
Otx2	SacI	T7	Blitz and Cho, 1995 Dr. Ira Blitz
Lens1	SacI	T7	Accession: AF186464
Pax2	EcoRI	T3	Heller and Brändli, 1997
γ-crystallin	SacI	T3	Dr. Jonathan Henry
Krox20	EcoRI	T7	Dr. Marc Amoyel

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. S econdary 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 e mbryos 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 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 Spenser). Images were acquired using Northern Eclipse software (Empix, Canada).

Table 2.2: List of primers used for semi-quantitative RT-PCR analysis (supplementary table)

Gene	Primer Sequence	Annealing Temperature (°C)	Cycle Number	Amplicon size (bp)	Reference
Pax6	FP: GCAACCTGGCGAGCGATAAGC RP: CCTGCCGTCTCTGGTTCCGTAGTT	57	28	450	Zuber et. al., 2003
Rx1	FP: CCCCAACAGGAGCATTTAGAAGAC RP: AGGGCACTCATGGCAGAAGGTT	67	27	416	Zuber et al., 2003
Sox2	FP: GAGGATGGACACTTATGCCCAC RP: GGACATGCTGTAGGTAGGCGA	68	27	214	Nitta et. al., 2006
BMP4	FP: GAGATTGTCCATTTCCCTTGGC RP: TCAGTGGAAAGAAGTCCAGCCG	62	26	262	Malarte et. al., 2006
Otx2	FP: GGATGGATTTGTTACATCCGTC RP: CACTCTCCGAGCTCACTTCCC	57	27	315	Zuber et. al., 2003
Six3	FP: TTGTCTGTCTGTCTCTTGTT RP: TTCTGTGTTTGGTTTATCTC	57	28	369	Zuber et. al., 2003
MafA	FP: CTTGCTCCTCCTCAATCTCTGG RP: CCGACAAAGGCGAAAGCTGGTG	57	30	331	Ishibashi et. al., 2001

Lens1	FP:CCTCTGGAGGCAGGAGAAGAAAACG RP: TCTGAGGGTTATATCCAGAGCCAA	60	30	462	Kenyon et. al., 1999
Pitx3	FP: AAGTCCGTTGTCATCAC RP: CTTCTGGAAAGTGGAGC	57	32	560	Khosrowshahian et. al., 2005
γ-crystallin	FP: CAAGGGCAGATGATGGAGTT RP: GAGGCTCCCCAGTCACTGTA	57	30	185	U48901 (Unigene)
Pax2	FP: GCAATGCAGACCTAGGAAGC RP: CATCTGGAAAGGCTGGATGT	60	28	150	Park and Saint-Jeannet, 2008
Krox20	FP: AACCGCCCCAGTAAGACC RP: GTGTCAGCCTGTCCTGTTAG	57	28	448	Xenopus Resource Centre (Xenbase)
EF1a	FP: CAGATTGGTGCTGGATATG RP: ACTGCCTTGATGACTCCTA	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).

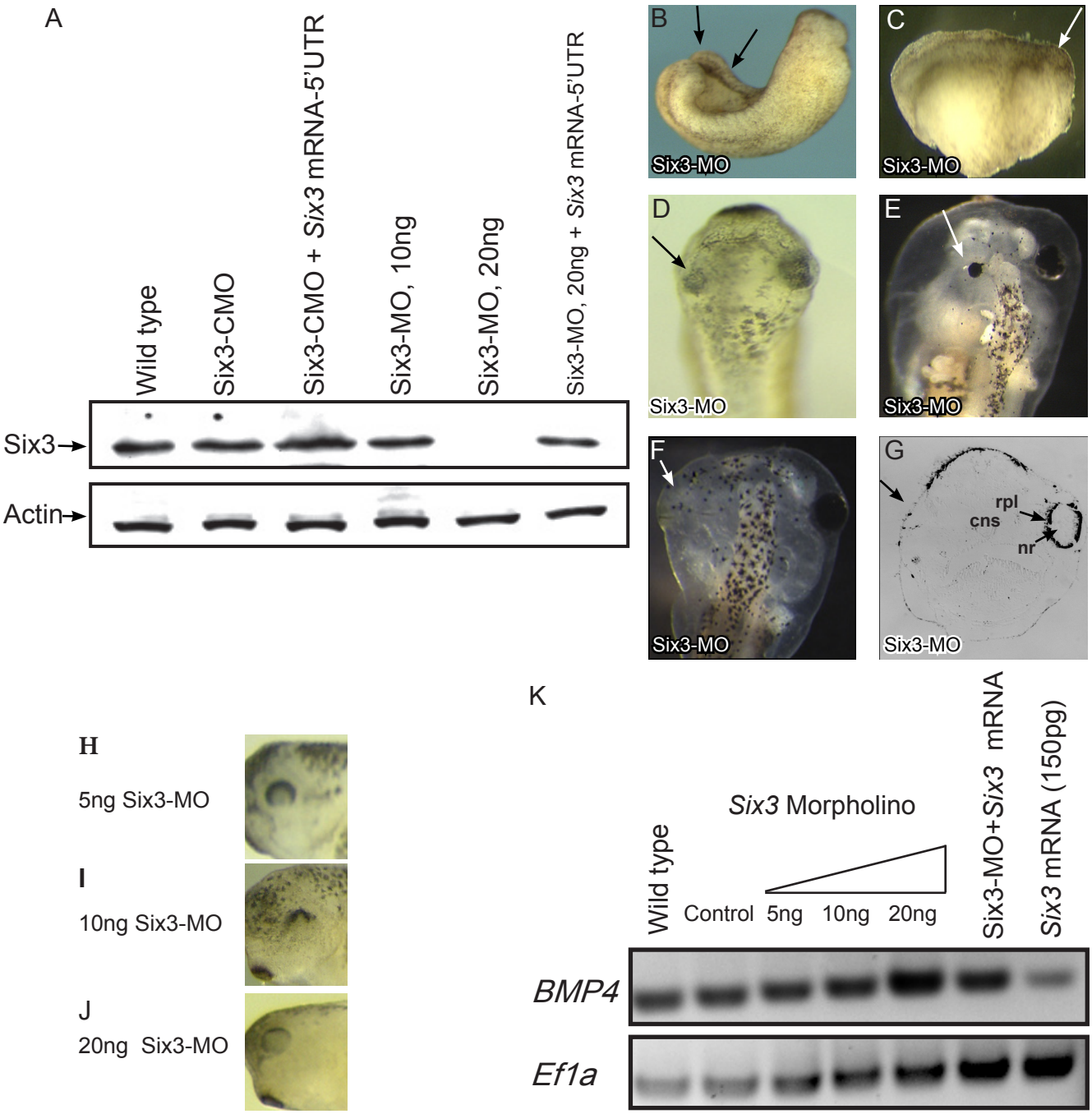

A
Wild type
Six3-CMO
Six3-CMO + Six3 mRNA-5'UTR
Six3-MO, 10ng
Six3-MO, 20ng
Six3-MO, 20ng + Six3 mRNA-5'UTR
Six3
Actin
B
Six3-MO
C
Six3-MO
D
Six3-MO
E
Six3-MO
F
Six3-MO
G
rpl
cns
nr
Six3-MO
H
5ng Six3-MO
I
10ng Six3-MO
J
20ng Six3-MO
K
Six3 Morpholino
Wild type
Control
5ng
10ng
20ng
Six3-MO+Six3 mRNA
Six3 mRNA (150pg)
BMP4
Ef1a

Table 2.3: Effect of *Six3* morpholino mediated knockdown with percentages of phenotypes observed at different concentrations of the Morpholino injected.

Morpholino Concentration (*Six3*)	**5ng**	**10ng**	**20ng**	**30ng**	**20ng *Control* MO**
n	165	142	288	121	231
Severe Phenotype	0%	2%	34%	36%	1.3%
Moderate Phenotpye	0%	3.5%	29%	4%	1%
Mild Phenotype	11%	54%	17%	8.5%	5.2%
Normal eye	77%	22.5%	7%	3.5%	79%
Dead	12%	18%	13%	48%	13.5%

***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.

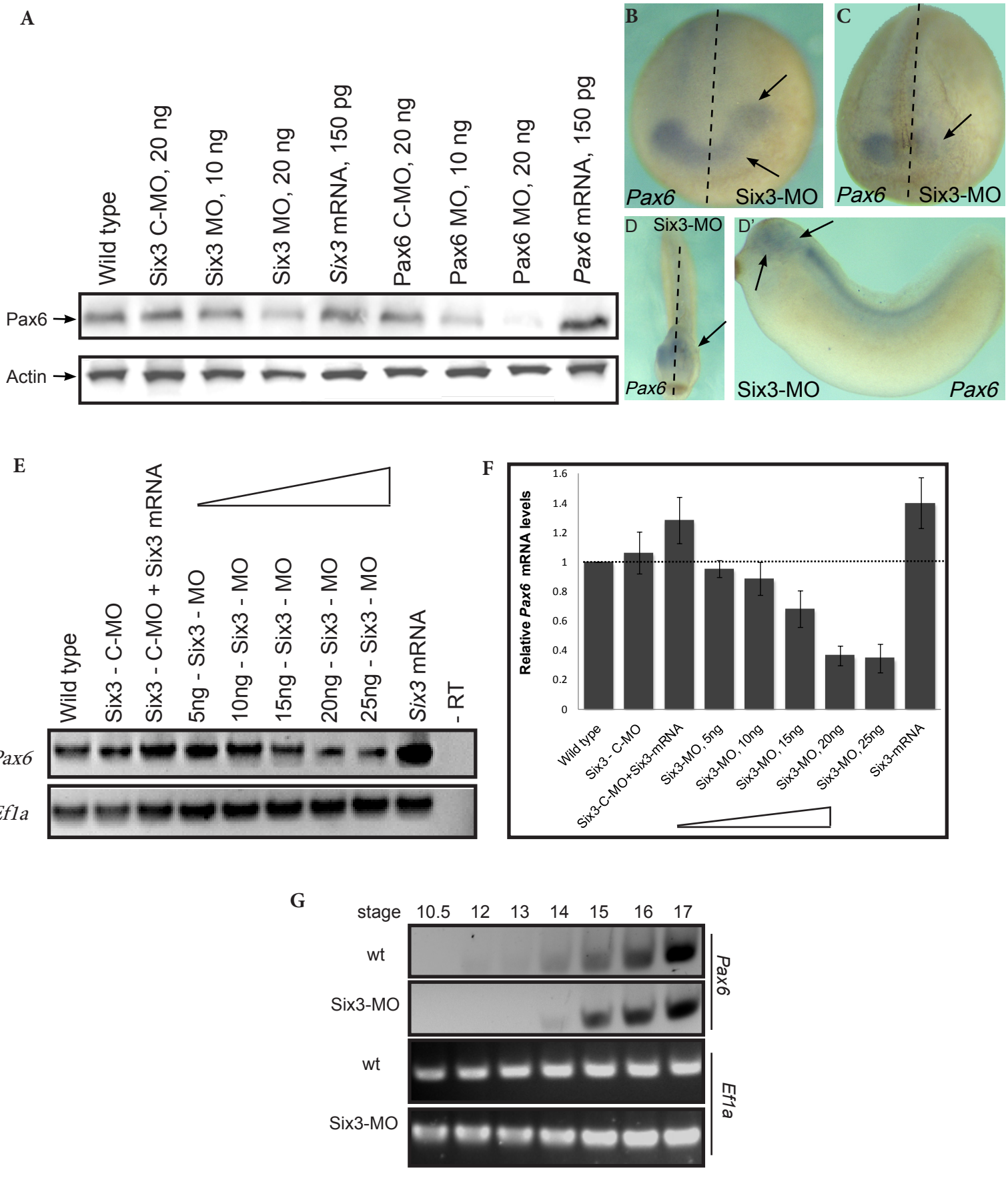
Figure 2.2: Six3 is essential for early Pax6 activation and maintenance

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).


A
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
Actin
B
Pax6
Six3-MO
C
Pax6
Six3-MO
D
Six3-MO
Pax6
D'
Six3-MO
Pax6
E
Wild type
Six3 - C-MO
Six3 - C-MO + Six3 mRNA
5ng - Six3 - MO
10ng - Six3 - MO
15ng - Six3 - MO
20ng - Six3 - MO
25ng - Six3 - MO
Six3 mRNA
- RT
Pax6
Ef1a
F
Relative Pax6 mRNA levels
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Wild type
Six3 - C-MO
Six3-C-MO+Six3-mRNA
Six3-MO, 5ng
Six3-MO, 10ng
Six3-MO, 15ng
Six3-MO, 20ng
Six3-MO, 25ng
Six3-mRNA
G
stage 10.5 12 13 14 15 16 17
wt
Six3-MO
wt
Six3-MO
Pax6
Ef1a

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.J). Compared to the uninjected side, ectopic *Pax6* expression later expanded the *Six3*

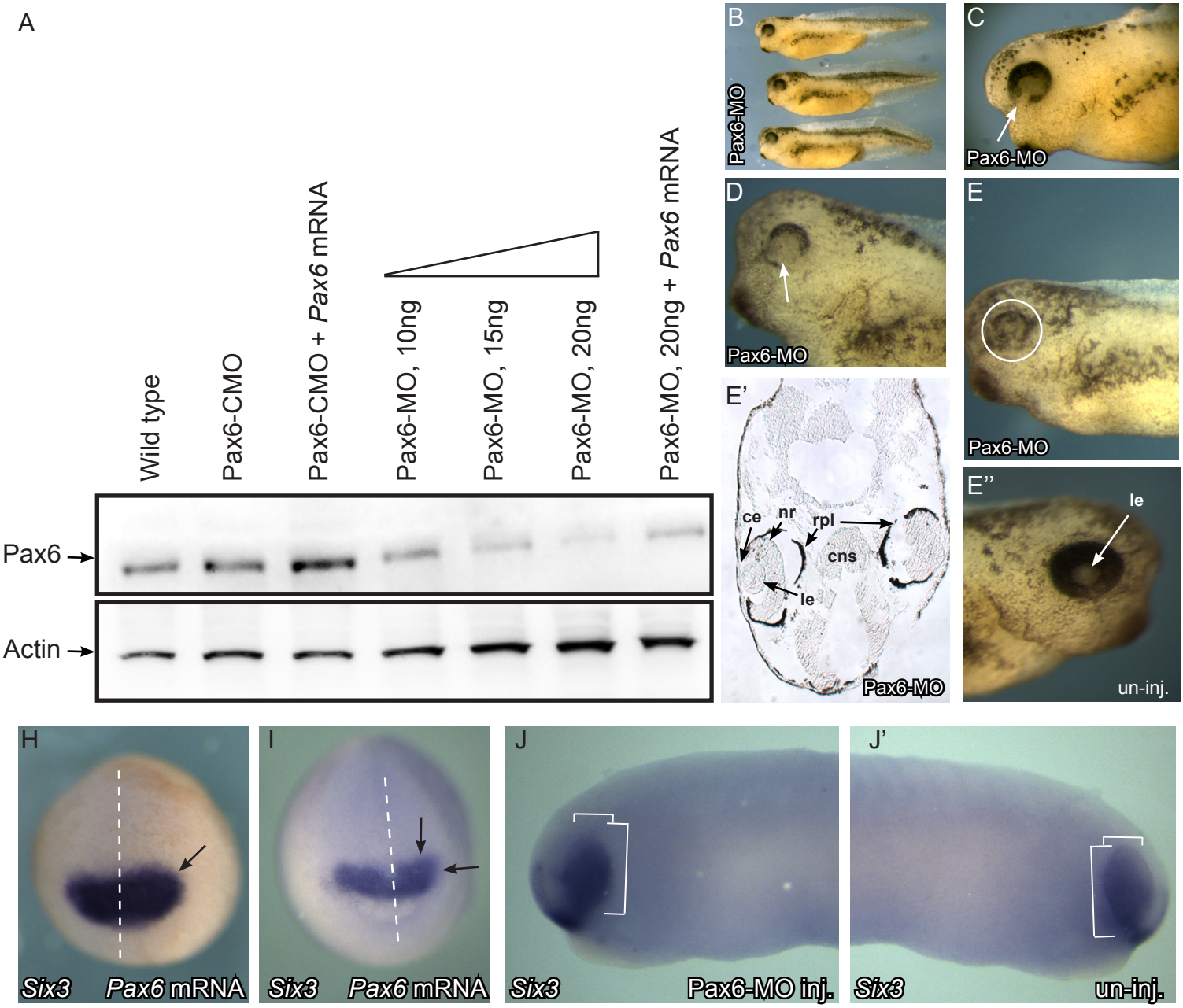
expression domain at early to mid neurula stages – this suggests *Pax6* feedbacks upon *Six3* very early in development (Figure 2.3 H,I)

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').


A
Wild type
Pax6-CMO
Pax6-CMO + Pax6 mRNA
Pax6-MO, 10ng
Pax6-MO, 15ng
Pax6-MO, 20ng
Pax6-MO, 20ng + Pax6 mRNA
Pax6
Actin
B
Pax6-MO
C
Pax6-MO
D
Pax6-MO
E
Pax6-MO
E'
ce
nr
rpl
cns
le
Pax6-MO
E''
le
un-inj.

H
Six3
Pax6 mRNA
I
Six3
Pax6 mRNA
J
Six3
Pax6-MO inj.
J'
Six3
un-inj.

Table 2.4: Effect of *Pax6* morpholino mediated knockdown with percentages of phenotypes observed at different concentrations of the Morpholino injected.

Morpholino Concentration *(Pax6)*	**5ng**	**10ng**	**20ng**	**30ng**	**20ng *Control* MO**
n	133	101	156	84	136
Eyes absent	2%	3%	10%	6%	1%
RPE deformities	5%	8%	40%	30%	2%
Lens absent or reduced	2%	33%	38%	32%	3%
Normal eye	83%	44%	12%	5%	86%
Dead	8%	12%	10%	27%	8%

***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 γ-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 in 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.

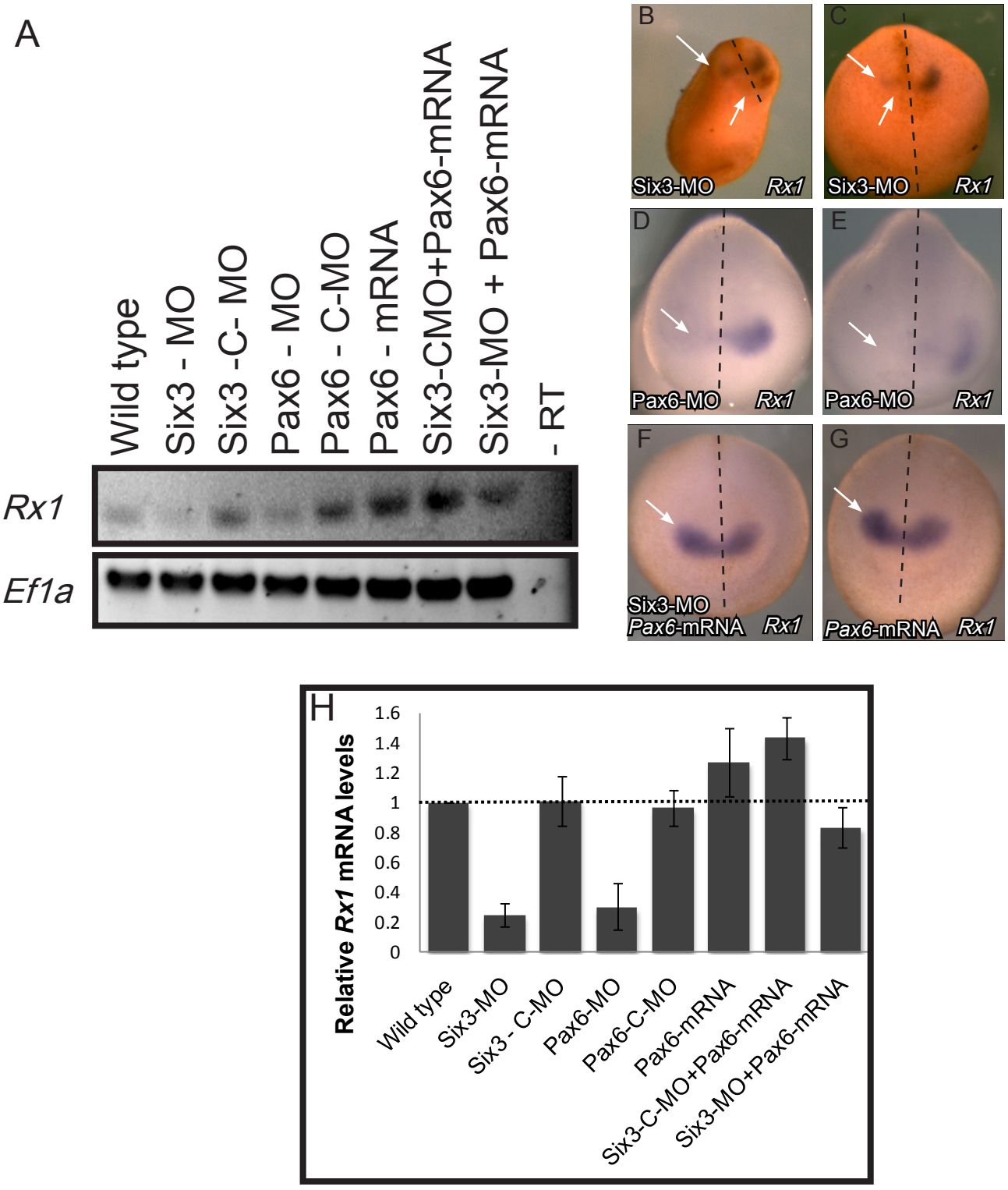
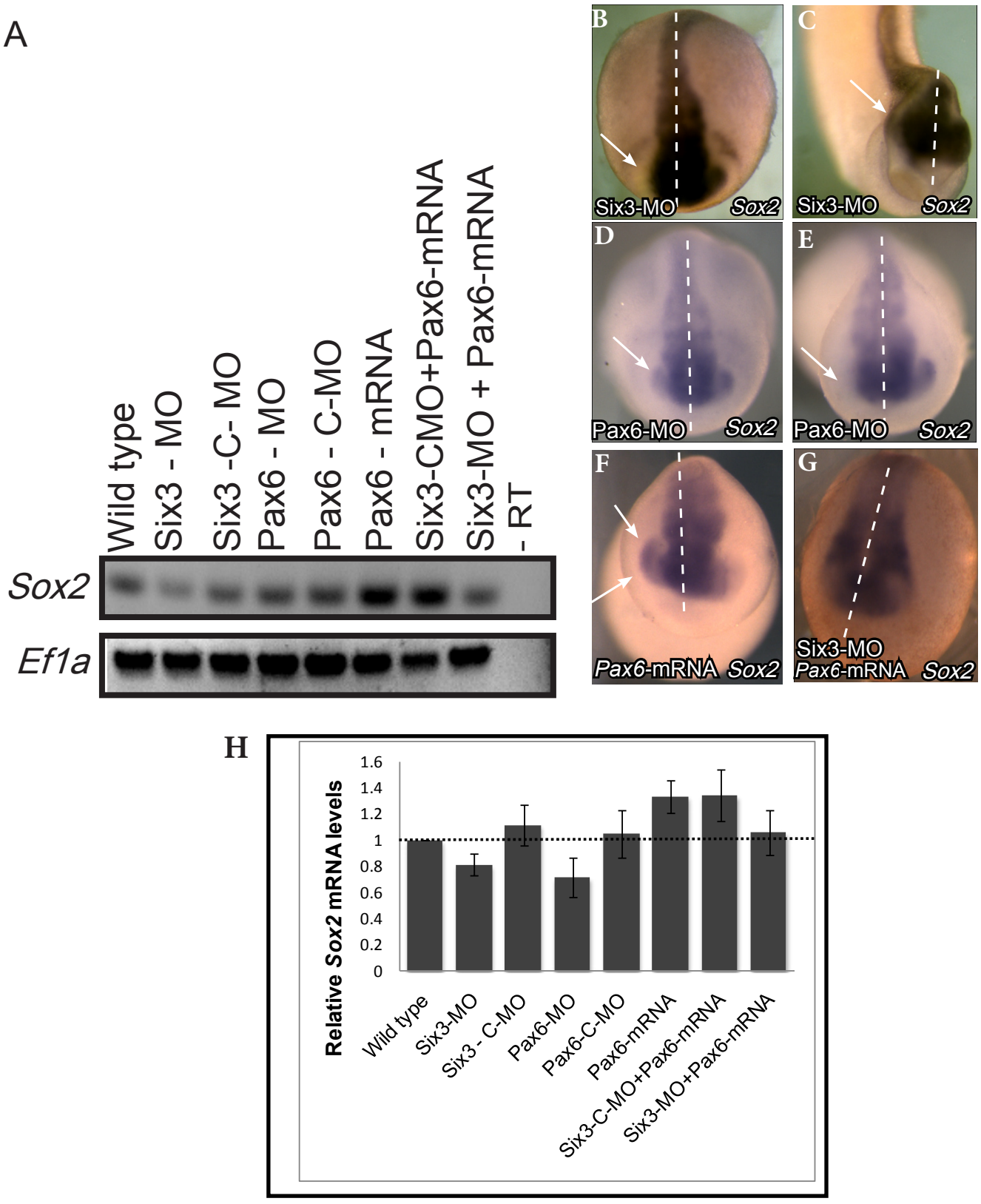

A
Wild type
Six3 - MO
Six3 -C- MO
Pax6 - MO
Pax6 - C-MO
Pax6 - mRNA
Six3-CMO+Pax6-mRNA
Six3-MO + Pax6-mRNA
- RT
Rx1
Ef1a
B
Six3-MO Rx1
C
Six3-MO Rx1
D
Pax6-MO Rx1
E
Pax6-MO Rx1
F
Six3-MO
Pax6-mRNA Rx1
G
Pax6-mRNA Rx1
H
Relative Rx1 mRNA levels
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Wild type
Six3-MO
Six3 - C-MO
Pax6-MO
Pax6-C-MO
Pax6-mRNA
Six3-C-MO+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
Wild type
Six3 - MO
Six3 -C- MO
Pax6 - MO
Pax6 - C-MO
Pax6 - mRNA
Six3-CMO+Pax6-mRNA
Six3-MO + Pax6-mRNA
- RT
Sox2
Ef1a
B
Six3-MO
Sox2
C
Six3-MO
Sox2
D
Pax6-MO
Sox2
E
Pax6-MO
Sox2
F
Pax6-mRNA
Sox2
G
Six3-MO
Pax6-mRNA
Sox2
H
Relative Sox2 mRNA levels
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Wild type
Six3-MO
Six3 - C-MO
Pax6-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 were 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*;

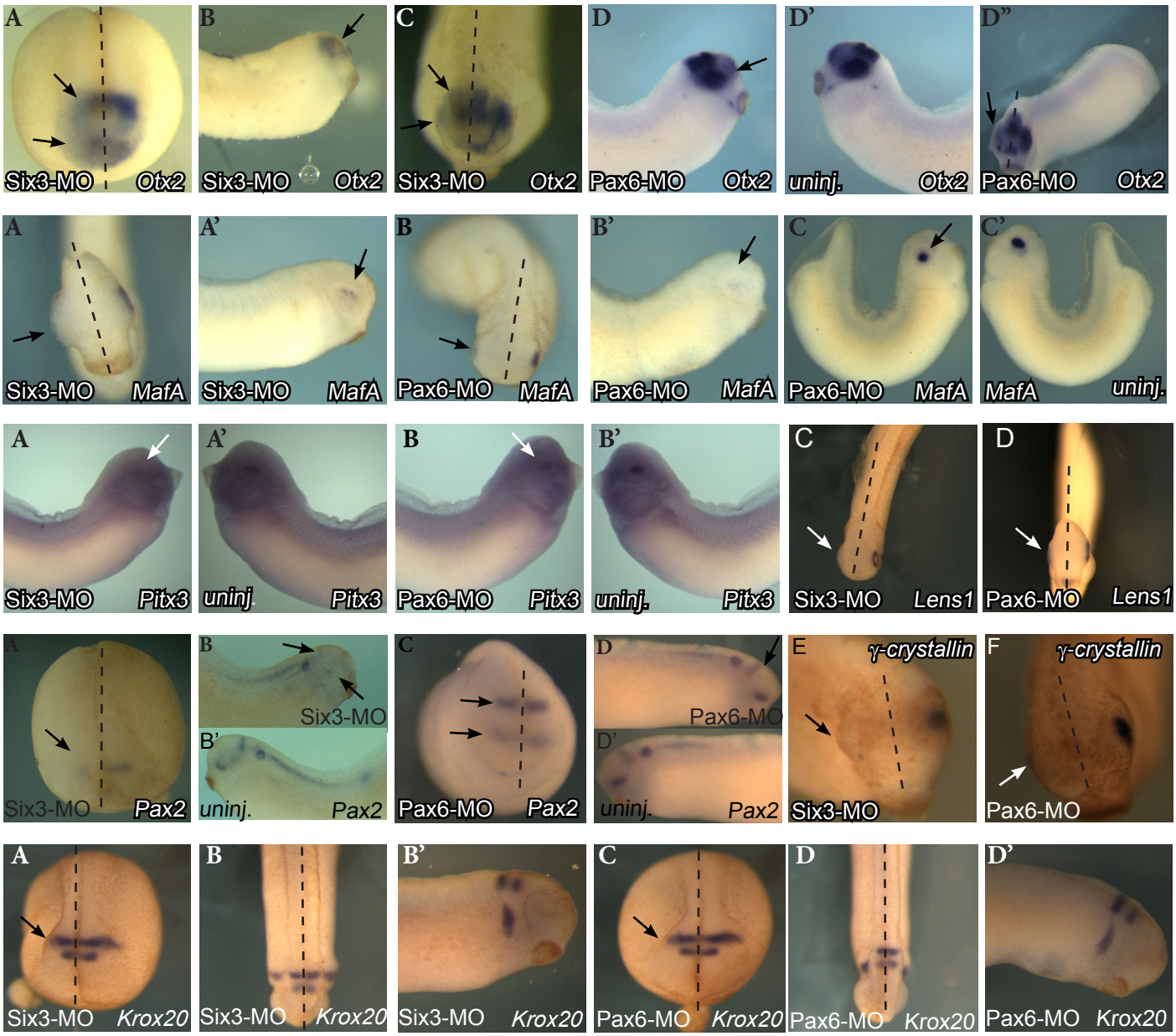
2.6.2: A-C' – *MafA*;

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


A
Six3-MO Otx2
B
Six3-MO Otx2
C
Six3-MO Otx2
D
Pax6-MO Otx2
D'
uninj. Otx2
D"
Pax6-MO Otx2
A
Six3-MO MafA
A'
Six3-MO MafA
B
Pax6-MO MafA
B'
Pax6-MO MafA
C
Pax6-MO MafA
C'
MafA uninj.
A
Six3-MO Pitx3
A'
uninj. Pitx3
B
Pax6-MO Pitx3
B'
uninj. Pitx3
C
Six3-MO Lens1
D
Pax6-MO Lens1
A
Six3-MO Pax2
B
Six3-MO
B'
uninj. Pax2
C
Pax6-MO Pax2
D
Pax6-MO
D'
uninj. Pax2
E
γ-crystallin
Six3-MO
F
γ-crystallin
Pax6-MO
A
Six3-MO Krox20
B
Six3-MO Krox20
B'
Six3-MO Krox20
C
Pax6-MO Krox20
D
Pax6-MO Krox20
D'
Pax6-MO Krox20

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*.

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 here 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).

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*$^{-/-}$ 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

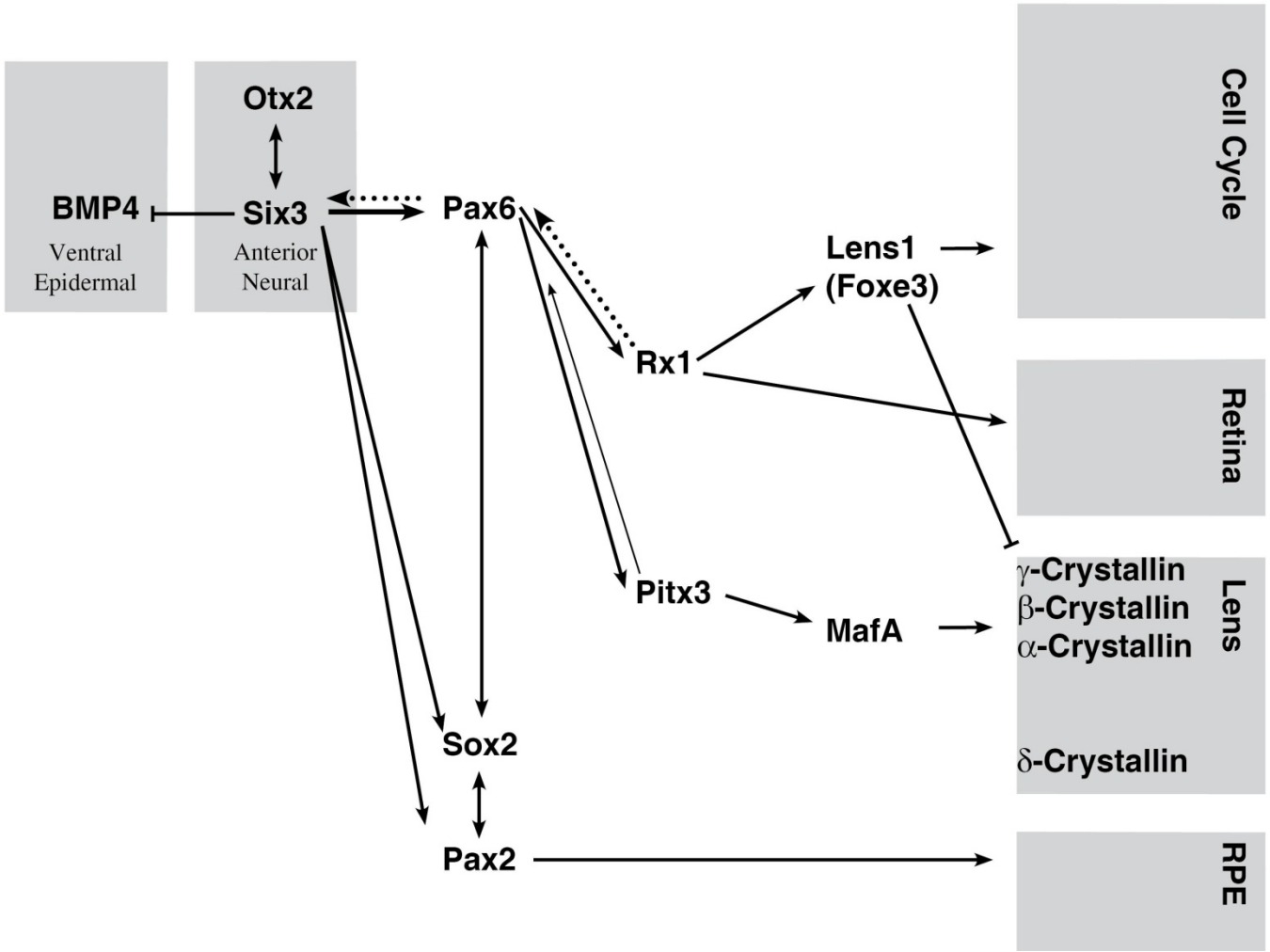
the foundation for eye development and morphogenesis. A similar permissive interaction between *BMP4* and *Otx2* is observed in early development of the cement gland (Gammill and Sive, 1999). Diminished *Six3* activity further relaxes constraints upon the influence of ventral genes like *BMP4*, resulting 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.


Otx2
BMP4
Six3
Pax6
Ventral
Epidermal
Anterior
Neural
Lens1
(Foxe3)
Rx1
Pitx3
MafA
Sox2
Pax2
γ-Crystallin
β-Crystallin
α-Crystallin
δ-Crystallin
Cell Cycle
Retina
Lens
RPE

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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 co nsequence 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).

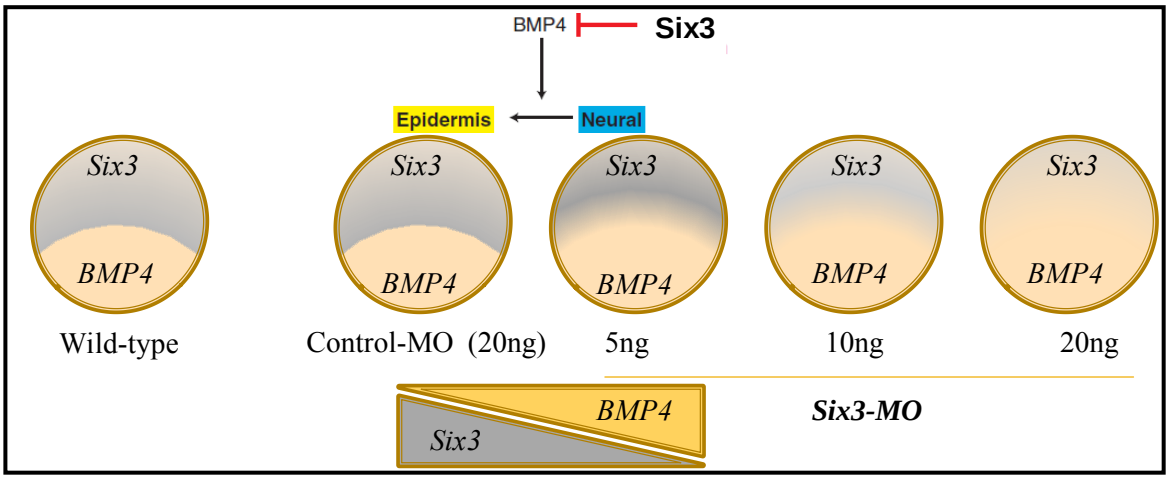


Figure 3.1: Antagonistic relationship shared between Six3 and BMP4.

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 downregulatd 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 medakafish, *Pax6* activates the expression of *Prox1* which activates inhibitors of cell cycle – *Cdkn1b* ($p27^{Kip1}$) and *Cdkn1c* ($p57^{Kip2}$) 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 stimulatiing 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 primoridum 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 over-lap, the effects of *Six3* and *Pax6* mis-regulation 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).

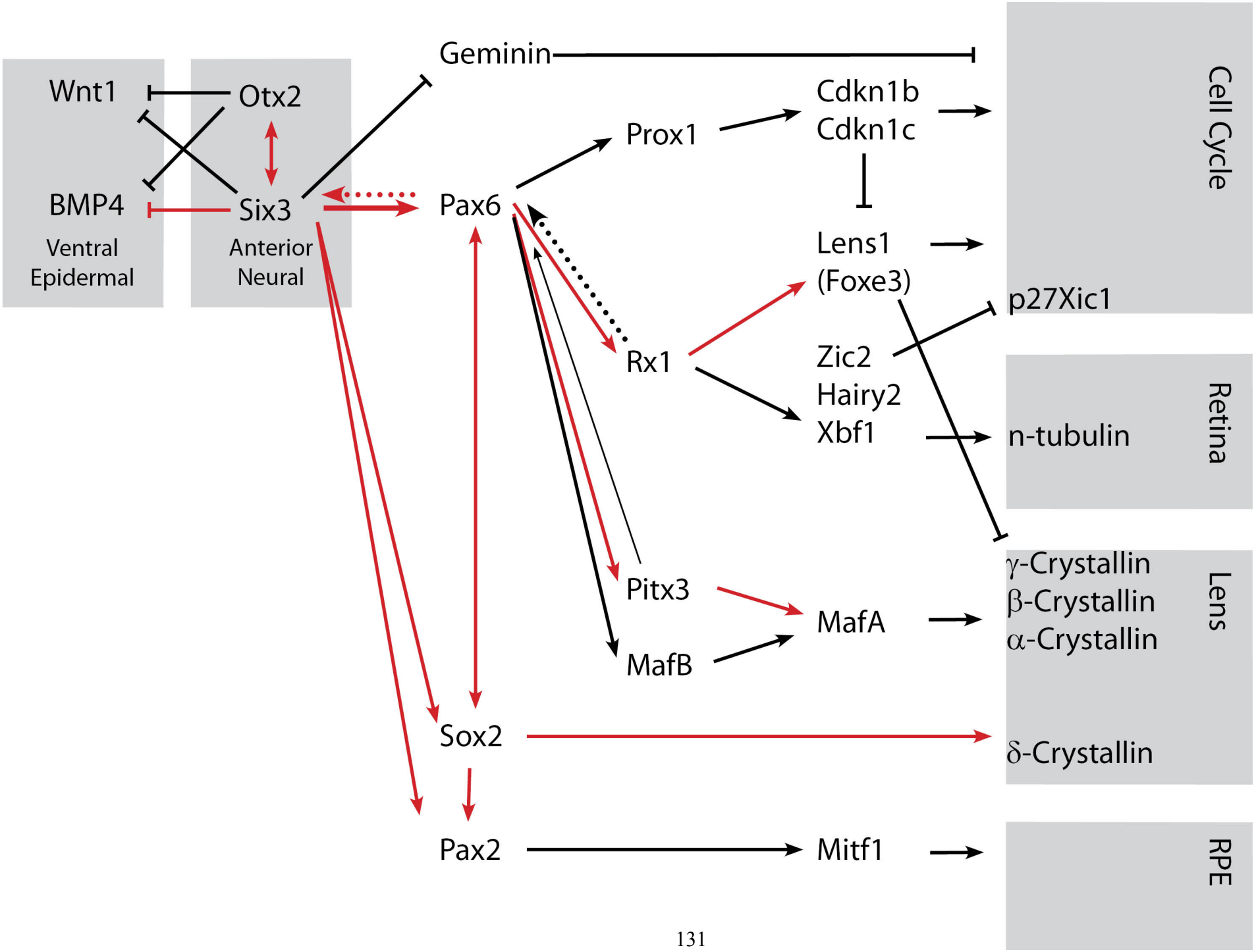
Proposed eye field signaling model

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 *Rx1* by *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 *in vivo*. For example, if over-expression of *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: *Six3* inactivation impairs *Rx1* expression, and *Pax6* can rescue it – but does not rescue the eye development process. This clearly suggests that *Six3, Pax6* and *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.


Wnt1
Otx2
BMP4
Six3
Ventral Epidermal
Anterior Neural
Geminin
Prox1
Cdkn1b
Cdkn1c
Pax6
Lens1 (Foxe3)
Rx1
Zic2
Hairy2
Xbf1
p27Xic1
n-tubulin
Pitx3
MafB
MafA
γ-Crystallin
β-Crystallin
α-Crystallin
δ-Crystallin
Sox2
Pax2
Mitf1
Cell Cycle
Retina
Lens
RPE

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 *in situs*, 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 *in vitro* and *in vivo*. *Six3* expression is required to activate the expression of *Pax6* (Liu et. al., 2006; current study). *Six3* but it also plays two additional roles – first it maintains a neural bias in the anterior neural plate by inhibiting expression of *BMP4* and *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 *Pax6*, and helping to sustain its level, its promotes differentiation at later stages (Figure 3.2)

At later stages of eye development, *Pax6* limits the expression of *Six3* in the eye field (Liu et. al., 2006; current study), possibly by regulating the expression of *Rx1* which controls proliferation of the retinal progenitor cells in which *Six3* expresses (Harris et. al., 2003; current study; Figure 3.2). Over-expression of *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).

Pax6 mediated activation of *MafA* is critical for crystallin expression (Takeuchi et. al., 2009). *Pitx3* regulation by 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.

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APPENDICES

APPENDIX A

Normalized Pax6 protein levels with respect to Actin levels

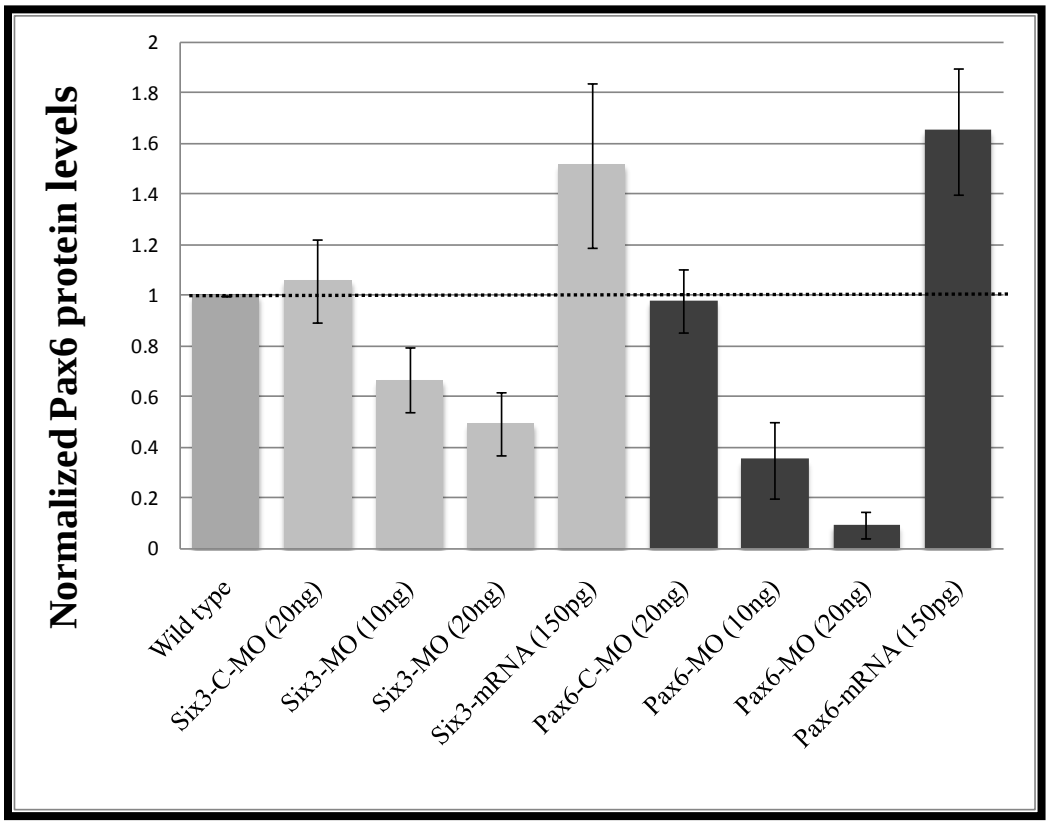


Figure A1: Normalized Pax6 protein levels.

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

APPENDIX B

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

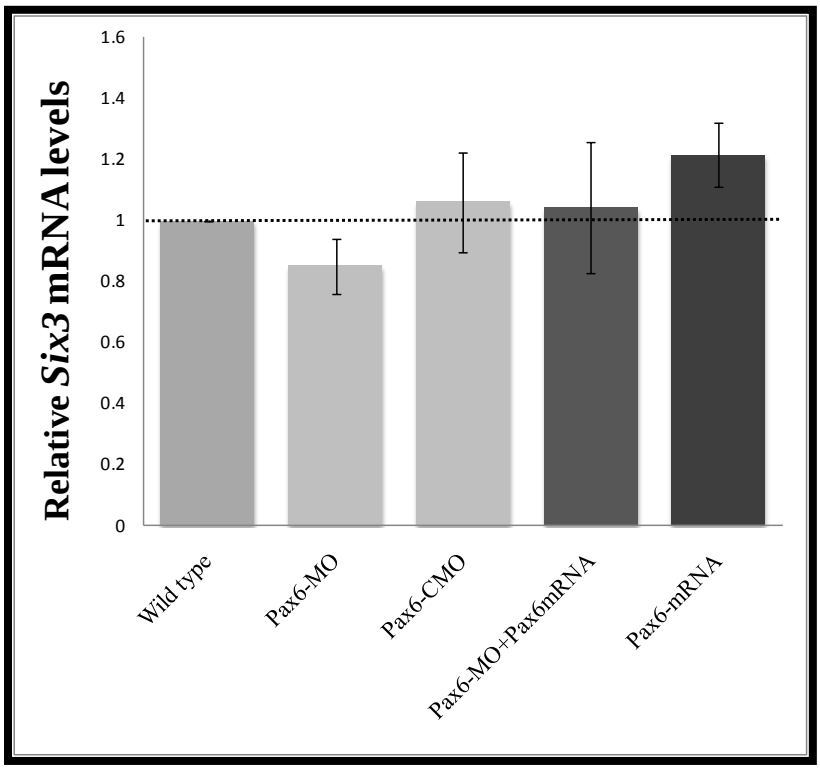


Figure B1: Relative *Six3* mRNA levels upon *Pax6* knockdown.

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.

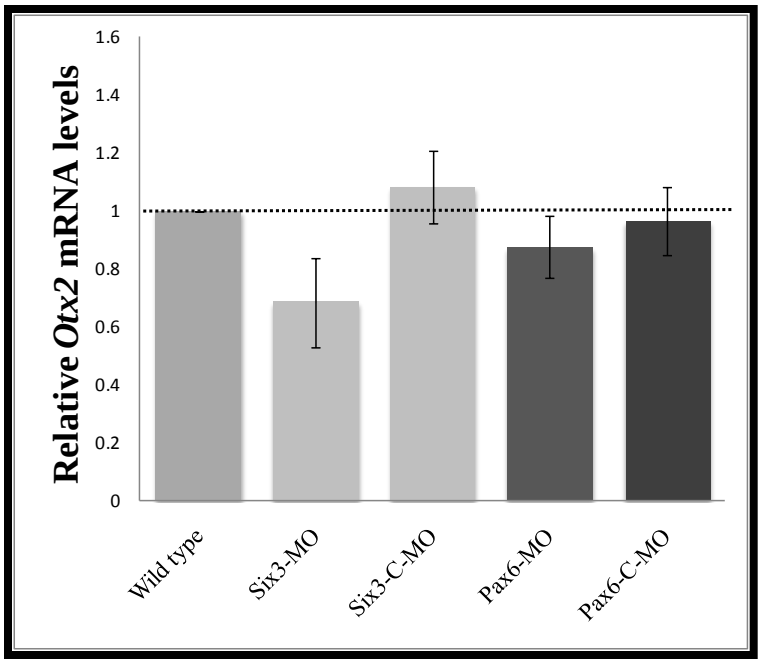


Figure B2: Relative *Otx2* mRNA levels upon *Six3* and *Pax6* knockdown

Upon Six3 and Pax6 knockdown, Otx2 levels are observed to decline. The severity is more in Six3 knockdown as compared to Pax6 knockdown.

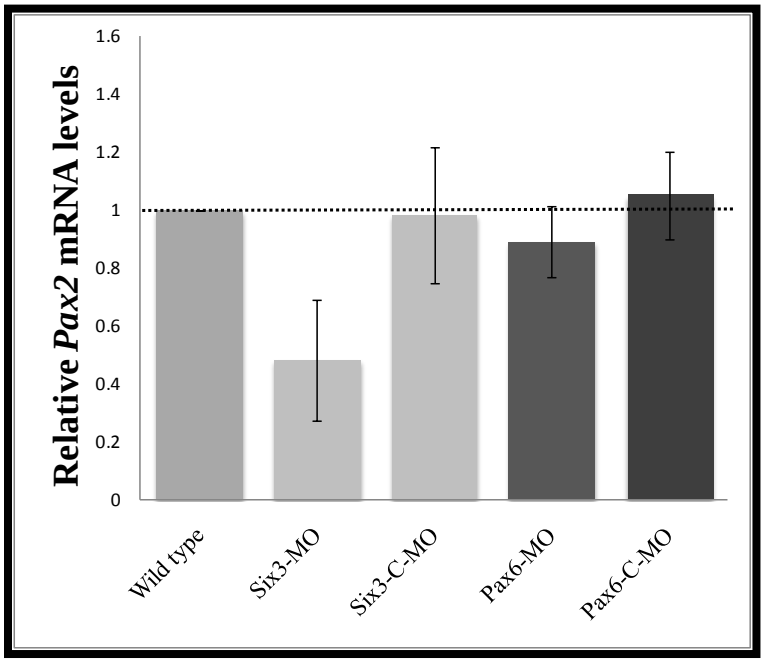


Figure B3: Relative *Pax2* mRNA levels upon *Six3* and *Pax6* knockdown

Pax2 expression level is abrogated upon *Six3* knockdown in the anterior neural plate. Upon *Pax6* knockdown expression is slightly diminished.

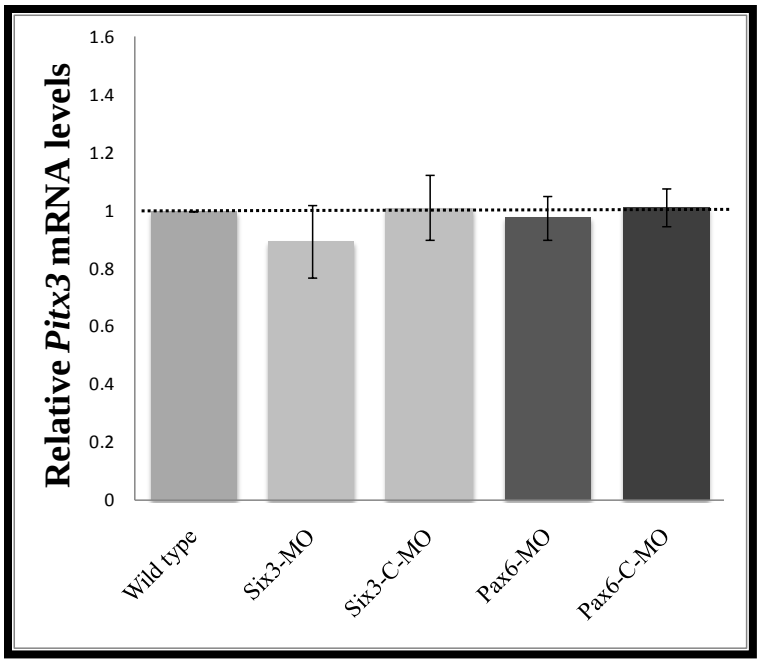


Figure B4: Relative *Pitx3* mRNA levels upon *Six3* and *Pax6* knockdown

Upon *Six3* knockdown, the expression of *Pitx3* is completely abolished in the PLE as confirmed by in situ hybdridization. However, *Pitx3* expression in the other regions (somites, heart and brachial arches) are unaffected upon *Six3* knockdown.

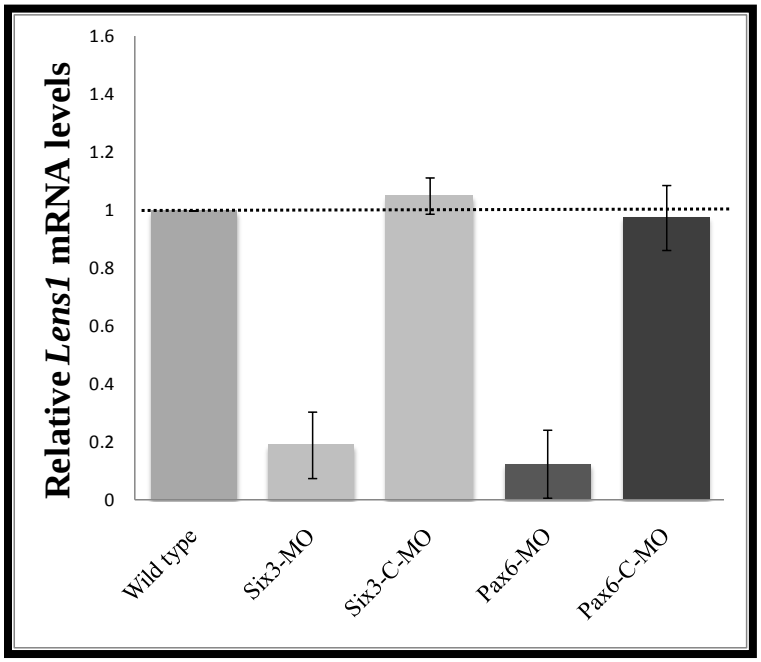


Figure B5: Relative *Lens1* mRNA levels upon *Six3* and *Pax6* knockdown

Lens1 levels are completey abolished upon *Six3* and *Pax6* knockdowns.

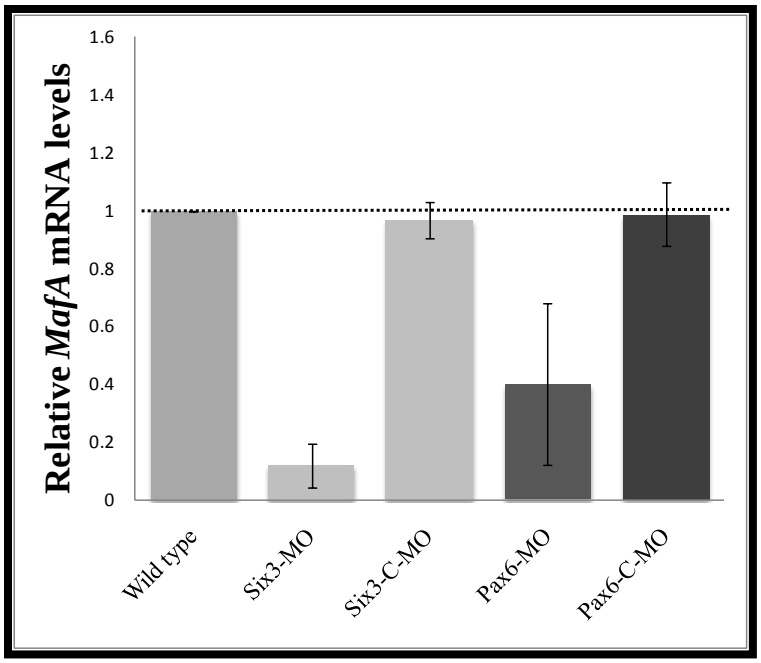


Figure B6: Relative *MafA* mRNA levels upon Six3 and Pax6 knockdown

MafA levels are completey downregulted in the eye field upon *Six3* and *Pax6* knockdown as confirmed by in situ hybridization.

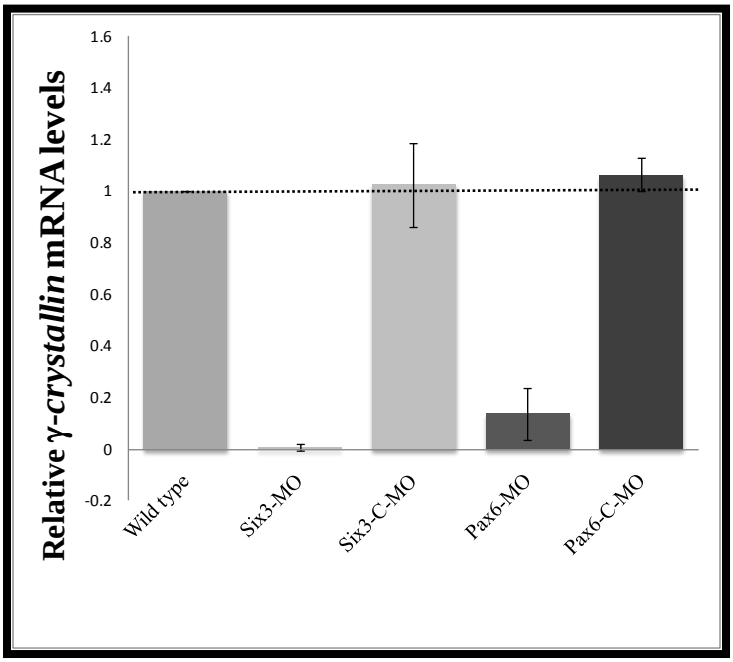


Figure B7: Relative *γ-crystallin* mRNA levels upon Six3 and Pax6 knockdown

γ-crystallin expression – a lens differentiation marker is almost deleted upon *Six3* and *Pax6* knockdown

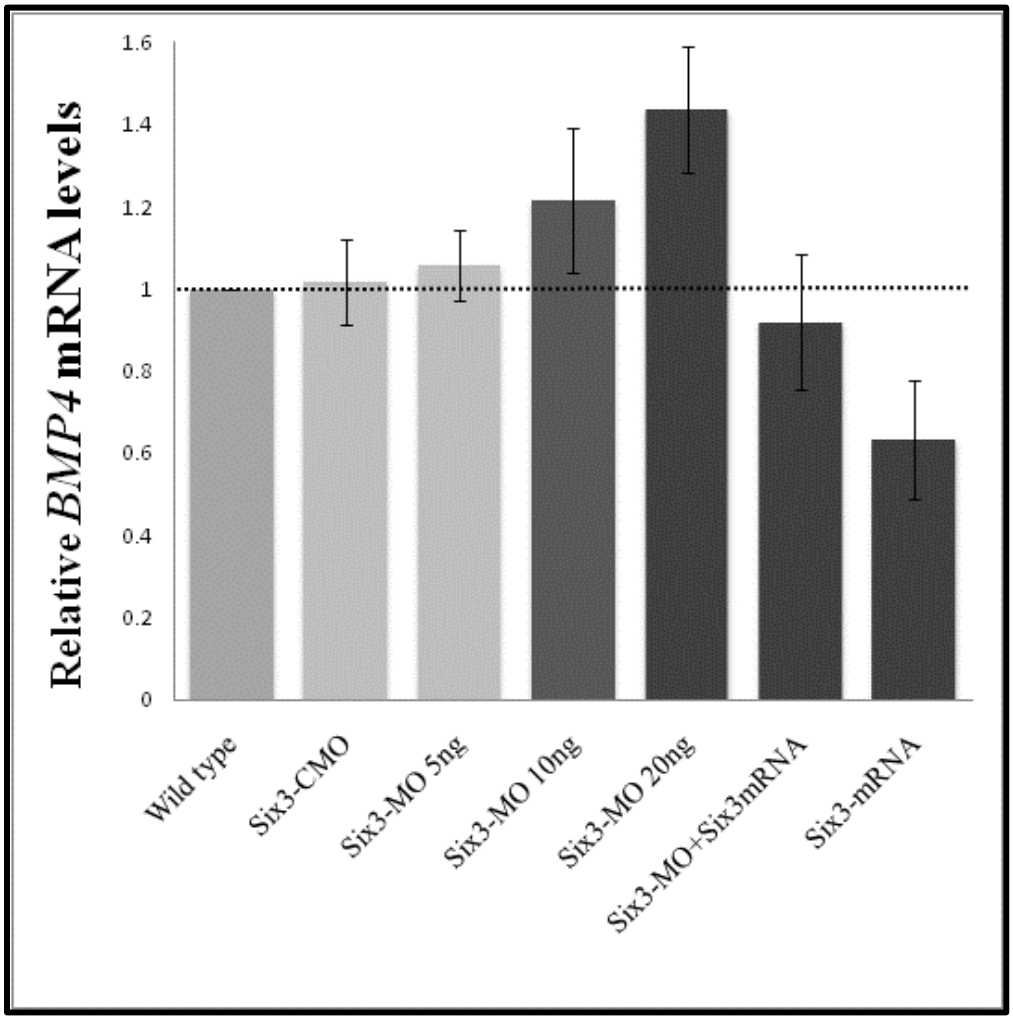


Figure B8: Relative *BMP4* mRNA levels upon Six3 knockdown and overexpression

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 over-expression, *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.

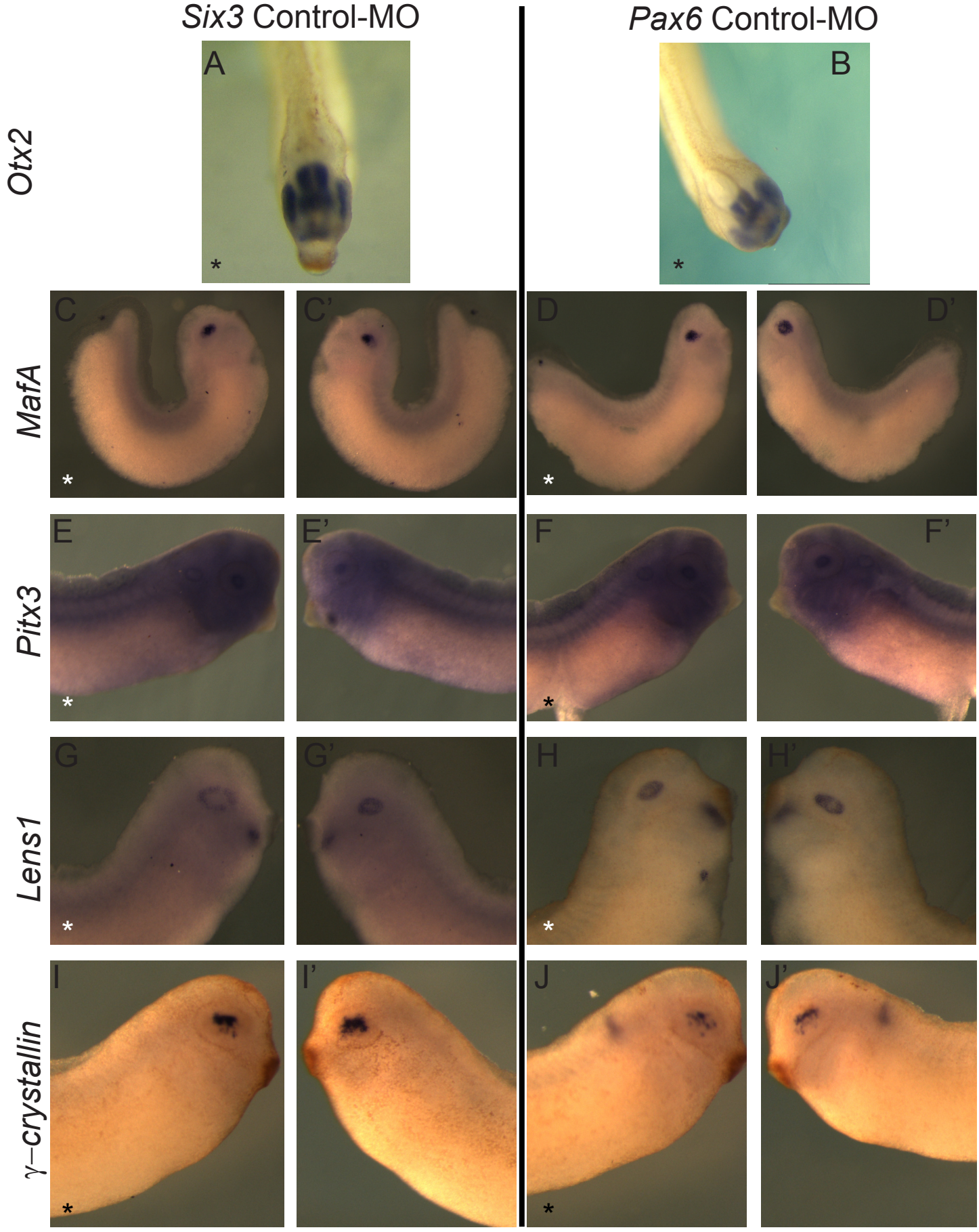
Otx2: A, (*Six3* MO), B (*Pax6* MO)

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
A
B
C
C'
D
D'
E
E'
F
F'
G
G'
H
H'
I
I'
J
J'
*
* : control morpholino injected side

APPENDIX D

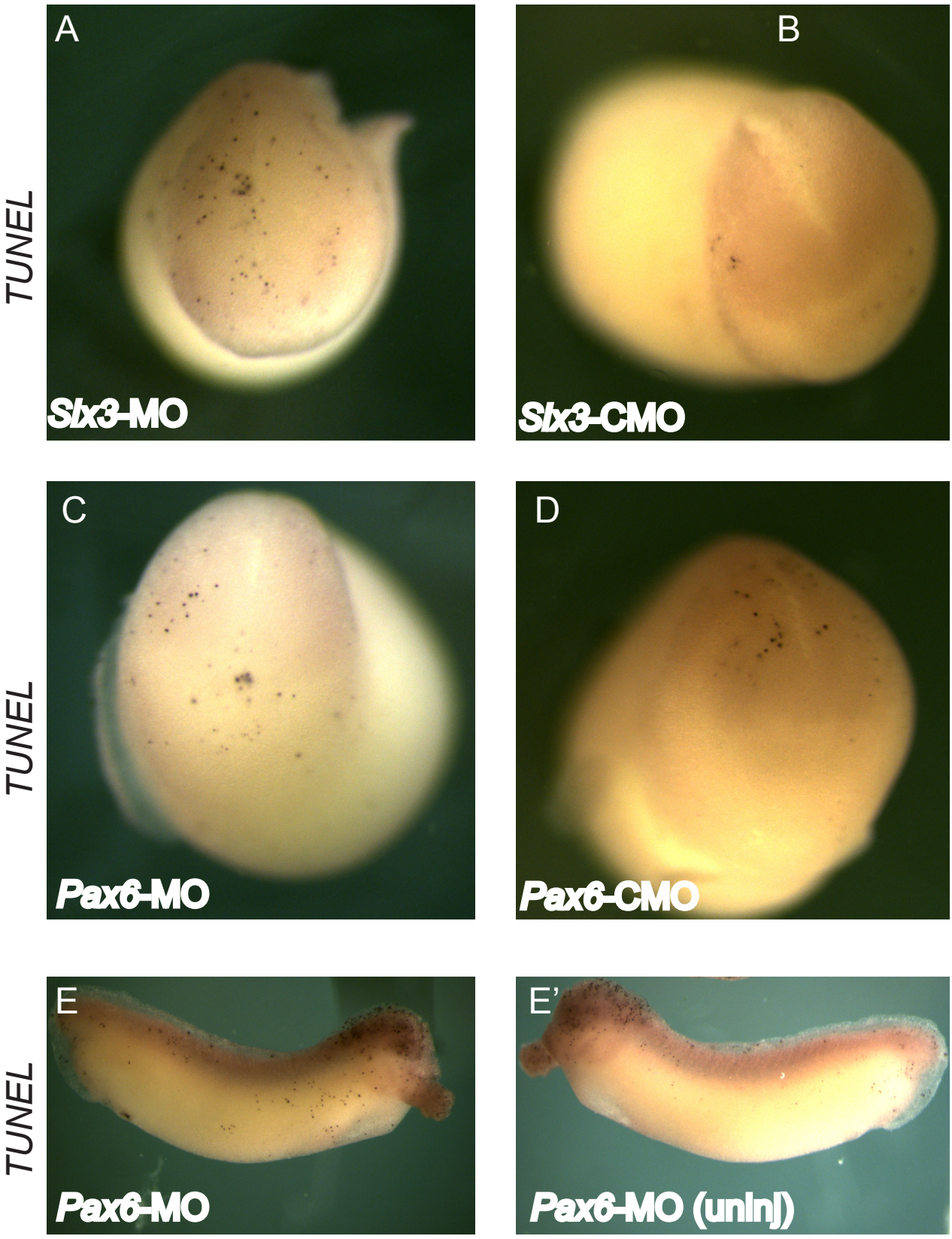
TUNEL ASSAY PROTOCOL MODIFICATIONS

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
4. Day3: Subsequent Fixing and Methanol Gradient – done in cold room.

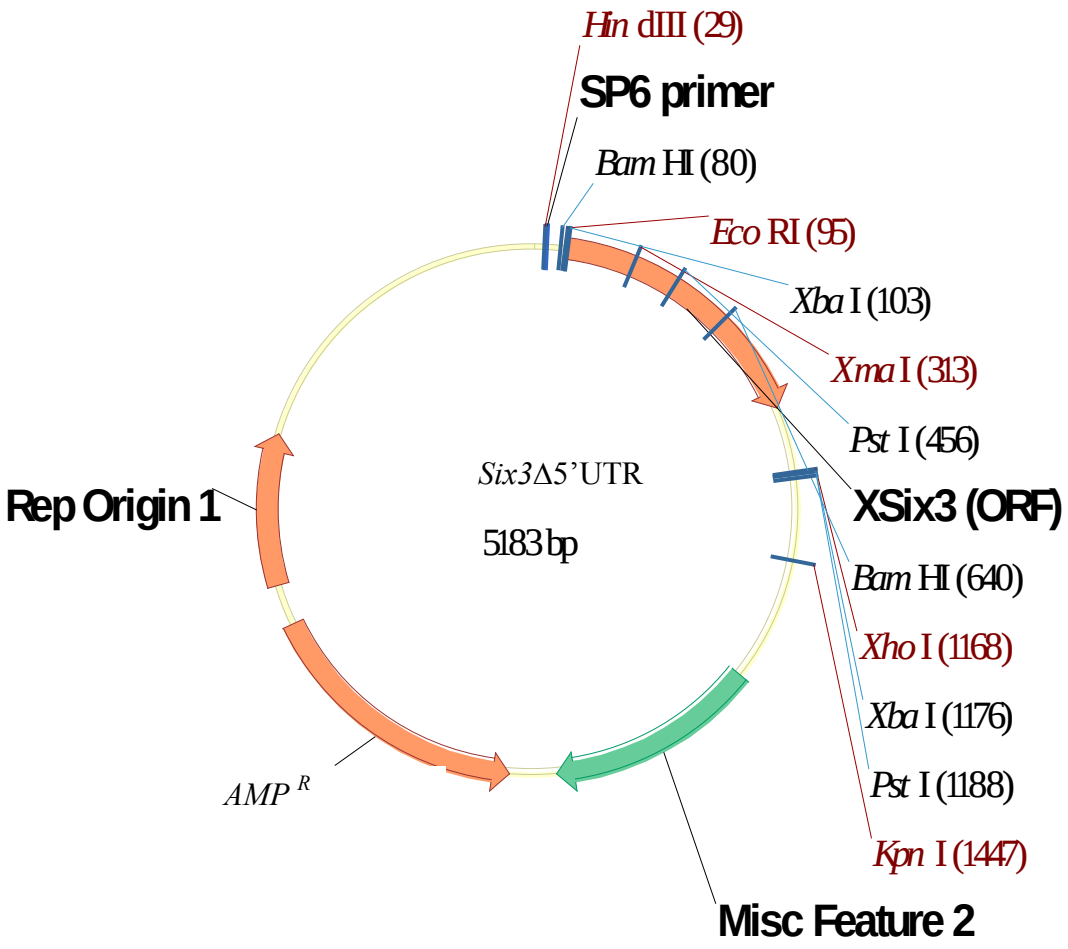
Figure D1: TUNEL Assay to detect apoptotic positive cells upon Six3 and Pax6 knockdown. Left side injected. Six3 MO (A), Six3 CMO (B), Pax6 MO early stages (C), Pax6 CMO early stages (D), Pax6 MO - at late lens differentiation stages (E) and uninjected side (E'). No significant difference in number of *TUNEL* positive cells.


TUNEL
A
Slx3-MO
B
Slx3-CMO
TUNEL
C
Pax6-MO
D
Pax6-CMO
TUNEL
E
Pax6-MO
E'
Pax6-MO (uninj)

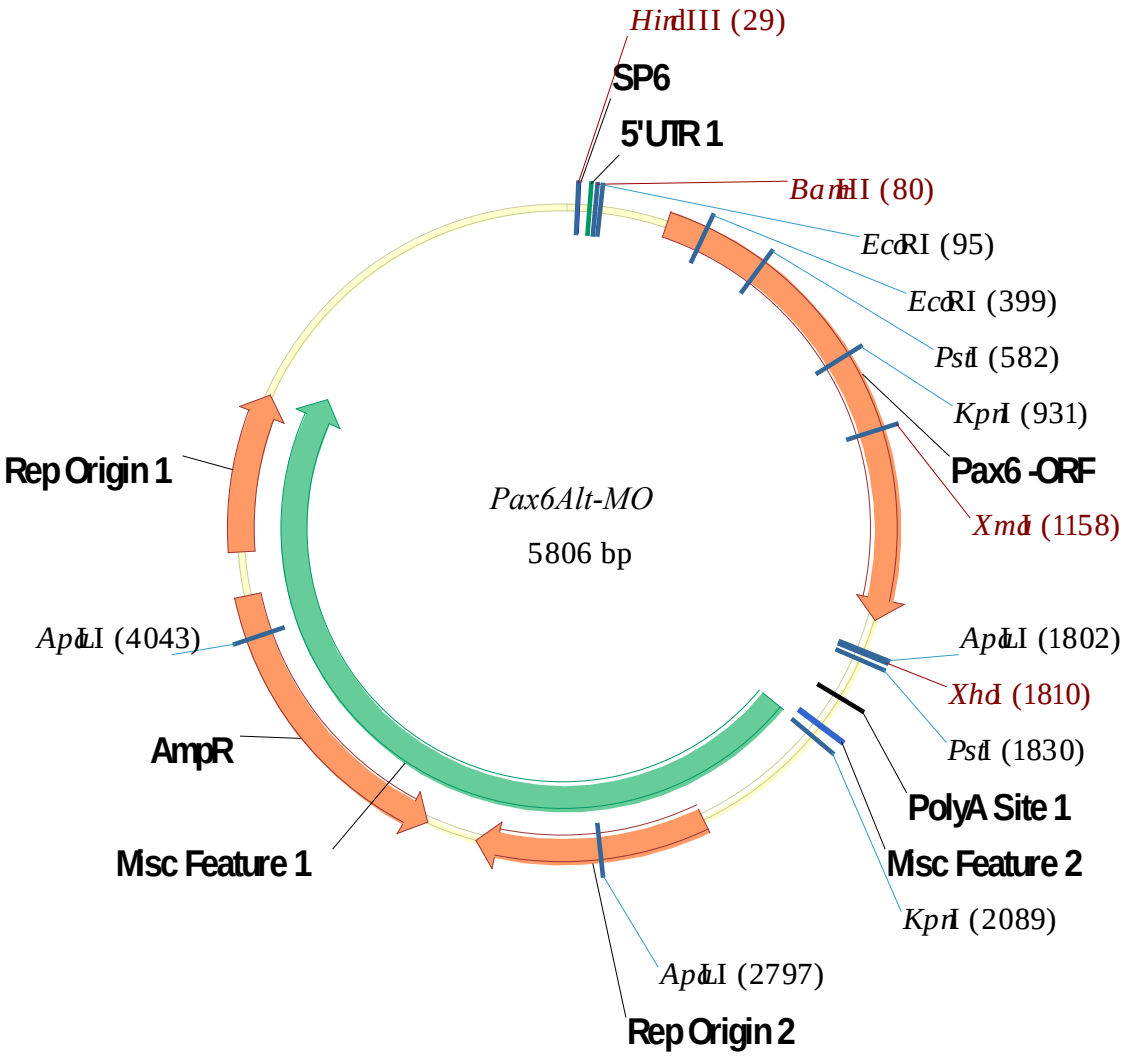
APPENDIX E

Plasmid Maps

***Six3*Δ5'UTR:**



Pax6AltMO:


HindIII (29)
SP6
5'UTR 1
BamHI (80)
EcoRI (95)
EcoRI (399)
PstI (582)
KpnI (931)
Pax6 -ORF
XmaI (1158)
Pax6Alt-MO
5806 bp
ApaLI (1802)
XhoI (1810)
PstI (1830)
PolyA Site 1
Msc Feature 2
KpnI (2089)
ApaLI (2797)
Rep Origin 2
ApaLI (4043)
AmpR
Msc Feature 1
Rep Origin 1

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SAQIB S. SACHANI

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Date of birth: 1987

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