The role of homeobox transcription factor *six3* in vertebrate eye development

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ABSTRACT

Six3, a homolog of *Drosophila optix*, is a homeobox-containing transcription factor critical for early anterior patterning and forebrain/eye formation. In humans, haploinsufficiency of *SIX3* has been associated with holoprosencephaly. *Six3* is a key component of the gene regulatory network (GRN) controlling eye formation along with genes such as *Pax6*, *Rax*, *Lhx2* and *Otx2*. However, the position of *Six3* in the eye GRN is unclear, in part because mouse homozygous *Six3* mutants are headless and conditional mutants targeting *Six3* make definitive epistatic relationships difficult to determine.

We have generated a *six3* mutant line in *Xenopus tropicalis* that has been uniquely valuable in revealing a central role for *six3* in regulating eye development. In contrast to mouse, *Xenopus six3* mutants retain a head but have malformed eyes with missing or undifferentiated lenses and a disorganized retina. We describe several key findings. 1) *six3* and *pax6* are regulated independently of each other in the lens ectoderm and early lens induction is not affected by the loss of *six3* while later phases of lens induction are severely impacted in the mutant. 2) *six3* activity is independent and additive to the related eye gene *six6* in eye formation. 3) The missing and defective lenses in the *six3* mutants are primarily due to its non-autonomous function mediated by the optic vesicle. 4) Our data here supports an essential role for the *mab21* genes in regulating lens and eye formation downstream of *six3*. 5) *six3* regulates lens and retina formation by impinging on the BMP, Notch and Wnt signaling pathways. 6) Our preliminary evidence also supports an important role for *six3* in patterning the neuroretina. Taken together, our findings present a new perspective on the eye GRN with *six3* at the top of this gene hierarchy in parallel with *pax6* and likely in concert with other eye field transcription factors regulating lens and retina formation.

TABLE OF CONTENTS

FIGURES AND TABLES

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Chapter I Overview of lens induction, current model and the role of transcriptional and signaling networks during lens commitment

Early studies in lens induction

The study of induction and determination began over a century ago with the early work done in amphibians by Hans Spemann and Warren H. Lewis whose studies in amphibians wherein they either ablated or transplanted optic vesicle from early or late neurula stages suggested that the optic vesicle was sufficient for lens induction from the overlying ectoderm (Spemann, 1901; Lewis, 1904). However, the conclusions of Spemann and Lewis as reviewed extensively by Margaret S. Saha and colleagues that suggested initially that there might be species specific differences with Spemann's and Lewis's work. However, it has become apparent that they were most likely hindered largely by the lack of precise techniques – initially by not using host and donor tissue marking to control experiments more precisely to avoid tissue contamination during transplants and forming conclusions from artifacts of transplantation. In addition in these early experiments there were no gene assays to accurately assess the presence or absence of an induced lens in their experiments (Saha, Spann and Grainger, 1989).

However, the idea of the optic vesicle being sufficient for inducing lens from the surface ectoderm persisted until the 1960's and even the 1980's when Antone G. Jacobson showed that during early and mid-gastrulation stages the underlying pharyngeal endoderm and heart forming mesoderm might be very early signaling factors that could initiate the induction of lens (Jacobson, 1963). Studies in the 1980's and 1990's from Jonathan J. Henry and Robert M. Grainger have shown that the optic vesicle is not sufficient (Henry and Grainger, 1987; Grainger, Henry and Henderson, 1988) and further work using animal cap transplant experiments that the animal cap during early blastula stage have a competence to form mesoderm initially, then gain neural competence followed by a short

time where the animal cap gains competence to form lens tissue. Even though this competency period is short lived it is sufficient for this tissue to become neural placode and otic tissues during early neurulation (Servetnick and Grainger, 1991). The Grainger lab further has shown through transplant experiments that these early signals might be due to planar signaling activity from the neural ectoderm rather than surrounding endo-mesoderm as suggested by Jacobson (Grainger, 1992; Grainger *et al.*, 1997).

Current model of lens induction

Grainger and colleagues describe five stage model for lens formation – competence, bias, specification, determination and differentiation (Fig.1-1) (Fisher and Grainger, 2004). Bias is defined as the acquired, preferential ability of cells to respond to specific inductive signals based on earlier inductive signals. Specification is defined as group of cells capable of generating a specific tissue in an autonomous fashion, in this case occurring in *Xenopus* stage 18 (all stages according to (Nieuwkoop and Faber, 1994)) when the presumptive lens ectoderm (PLE) is isolated and cultured in saline solution (Jin, Fisher and Grainger, 2012). Determination, the immutable commitment to certain cell fate and ultimately differentiation, is acquired at *Xenopus* stage 21 as shown by transplantation of the PLE to the posterior of an embryo, which inhibits lens formation in PLE that is not determined (e.g. stage 18 specified ectoderm) (Jin, Fisher and Grainger, 2012). A pictorial representation of the stages of lens determination and the corresponding stages in *Xenopus* are shown below (Fig. 1-1).

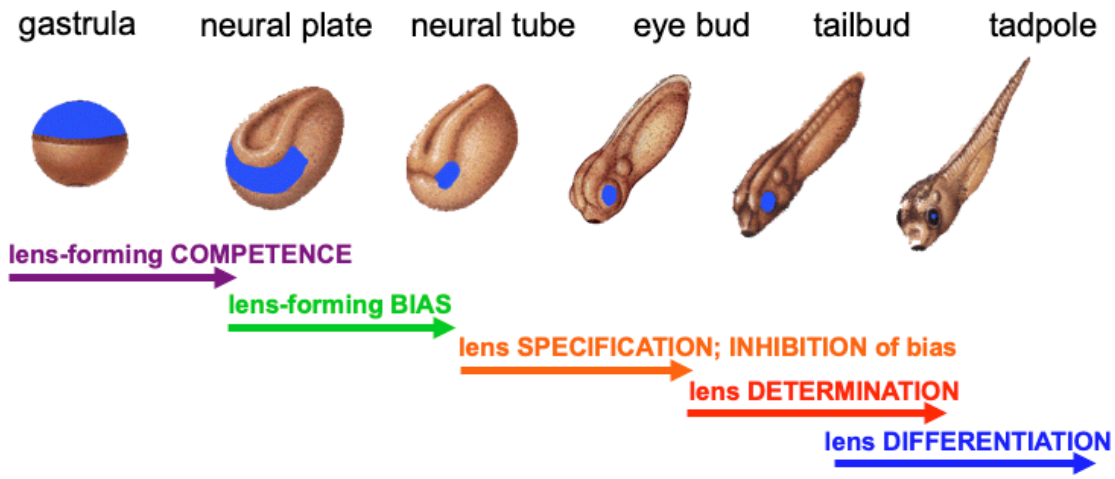

Figure. 1-1 Five stage model for the process of lens commitment.

Depiction of the 5 stage model of lens commitment starting at the gastrula stage where the animal cap develops competency to form lens (highlighted in blue); this is further refined by the neural plate stage when cells in the anterior placodal region (blue) have acquired a lens forming bias; by the end of neurulation the ability to make a lens is specified in regions overlying the area where the optic vesicles will contact the lens ectoderm; lens determination occurs slightly after neural tube closure and the lens begins to differentiate by cell elongation in the lens ectoderm and expression of tissue-specific crystallins in this tissue by the tailbud stages. Fig. 1-1 – Provided by the Grainger Lab.

Competence is a poorly understood process but is defined as the stage where a tissue starts on the process of commitment wherein it has acquired the ability to make a lens (Fisher and Grainger, 2004). However, this tissue at this stage has not been induced to continue on this path and required the presence of subsequent signals to do so. In the case of the lens induction, early studies suggested that the optic vesicle provided the inductive signal to turn the ectodermal tissue into lens (Spemann, 1901; Lewis, 1904). There is evidence in *Xenopus* to suggest that this competency process begins much earlier than the time when the optic vesicle makes contact with the lens ectoderm. In fact when neural plate stage retinal tissue is ablated, free lenses still form even in the absence of retinal tissue (Grainger, 1992). With regards to competence as mentioned above there appears to be a short window of 2 to 3 hours during gastrulation that the animal cap gains competence to form a lens (Servetnick and Grainger, 1991). This short inductive period is sufficient to make changes to the induced cells to proceed to the next step of commitment process. This change primes the cells to be receptive to subsequent inductive signals indicating that signals from outside this region might be driving the change in fate for these cells (Servetnick and Grainger, 1991).

Bias is a stage in the commitment process which follows competency. It is defined as a state in which a tissue has gained initial inductive signals and therefore can act on receiving subsequent inductive signals (Fisher and Grainger, 2004). In *Xenopus* the bias stage in the lens ectoderm occurs during the neural plate stage (stage 14). The gaining of bias involves the activation of transcriptional regulators like *otx2* and *pax6* (Zygar, Cook and Grainger, 1998). Some of the early work performed involved reciprocal reverse transplantation of the placodal region into a host resulting in generation of the placodes

suggesting that by the neural plate stage the placodes already had received some inducing signal (Jacobson, 1963). The PLE during this stage shares a region located at the border region of the anterior neural plate (as approximately shown in Fig. 1-1 as the "biased" region of ectoderm in neural plate stage embryos). In this pre-placodal region, it is located posterior to the adenohypohesyal and olfactory placode and anterior to the otic placode (Pieper *et al.*, 2011). This pre-placodal region consists of the non-neural ectodermal region wherein the sensorial placodes and in the lateral region lies the neural crest placodes. It is suggested that at the neural plate stage anterior regions of the placode have a basal ability to form a lens (Bailey *et al.*, 2006). The dispersion of the placodal region and the prospective tissues are thought to be more intermingled in chick and zebrafish and further movement of the cells result in distinct placode formation (Bhattacharyya *et al.*, 2004; Toro and Varga, 2007). However, studies in *Xenopus* suggests that this region might be overlapping to a lesser extent and with localized cell movement compared to the chick studies (Pieper *et al.*, 2011). Expression patterns of transcription factors at this stage begin to mark different placodal regions as mentioned above with *otx2* and *pax6* (Zygar, Cook and Grainger, 1998; Ogino *et al.*, 2012). For a partial list of the key transcription factors involved in lens formation, see Fig. 1-3. Overlapping expression patterns of different transcription factors mark and, in some cases, help to differentiate the location of the different placodes. For example, expression of *pax6* is limited to the anterior placodal and presumptive retina regions. Since *pax6* is expressed in both retina and the lens and is thought to be a primary regulator of eye development, its expression in the presumptive lens ectoderm (PLE) at the neural plate stage is a key determinative factor for lens induction based on previous inductive signals (Fujiwara *et al.*, 1994). The retina and lens are

distinguishable due to the fact that they are not yet under/over lying each other, therefore making identification much easier in *Xenopus* as shown in Fig. 1-2.

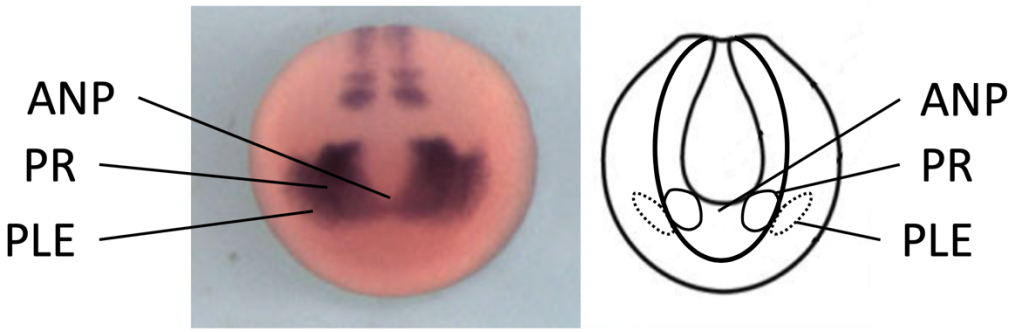

Figure. 1-2 Image of neural plate stage (stage 15) embryo with *pax6* expression domain and a schematic depicting its expression. Schematic showing the expression of domain of *pax6* distinct in the presumptive retina (PR) distinct from the presumptive lens ectoderm (PLE), ANP - Anterior neural plate. Fig. 1-2 – Modified from H. Jin, PhD thesis, University of Virginia, 2008.

Specification is defined as the state where a group of cells are able to autonomously generate a specific tissue when isolated from the embryo (Slack, 1991; Fisher and Grainger, 2004). The timing of lens specification is thought to involve signaling from surrounding tissue that generates a change in the PLE that is biased to become a lens. Species specific differences are seen when comparing integrating studies in chick, zebrafish and *Xenopus*. Lens specification appears to occur much earlier in chick when compared to *Xenopus*. In chick, explant assays suggest lens tissue is specified during late gastrulation (Sullivan *et al.*, 2004; Sjödal, Edlund and Gunhaga, 2007; Jin, 2008; Jin, Fisher and Grainger, 2012). However, Grainger and colleagues have shown that this specification occurs much later in *Xenopus* closer to neural tube closure (Ogino, Fisher and Grainger, 2008; Jin, Fisher and Grainger, 2012). Studies in mammalian systems have been sparse since access to early development embryonic tissue has been difficult (Cvekl and Zhang, 2017). However, data from the Grainger suggests that the PLE becomes specified in mouse at E9.5, just after neural tube closure (Enwright et al, unpublished).

Studies in *Xenopus* have shown that Delta/Notch signaling from the optic vesicle in co-ordination with the expression of *otx2* is required for lens specification along with a co-activator *rbpj* to activate the expression of transcriptional regulator *foxe3* expressed during the lens specification stage (Ogino, Fisher and Grainger, 2008). However, this function may not be conserved in mouse since disruption of Delta/Notch activity by conditional deletion of *Jag1* or *Rbpj* does not appear to affect early lens formation (Le *et al.*, 2012). However, this result does not consider possible redundancies or the requirement of other Delta/Notch family members. Further evidence regarding the specification of lens ectoderm in *Xenopus* comes from explant and transplantation experiments which

demonstrated that the PLE isolated from neural tube stage (stage 18) was able to express *cryg1* (a gene turned on during lens differentiation) in the majority of the cases i.e. is specified, through transplantation experiments show that the tissue at this stage while specified is not yet determined (Jin, Fisher and Grainger, 2012).

Determination and differentiation are the final two steps in the commitment process giving rise to terminally differentiated lens tissue. The timing of determination was established in a series of transplantation experiments that distinguish the similar, but distinct properties of specification and determination. During transplant experiments specified lens tissue as expected was able to generate a lens (assayed by expression of *cryg1*) when transplanted to the head regions of the donor embryo (Jin, Fisher and Grainger, 2012). However, these transplants failed to express *cryg1* when transplanted to regions in the posterior of the embryo where we would expect it to be located in an inhibitory environment because of strong signals produced there, e.g. Wnt's that are known to be inhibitory toward lens formation (Kiecker and Niehrs, 2001). Similar transplant experiments carried out post neural tube closure demonstrated that the lens ectoderm at this stage expressed *cryg1* when transplanted not only to the head ectoderm of the host but also in all regions of the host embryo indicating that there was a change which lead to the immutable state (determination) of lens tissue (Jin, Fisher and Grainger, 2012).

Differentiation of lens tissue is the terminal step in the commitment process. Primary function of the lens is to provide a clear path for light to pass through to the retina. A major protein component of the lens are the crystallins (Cvekl, McGreal and Liu, 2015). They are a group of water-soluble proteins which form majority of the proteins in mature lens (Cvekl, McGreal and Liu, 2015). There are species specific differences in the ratios

and type of crystallin being produced. In *Xenopus* β-crystallin is one of the earliest crystallin genes detected as early as the neural tube stage post determination. γ-crystallin s form the majority of the crystallins in the lens and are expressed at the onset of differentiation in tailbud stage embryos (stage 28 in *Xenopus*). The lens is composed of an anterior layer of epithelium which at the equatorial regions differentiate into elongated fiber cells which eventually undergo enucleation to make transparent structures in order to let light pass through (Cvekl and Zhang, 2017). Both intrinsic and extrinsic signaling, some of which are prevalent during earlier stages of lens formation are re-purposed for inducing differentiation of the lens. Activity of *pax6*, *six3*, *nrl* and *mafb* are all transcription factors all of which are important for activation of crystallin expression (Cvekl, McGreal and Liu, 2015). Signaling molecules like WNT, BMP and FGF have been shown in mouse to be relevant for lens epithelial differentiation, activation of *nrl* and lens fiber cell differentiation respectively (Lovicu and McAvoy, 2001; Stump *et al.*, 2003; Pandit, Jidigam and Gunhaga, 2011).

Role of transcription factor cascades and signaling molecules in lens induction

With the identification of multitude of signaling molecules and transcription factors in the past several decades, it has become possible to begin to clarify the non-molecular research laying out the conceptual framework of lens induction from earlier times by attaching distinct molecular signals and responses that could be linked to the various stages of the determination and differentiation processes for lens induction as observed in *Xenopus*. Molecular studies over the years in *Xenopus* along with zebrafish, chick, mouse and Drosophila embryos have identified several key transcription factors

involved in lens determination. Many of these belong to a larger group of eye field transcription factors (EFTF) that are localized to the prospective eye region and whose expression begins early during the blastula stages (Zuber *et al.*, 2003).

The expression of some of these eye field transcription factors are also maternally inherited at low levels as detected by RT-PCR analysis (Zuber *et al.*, 2003). In *Xenopus*, embryos undergo a mid-blastula transition (MBT) which occurs after cleavage 12 or stage 8 leading to the beginning of zygotic transcription during this transition event (Newport and Kirschner, 1982). Following gastrulation during the early to mid-neural plate stage, expression of genes responsible for the establishment of the eye field increases and gradually becomes restricted to specific regions of the eye.

A review of key transcription factors relevant to this proposal and expressed early in the pre-placodal ectoderm are reviewed here and others in more detail by (Ogino *et al.*, 2012). As mentioned in previous section, the placodal region is located at the anterior border of the neural plate and during the neural plate stage (stage 15), and expression of various transcription factors begins to mark this region. These transcription factors are expressed in overlapping regions and gradually become restricted to specific placodes. Some of the early genes expressed in the pre-placodal ectoderm but not exclusively are the *Dlx* family, *Otx2* and the *Hes* family of transcription factors (Ogino *et al.*, 2012). The *Dlx* family has been shown to define boundaries of neural tissue and have a broader expression domain than just the placodal region (Ogino *et al.*, 2012). Previous research in *Xenopus* has shown that BMP signaling from the endo-mesoderm and non-neural ectoderm plays a role in regulating expression of the *Dlx* family of transcription factors (Feledy *et al.*, 1999; Luo *et al.*, 2001). Given their role in early embryonic patterning loss of function of multiple

Dlx family genes results in severe abnormalities which includes the loss of eye structures (Ogino *et al.*, 2012). Other transcription

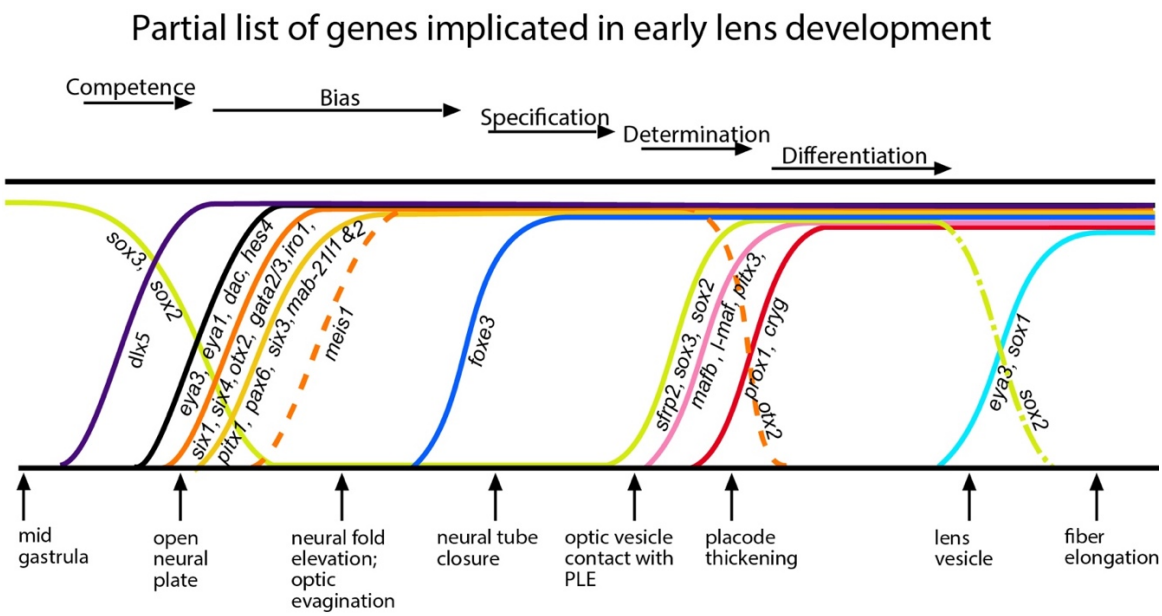

Figure. 1-3 – Partial list of transcription factors and the timing of their activity during lens formation with respect to key biological stages of lens formation. Key transcription factors from this list include – *otx2, dlx5, six3, pax6, mab21l1, mab21l2, foxe3, nrl (old name l-maf)* and *mafb.* Fig. 1-3 Modified by the Grainger lab from (Fisher and Grainger, 2004).

factors such as *Otx2* have been shown be involved in anterior head formation and formation of the eye (Matsuo *et al.*, 1995). However, its expression in the PLE during late neurulation is shown to activate downstream lens differentiation genes such as *foxe3* via the major *foxe3* enhancer (Ogino, Fisher and Grainger, 2008). Knockdown of *hes4* (old name: *xhairy2*) has been shown to regulate *pax6* and *six3* expression in the lens placode and the loss of lens field in *Xenopus* (Murato and Hashimoto, 2009).

Expression of *pax6* begins prior to the neural plate stage starting at stage 12.5 (Hirsch and Harris, 1997) during the time when lens tissue is shown to gain lens-forming bias and is limited to the PLE region. Expression of *pax6* marks the anterior part of the placode which includes the lens and olfactory placode (Nakayama *et al.*, 2015). Concurrently, *pax6* is also expressed in the presumptive retina region allowing us to clearly identify the presumptive retina and PLE regions during the neural plate stage in *Xenopus* (Nakayama *et al.*, 2015). In the pre-placodal ectoderm an overlap of *DLX5* and *PAX6* expression is observed in chick embryos (Bhattacharyya *et al.*, 2004). As development progresses expression of *DLX5* is lost in the lens ectoderm and expression of *PAX6* is lost in the olfactory region (Bhattacharyya *et al.*, 2004). This suggests that transcription factors are gained and/or lost in the placodal region as commitment progresses.

Pax6 was first identified in mice and humans as a homolog of *Drosophila eyeless* in small eye (Sey) mice (Hill *et al.*, 1992; Quiring *et al.*, 1994). Heterozygous mice present phenotypes similar to human aniridia, a rare autosomal dominant disorder characterized by the absence of an iris, coloboma and amongst other eye defects (Hill *et al.*, 1992; Plaisancié *et al.*, 2018), Homozygous *Sey* mice have an absence of eye and nasal passages (Hogan, Horsburgh and Cohen, 1986; Hill *et al.*, 1992; Grindley, Davidson and Hill, 1995). Mutants

in rat also show a similar phenotype to mice. Homozygous mutations in the *pax6* locus in *Xenopus* show a somewhat less severe phenotype, though they have small highly deformed eyes lacking a lens and showing abnormal retinal development and also die during larval development (Nakayama *et al.*, 2015). Loss of function studies in mouse, chick and *Xenopus* suggest that many key lens genes including but not limited to *mab21l1*, *mab21l2*, *foxe3*, *nrl*, *mafb* and *crystallin* genes are genetically downstream of *Pax6* which indicates to the importance of *Pax6* in lens formation. Recent studies have shown that the later effect of *Pax6* in regulating lens formation is driven by its expression in the optic vesicle. This is shown by the conditional knockout of *Pax6* in the optic vesicle and not the lens ectoderm leading to the loss of lens formation in these mutants (Klimova and Kozmik, 2014). How this effect is mediated is unknown as it does not involve the activities of BMP, FGF or Wnt signaling pathways (Klimova and Kozmik, 2014). However, not all of its targets have been described and the Grainger lab is in the process of determining the targets of *pax6* in both retina and lens development.

Another transcription factor that is expressed during early neural plate stage (stage 14) at the time when the PLE is in the bias stage is *six3* (Zhou *et al.*, 2000), the gene which is the primary topic of this thesis. It is a member of a family of genes that were identified in Drosophila as *sine oculis* (*so*) which much like *ey* or *pax6*, when lost resulted in loss of the compound eye (Cheyette *et al.*, 1994). Subsequent studies in vertebrates has shown that *Six3* mediates formation of the anterior forebrain and development of the retina and the lens (Lagutin *et al.*, 2003; Liu *et al.*, 2006, 2010; Liu and Cvekl, 2017; Diacou *et al.*, 2018). More detail on the role of *six3* in eye/lens formation in *Xenopus* is discussed in Chapters II and III.

Although *pax6* and from the work described in this thesis, *six3*, are major contributors to the lens induction process, a number of other transcription factor and nuclear proteins are expressed in the eye. An example of nuclear proteins whose function is relatively poorly understood are the *mab21* family – *mab21l1* and *mab21l2*. Both genes are highly conserved and have similar expression patterns being expressed strongly in the retina and the lens. However, there are slight differences in the expression patterns between species. For, example, expression in mouse suggests that *Mab21l1* is expressed in the lens and retina while *Mab21l2* is expressed much more strongly in the retina (Yamada *et al.*, 2003, 2004). Zebrafish expression shows stronger expression for *mab21l2* in the lens while *mab21l1* shows stronger expression in the retina compared to the mouse (Cederlund *et al.*, 2011). In *Xenopus*, both *mab21l1* and *mab21l2* RNA are expressed in the retina and the lens (our data). As mentioned, there are slight differences, but the expression pattern is largely conserved between vertebrates and mammals. Genetic mutants in mouse and zebrafish also show variable phenotypes due to the differing expression patterns. No *Xenopus mab2l1l1* or *mab21l2* mutants exists in the literature. However, we are currently raising F0 animals for *mab21l2* mutations. I also recently generated out of frame *mab21l1* homozygous mutants in *Xenopus*. Our *mab21l1* mutants, however, do not show any apparent embryonic phenotype possibly due to functional redundancy with *mab21l2* which share ~94% amino acid homology (De Oliveira Mann *et al.*, 2016).

Functionally *mab21* family are thought to be related to nucleotidyl transferase proteins with sequence homology with cyclic GMP-AMP synthase (cGAS) (De Oliveira Mann, Kiefersauer, Witte, & Hopfner, 2016a). They are thought to bind with *Smad1* and antagonize BMP4 signaling (Baldessari *et al.*, 2004). In *C.elegans*, loss of *mab21* also

regulates the formation of the posterior sensorial rays and is shown to be interacting with genes homologous to vertebrate BMP and *Smad– dbl-1* and *sma2*, *sma3* and *sma4* (Chow, Hall and Emmons, 1995; Morita, Chow and Ueno, 1999). However, their molecular function still remains largely unknown.

foxe3 is a member of the forkhead family of transcription factors (Brownell, Dirksen and Jamrich, 2000)whose expression in the PLE is corelates with the timing of lens specification and is activated by the coordinated action of *otx2* and *delta2*/*notch* signals originating in the optic vesicle (Ogino, Fisher and Grainger, 2008). Along with influence from notch signaling pathway, this gene is also thought to be regulated by the interaction of *Sip1* and *Smad8* on the proximal promoter region of *Foxe3* (Yoshimoto *et al.*, 2005). *foxe3* expression is observed in the pre-placodal ectoderm and becomes specific to the PLE by the time it is specified (Ogino, Fisher and Grainger, 2008). It's importance for lens formation was originally identified in a spontaneous mutation in mouse called *dyl*, which showed a smaller lens (Sanyal and Hawkins, 1979). The relatively later activity of *Foxe3* is thought to be involved in lens differentiation, although in the *dyl* mutants, expression of *crystallin* genes is not lost indicating that it is not a necessity for their expression (Brownell, Dirksen and Jamrich, 2000). However, the Grainger lab has shown that *foxe3* expression early is important for lens formation and mutations in humans show loss of lens (aphakia) phenotypes (Valleix *et al.*, 2006).

Although the list of transcription factors involved in lens formation listed in this overview is not all inclusive, as the transcription factor cascade continues during development, two other key regulators that are activated are *mafb* and *nrl* (old name: *l-maf*). Both *maf* genes are involved in activation of *crystallin* genes as shown by the

activation of *crystallin* upon overexpression (Yoshida and Yasuda, 2002). However, loss of function of *Nrl* and *Mafb* does not appear to show any loss of crystallin expression in mice (Takeuchi *et al.*, 2009) but loss of *C-maf,* expressed in chick, mouse and zebrafish, but not in *Xenopus,* does show reduced expression of crystallin genes (Kawauchi *et al.*, 1999). In *Xenopus*, *nrl* and *mafb* are the most abundant *maf* proteins in the eye (Coolen *et al.*, 2005). Expression of *mafb* precedes activation of *nrl* corelating with the onset of determination of the lens, with expression of *nrl* corelating with lens differentiation and being reliant on the presence of the optic vesicle (Ishibashi and Yasuda, 2001; Jin, 2008).

Signaling molecules play a vital role in early embryonic development, and in particular in the lens induction process, as has been shown in various model systems (Patthey and Gunhaga, 2014). The modulation of these signaling molecules are critical for development. In lens induction, wingless integration-1 (Wnt), bone morphogenetic protein (BMP), fibroblast growth factor (FGF) and retinoic acid (RA) play varied roles during different time points over the course of commitment (Enwright and Grainger, 2000; Wilson *et al.*, 2000; Lovicu and McAvoy, 2005; Smith *et al.*, 2005; Fuhrmann, 2008; Steventon *et al.*, 2009; Garcia *et al.*, 2011). Amphibian model systems have clearly demonstrated the gradient of Wnt being high in the posterior and low in the anterior responsible to establish the anterior posterior gradient (Fuhrmann, 2008). Likewise, BMP signaling establishes the dorsoventral gradient with inhibition of BMP signaling crucial for neural development.

Early in pre-placodal region formation, inhibition of WNT and BMP signaling is required for the positioning of the anterior placodal and neural crest regions adjacent to the neural tissue. The neural crest derivative which form the lateral interior region of the placodes require WNT signaling for gastrula stage induction with inhibition of BMP

signaling (Steventon *et al.*, 2009). Studies in zebrafish have also shown low levels of BMP signaling act to specify neural crest fates and high levels of BMP acting to restrict the lateral regions to have a non-committed fates (Kwon *et al.*, 2010). Considering this is a border region between the neural plate and epidermis, the action of FGF is thought to maintain balance of BMP signaling in this region leading to the precise induction and localization of pre-placodal and neural crest fates (Wilson *et al.*, 2000; Kwon *et al.*, 2010; Patthey and Gunhaga, 2014). A schematic of the induction events leading to the formation of the pre-placodal ectoderm is shown in Fig. 1-4.

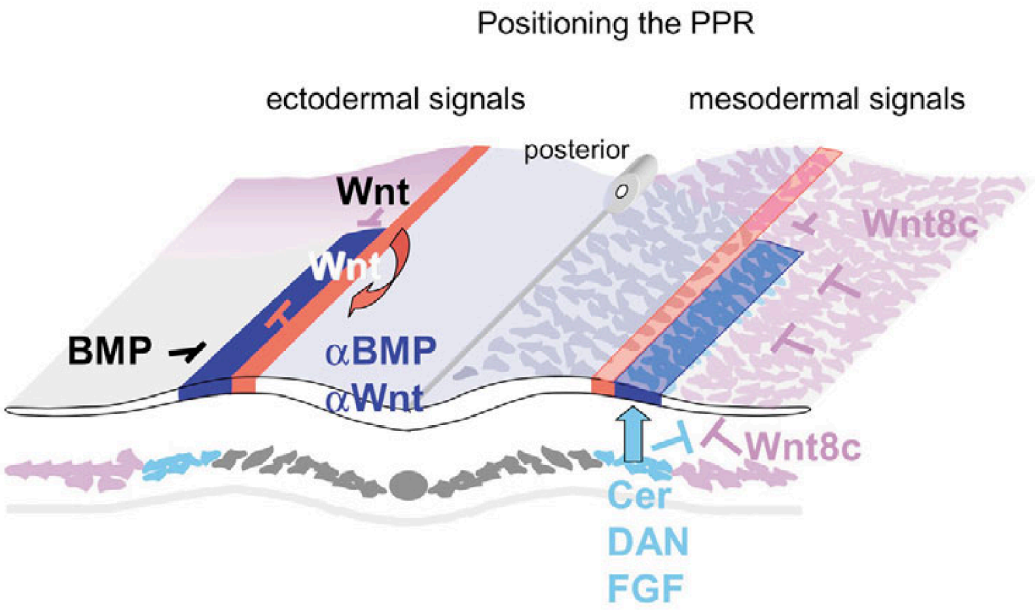

Figure. 1-4 Signaling factors influencing the formation of pre-placodal ectoderm. This schematic shows a flattened sections dorsal half of a chick embryo with the anterior end at the front of the image. The dark blue regions depict the location of the pre-placodal region (PPR) flanking the neural plate (light purple) and immediately adjacent to the neural crest (red). Inhibitory BMP and Wnt along with inducing FGF signals (light blue) from the mesoderm specify the location of the pre-placodal region. Fig. 1-4 Modified from (Litsiou, Hanson and Streit, 2005).

As the pre-placodal region has gained regional properties, the signaling networks are also re-used in different ways to generate specific placodes in co-ordination with the transcription factor networks. Progressively, BMP and Wnt antagonists along with FGF induce the activity of pre placodal transcription factors like *Six1*, *Eya2*, *Eya4* which define the boundaries of the region (Litsiou, Hanson and Streit, 2005). The early role of FGF signaling was demonstrated by the use of transgenic mouse line expressing dominant negative *Fgf* receptors which diminished the expression of *Pax6*. However, lenses were not completely abolished in these transgenic mice (Faber *et al.*, 2001). Establishing precise timing of these signaling events is difficult given the differences in the timing of the commitment process and accessibility of early embryos as demonstrated earlier studies in chick of lens specification. In chick, FGF signaling directed epidermal fates for olfactory and lens placodes while BMP induced the formation of olfactory and lens placodal fates even in neural tissue explants (Sjödal, Edlund and Gunhaga, 2007). However, there is difference in timing of this specification activity since lens tissue in *Xenopus* does not get specified until the end of neurulation (Ogino, Fisher and Grainger, 2008). The *Xenopus* model has specific advantages using explants which enables studies in competence and specification using a neutral salt based medium unlike studies in chick explants. Further, transplant assays allow for the study of determination which is difficult to achieve in other model systems.

As described above both inducing and inhibitory BMP signals from neighboring tissues position and drive the formation of the preplacodal region (PPR). As the PPR becomes regionalized into placodal fates, activity of signaling networks are appropriately modified and BMP activity becomes important for lens formation. Studies in mouse have

shown that expression of *Bmp4* is required for lens induction mediated by the optic vesicle as shown in experiments where the presence of *BMP4* coated beads along with an optic vesicle could drive formation of lens in mouse explant cultures (Furuta and Hogan, 1998). Another member of the TGF-b family of signaling molecules, *Bmp7* (homolog of *bmp7.1* in *Xenopus*) is also expressed transiently in the optic vesicle and lens tissues. Genetic mutants of *Bmp7* in mouse lacked lenses in most cases (Wawersik *et al.*, 1999). Signaling molecules utilize a range of receptors and signal transducers to modulate gene expression and therefore affect the determination states of the cells or group of cells it acts on. In the case of BMP signaling in the lens, type I receptors - *Bmpr1a* and *Acvr1* are shown to mediate BMP activity in the lens. The mothers against decapentaplegic or *Smad* family of transcription factors act downstream of receptor activation and form complexes that control gene expression.

Modulation of these downstream factors of BMP signaling also affects lens formation. Loss of function of type I BMP receptors *Bmpr1a* and *Acvr1* individually resulted in abnormal lens formation (Rajagopal *et al.*, 2009). However, loss of both receptors completely ablated lens formation (Rajagopal *et al.*, 2009) suggesting redundancy of receptor function. The loss of function of receptor regulated *Smads* - *Smad1* and *Smad5* does not appear to ablate lens formation in mice (Rajagopal *et al.*, 2009). This suggests the possibility of a receptor regulated but *Smad* independent pathway involved in BMP mediated lens formation. *Smad7*, an inhibitory *Smad* gene, which interferes with *Smad*-*Smad* or *Smad*-receptor interaction (Massagué, Seoane and Wotton, 2005) is known to be involved in a negative feedback loop to fine-tune the *bmp4* activity in *Xenopus* (Christian and Nakayama, 1999). Loss of function of *Smad7* in mouse leads to defective

retina and lens indicating that some *Smad* activity either acting to modulate BMP signaling or regulated by other TGF-β signaling pathway is important for lens formation (Zhang *et al.*, 2013). However, these studies were based on lens specific loss of function or germline loss of function studies in the mouse, no analysis has so far been performed in *Xenopus*.

Post lens placodal stage, BMP activity is also shown to be important for lens differentiation process. BMP signaling is required for initial activation of *NRL*, studies in chick explants have shown that specified explants exposed to *BMP4* expressed *NRL* whereas when these explants were exposed to the BMP inhibitor *noggin*, expression of *NRL* was inhibited (Pandit, Jidigam and Gunhaga, 2011). These data generated from different species indicates that BMP signaling is important for lens formation through the developmental process. The lack of studies in *Xenopus* however limits our abilities to draw conclusions as to its specific role in lens formation in this species.

As mentioned earlier in the chapter, FGF and Wnt signaling also play varied roles during lens formation. Later during lens formation, FGF signaling is required for the differentiation of lens epithelium to fiber cells during which levels of FGF activity tilts the balance between proliferation and differentiation (Lovicu and McAvoy, 2001). Wnt signaling is normally inhibitory for early lens induction (Fujimura, 2016). However, its activity later is required for morphogenesis of the lens. Loss of function of β-catenin in the surrounding peri-ocular mesenchyme (POM) results in ectopic activation of crystallin whereas loss in the lens ectoderm affects late lens morphogenesis (Smith *et al.*, 2005). Loss of WNT co-receptor *Lrp6* also shows a similar phenotype affecting late lens epithelial differentiation (Stump *et al.*, 2003).

Other signaling molecules such as retinoic acid, notch, sonic hedgehog also play important roles in the process of eye and lens formation (Ogino, Fisher and Grainger, 2008; Cvekl and Wang, 2009; Kerr *et al.*, 2012). Our unpublished and previously published data by others suggests that there might be an early and late inducing effect from the activity of the transcription factors mediated either autonomously or non-autonomously (our data, Klimova and Kozmik, 2014). Early expression of *six3* and *pax6* are independent of each other and in the loss of *six3* early lens induction is apparently normal (our data, unpublished). The traditional notion that *Pax6* is a primary regulator might need to be revised to include *six3* and perhaps other transcriptional regulators as being equally important for lens formation. Additionally, understanding the role of other non-transcription factor genes such as *mab21l1* and *mab21l2* are important to help construct the gene regulatory networks that are contributing to lens formation. Taken together, the signaling molecules and the activation of transcription factors result in the formation of the lens. Another process that is likely to be equally important and is discussed briefly in Chapter IV is the dynamic nature of the chromatin that provides timely accessible and closed regions in the genome hypothesized to drive the process of commitment.

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Chapter II The role of homeobox transcription factor *six3* during lens formation in *Xenopus*

Summary

Six3 is a homeobox-containing transcription factor critical for early anterior patterning and forebrain and eye formation. In humans, haploinsufficiency of *SIX3* has been associated with holoprosencephaly. Here we describe a *Xenopus tropicalis six3* frameshift mutant, generated using CRISPR technology which results defective forebrain and malformed eyes. The presence of rudimentary eyes, in contrast to similar mouse mutants where the eye is missing altogether, allows us to investigate the role of *six3* in eye formation from the time this gene is first activated. The weaker phenotype appears, at least in part, due to the fact that in *Xenopus* the Six gene family member *six6*, which contributes to eye formation as well, is unaffected in our *Xenopus* mutant while it is down-regulated in the mouse *Six3* mutant, thereby accounting for a stronger phenotype in the mouse. Using *in situ* hybridization analysis, we have made several key novel insights into the role of *six3* in lens formation: early stages of the lens gene regulatory networks (GRN) are largely not perturbed in the *six3* mutant as evidenced by essentially unchanged expression of *pax6* and other eye field transcription factors such as *six6, rax and lhx2* in the presumptive eye region. The lens GRN is perturbed, however, later during development as evidenced by the lack of lens or the formation of generally smaller lens or lens like structure. This is also accompanied by the variable and extensively reduced expression of retinal and lens genes associated with later stages of embryonic commitment and differentiation (for example, *bmp4*, *smad7*, *foxe3, mafb* and *cryg1*). Transplant assays suggest a primarily non-autonomous role (albeit not exclusively) for *six3* in mediating the lens program through inductive signals from the neuroretina, not surprising since gene expression and patterning in the *six3* mutant retina is severely perturbed. Expression of two other genes, *mab21l1* and

mab21l2 is reduced or not activated in the neurula stage *six3* mutant retina and was of particular interest since both genes have been associated with eye formation previously, in moue mutants (Yamada *et al.*, 2003, 2004) and when mutated in humans both are associated with eye defects (Rainger *et al.*, 2014; Rad *et al.*, 2019). However, until our studies, neither gene had been shown to be regulated by *six3.* The importance of these genes in regulating lens development was demonstrated by injection of *mab21l1* mRNA into *six3* mutants, resulting in restoration of significantly more normal lens formation, including increased expression of a primary lens differentiation gene *cryg1* indicating that *mab21l1* (and/or the closely related family member *mab21l2*) is required downstream of *six3* for lens formation. Furthermore, we also show recovery of *smad7*, an intermediary in BMP signaling and regulator of later stages of lens formation, in the developing eye-field of *mab21l1*-injected *six3* mutants, leading us to hypothesize a primarily non-autonomous role for *six3* in lens formation being mediated by *bmp4* activity in the optic vesicle. Our data here reveals a previously unrecognized primary role for *six3* in the eye GRN involved in lens formation, placing it in a key position in the GRN, along with *Pax6* in regulating lens formation. We present evidence to show that *mab21l1* functions as a novel regulator of *six3* activity in lens formation. Future work would involve functional assays to determine the precise roles of BMP and Notch signaling pathway regulating gene expression downstream of *six3* and the extent to which the *mab21l1* and *mab21l2* genes mediate these signaling events. Additionally, the availability of *Xenopus* mutants in other eye field specification genes (e.g. *rax* and *pax6*) will provide a framework to refine the relative roles of particular targets (transcriptional regulators and signaling networks) involved in the determination of the lens.

Progressive induction of the lens

Lens induction has been studied in amphibian systems for over a century starting with the pivotal work done by Spemann and collogues suggesting an important role for the optic vesicle inducing lens formation (Lewis, 1904; Saha, Spann and Grainger, 1989). However, their work was limited by the tools and technology available to them at that time. Over the years seminal work by Henry and Grainger have shown that lens induction is an earlier process than originally thought beginning at the gastrulation stage (Henry and Grainger, 1987). Subsequently, others have also shown that at later stages there are inductive signals operating between the optic vesicle and developing lens, as well as from surrounding tissues like the mesoderm or the neural crest which is an inhibitor play an important role in lens formation (Henry and Grainger, 1987; Furuta and Hogan, 1998; Grocott *et al.*, 2011; Gunhaga, 2011; Klimova and Kozmik, 2014; Huang, Liu, Filas, *et al.*, 2015). Although, studies of tissue interactions have revealed elements of this complex process, much remains to be characterized in the GRN that contributes to the formation of the lens both intrinsically and in signals arising from the adjacent retina and other nearby tissues. An in-depth review of lens induction is described in chapter I and therefore will not be presented in detail here.

Role of *six3* in brain and eye formation

Six3, a member of the *Six* family of homeobox transcription factors was initially identified during studies in *Drosophila* back in the 1990's as *sine oculis* (*so*). It is thought to be important for the development of the fly visual system (Cheyette *et al.*, 1994). Disruption of this gene caused the fly optic lobe to fail to invaginate resulting in arrest of the development of the fly visual system (Cheyette *et al.*, 1994). Upon its discovery in *Drosophila* studies began to identify and characterize the homologs in vertebrates. A few Murine homologs were identified with *Six3* and *Six6* which are closely related members of this family relevant to the studies presented here (Oliver *et al.*, 1995; Kumar, 2009). A pictorial representation of the Six family and their *Drosophila* homologs are shown in Fig. 2-1.

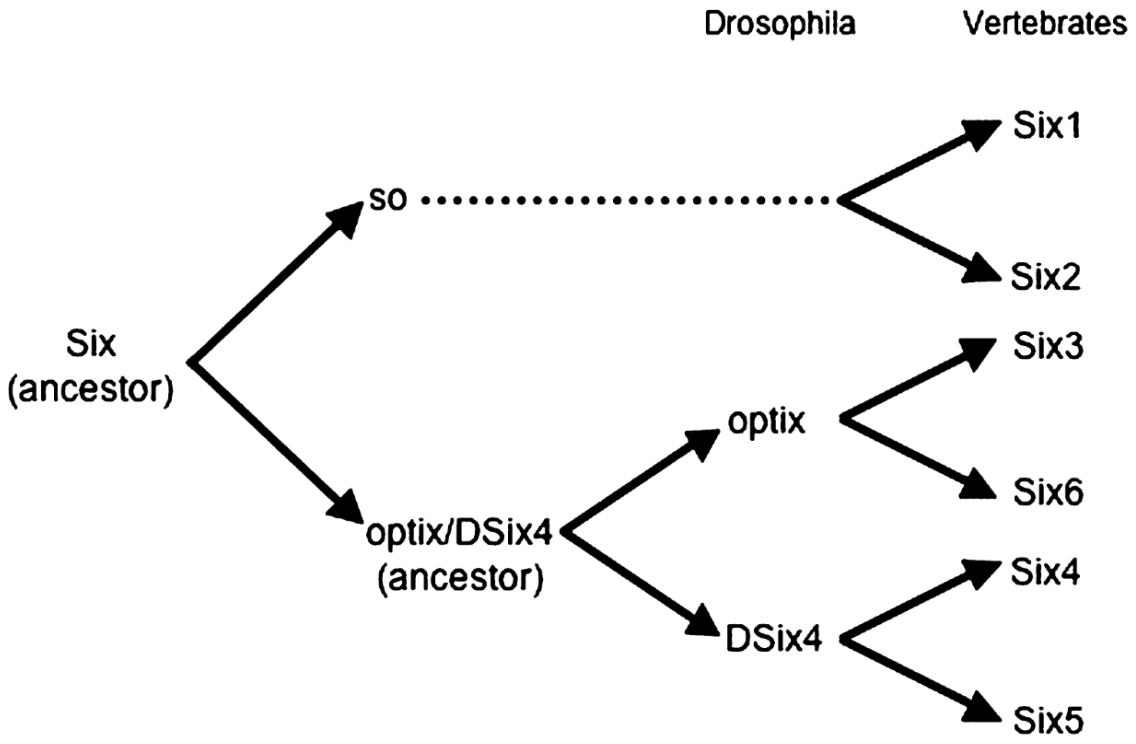

Figure. 2-1 Schematic of Vertebrate Six family of genes and their evolutionary relationship with *Drosophila* homologs. Fig. 2-1 Reprinted with permission of Springer Nature from (Kumar, 2009); permission conveyed through Copyright Clearance Center, Inc.

The members of this family contain a conserved protein-protein interacting Six domain and a DNA binding homeodomain (Kumar, 2009). The *Six3* expression domain was mapped to the anterior neural plate – the regions of the prospective forebrain and eye (Oliver *et al.*, 1995). Both *six3* and *six6* show overlapping expression domains in the anterior forebrain and eye fields in *Xenopus* (Ghanbari *et al.*, 2001). Previous evidence in literature shows that *six3* plays an important role in eye and forebrain patterning primarily being mediated initially by the inhibition of Wnt and BMP signaling in the anterior neural ectoderm (Gestri *et al.*, 2005; Liu *et al.*, 2010). *Six3* also regulates the ventral forebrain formation by direct regulation of *Shh* mediated by its binding to a conserved enhancer (SBE2) of *Shh* (Geng *et al.*, 2008; Jeong *et al.*, 2008). Zebrafish *six3* homologs are thought to regulate Nodal activity in order to establish the asymmetric development of the components of the epithalamus in the brain (Inbal *et al.*, 2007). In *Xenopus,* pull down assays suggest that *six3* interacts with several bHLH containing proteins which are important for neuroretina differentiation (Tessmar, Loosli and Wittbrodt, 2002). These early functions of *six3* combine with other regulators to pattern the anterior forebrain and eyes. Due to its role in forebrain formation *SIX3* mutations are associated with the human disease of holoprosencephaly (Solomon *et al.*, 2009).

In addition to its role in anterior forebrain patterning, *six3* is also expressed in the eye field and is thought to be one amongst the key eye field transcription factors that regulate the formation of the retina and the lens (Oliver *et al.*, 1995; Zuber *et al.*, 2003). Ectopic expression using both a plasmid and RNA constructs containing mouse *Six3* sequences in the fish medaka resulted in the generation of ectopic retina/lens and retina, respectively, in the anterior regions of the embryo, near the otic vesicle, in the head region,

though this is a region which is already relatively permissive for lens formation (Oliver *et al.*, 1995; Loosli, Winkler and Wittbrodt, 1999). Germline loss of function mutants in mouse show a severe phenotype with complete loss of the anterior forebrain with complete loss of the eyes (Lagutin *et al.*, 2003). Antisense morpholino knockdown in medaka fish show similarly disrupted forebrain and eyes in a dosage dependent fashion (Carl, Loosli and Wittbrodt, 2002). Antisense morpholino knockdown in *Xenopus* also shows defective anterior forebrain and eyes but present milder phenotypes compared to the headless phenotype observed in mouse (Gestri *et al.*, 2005), an interesting finding given the milder phenotype of the *six3 Xenopus* mutant studied here.

Conditional knockout of *Six3* in mouse lens ectoderm, while providing further insights into the role of this gene in lens formation, are also in some cases difficult to interpret. One such study showed failure of lens formation mediated primarily due to the loss of *Pax6* and *Sox2* activity (Liu *et al.*, 2006). The phenotypes were variable possibly due to the timing of activation of the *Cre* (Liu *et al.*, 2006) or other factors which could lead to artifactual reduction of *Pax6* expression (Dorà *et al.*, 2014). However, conditional knockout of *Pax6* in the lens also results in the loss of *Six3* expression in the presumptive lens ectoderm (Ashery-Padan *et al.*, 2000). Other conditional knockouts of *Six3* result in the removal of *Six3* from the retina and not the lens have missing lenses in the most severe phenotypes (Liu *et al.*, 2010; Liu and Cvekl, 2017). The most severe of these retinal knockouts ablate retina formation and therefore the loss of lens could be attributable to the absence of the retina and rather not to a specific activity of *Six3* in the retina. The non-autonomous role of *Six3* has been hinted since the overexpression studies where ectopic lenses were formed in the vicinity of *Six3* expressing cells (Oliver *et al.*, 1996). In mouse

embryonic stem (mES) cell cultures where transfection and expression of either *pax6* or *six3* containing plasmids in to mES resulted in expression of *γA–crystallin* in neighboring cells (Anchan *et al.*, 2014). A more recent study of conditional knockout of *Pax6* in the retina of the *Sey* mouse results in failure of lens formation suggesting even *Pax6* might have secondary non-autonomous role in lens formation (Klimova and Kozmik, 2014). However, this study again highlights the loss of the retina in these mutants which could contribute to the failure of lens formation.

The epistatic relationship between *Six3* and *Pax6* was determined based on the studies in the *Drosophila so* mutant where expression of *ey* is not lost and co-expression of *so* and *eya* are thought to regulate *ey* expression but with the existence of a secondary feedback loop with *eya* and *so* regulating *ey* expression (Pignoni *et al.*, 1997). In vertebrates, conditional loss of *Pax6* in the lens ectoderm results in the loss of *Six3* expression in the lens (Ashery-Padan *et al.*, 2000) and we see any inverse relationship when *Six3* is conditionally removed from the lens ectoderm (Liu *et al.*, 2006). Subsequent studies show that both *Six3* and *Pax6* mutually regulate each other (Goudreau *et al.*, 2002). However, their earliest analysis was done at E12.5 well past the early lens or later optic vesicle mediated lens induction processes. The interaction between *Six3* and *Pax6* is specific to the lens as their expression is established to be independent in the retina (Goudreau *et al.*, 2002). The over expression studies suggest *Six3* to be upstream of *Pax6* however those results are difficult to interpret in any endogenous context. Our data (unpublished) shown later in this chapter suggests that expression of *pax6* to be independent of *six3* in the lens ectoderm of *Xenopus.* In addition, our preliminary

unpublished analysis of the *Xenopus pax6* mutant also suggests that *six3* might also be independent of *pax6* expression. However, further tests are pending to verify this finding.

Background on *mab21* family of genes

Another member of the gene regulatory network expressed in the lens and shown to be important for lens and eye formation is the *mab21* family of genes. Their functional roles are poorly characterized. The *mab21* (male abnormal 21) family of genes were originally identified in *C.elegans* where it was identified as the causative gene in which mutations caused the defective ray sensilla in the male *C.elegans* (Chow, Hall and Emmons, 1995). Soon after, human, mouse and vertebrate homologs were identified (Margolis *et al.*, 1996; Mariani *et al.*, 1998; Wong, Wong and Chow, 1999). In *Xenopus* three *mab21 like* family genes have been identified (Lau *et al.*, 2001; Sridharan *et al.*, 2012). As described in Chapter I expression domains of *mab21l1* and *mab21l2* show significant overlap with expression detected in the retina and presumptive lens ectoderm (our data, (Lau *et al.*, 2001)). *mab21l1* and *mab21l2* are also highly similar with 94% protein homology ((De Oliveira Mann *et al.*, 2016). The expression domain of *mab21l3* appears to be primarily non-ectoderm and only shares 44% homology with *mab21l1* and *mab21l2* (Sridharan *et al.*, 2012). As mentioned earlier loss of *mab21* causes defects in the invertebrate sensory system (Chow, Hall and Emmons, 1995). In vertebrates, loss of function of murine *Mab21l1* causes loss of lens and defective retina (Yamada *et al.*, 2003). Similarly, loss of function of *Mab21l2* also causes loss of lens and an even more severe defect in the retina (Yamada *et al.*, 2004). These studies have shown that *Mab21l1* is upstream of lens genes such as *Foxe3* but downstream of *Six3* and *Pax6* (our data, Yamada

et al., 2003). *Mab21l2* is shown to be downstream of *Rax*, *Lhx2* and *Pax6* but upstream of *Vsx2* (Yamada *et al.*, 2004). Both *Mab21l1* and *Mab21l2* mouse mutants and chick *MAB21L2* RNA interference experiments suggest important role for these genes in eye formation (Sghari and Gunhaga, 2018). Mutations in *MAB21L1* and *MAB21L2* have been shown to cause a range of disorders which show defective eyes in humans (Rainger *et al.*, 2014; Rad *et al.*, 2019). Further studies in *C.elegans* has shown that *mab21* is involved downstream of the BMP homolog *dbl-1* by interacting with homologs of vertebrate *Smad*s *sma-2*,*sma-3*,*sma-4* (Suzuki *et al.*, 1999). In *Xenopus*, an early activity of *mab21l2* is suggested to antagonize BMP signaling via its interaction with *smad1* involved in dorso-ventral patterning (Baldessari *et al.*, 2004). Similar activity was also identified for *Xenopus mab21l3*, however it is not expressed in the eye as noted earlier (Sridharan *et al.*, 2012). In the *Xenopus* eye, expression of *mab21* genes are shown to be downstream of both *rax* and *pax6* expression as shown in their loss of function studies (Fish *et al.*, 2014; Nakayama *et al.*, 2015)A recent study characterized the structure of the *mab21l1* protein revealing similarities with cGAS – a DNA sensor which also has nucleotidyltransferase activity (De Oliveira Mann *et al.*, 2016). These data together with the human syndrome suggest that the *mab21* family, in particular *mab21l1* and *mab21l2* are important factors in lens and eye formation.

Results

CRISPR mediated mutation in *Xenopus tropicalis six3* locus results in malformed retina and loss or reduced size of the lens

The Grainger lab has generated a CRISPR mutant targeting coding region of the *six3* locus in *Xenopus tropicalis* that was used in the studies presented here (Nakayama *et al.*, 2013). In this mutant, a 19bp deletion causes a frameshift mutation resulting in truncation at the beginning near the N-terminus in the *six*-domain coding region (Fig. 2-2A). The expected protein is truncated at L60X containing only 17 amino acids of the Six domain (Fig. 2-2A). This mutant is highly unlikely to have any biological activity, based on experiments done in the zebrafish (Domené *et al.*, 2008) where RNA rescue assays show that wildtype *six3* RNAs will rescue a headless phenotype resulting from *tcf3* morpholino treatment, but mutants with similar truncations to ours show no rescue. The truncated SIX3 with either point mutations upstream or truncations downstream of our target site did not show significant activity in these bioassays. Only SIX3 constructs with mutations after position 129 retained some activity. Therefore, we propose that our mutant is nonfunctional. Homozygous animals for the 19bp del (hereafter referred to as *six3* mutant) do not survive past the tadpole stages. The embryos at stage 45 show a severely truncated and fused brain with loss of separation of the left and right hemispheres of the brain, an indication of holoprosencephaly (Fig. 2-2B and 2-2C). In addition, the nasal passages were also fused in the mutant tadpoles (Fig. 2-2B). The tadpoles also demonstrated malformed retinas with the loss of laminar structures typical of the wildtype lens (Fig. 2-2C) and a majority of the animals lack a lens or have an undifferentiated lens-like structure (Fig. 2-2C).

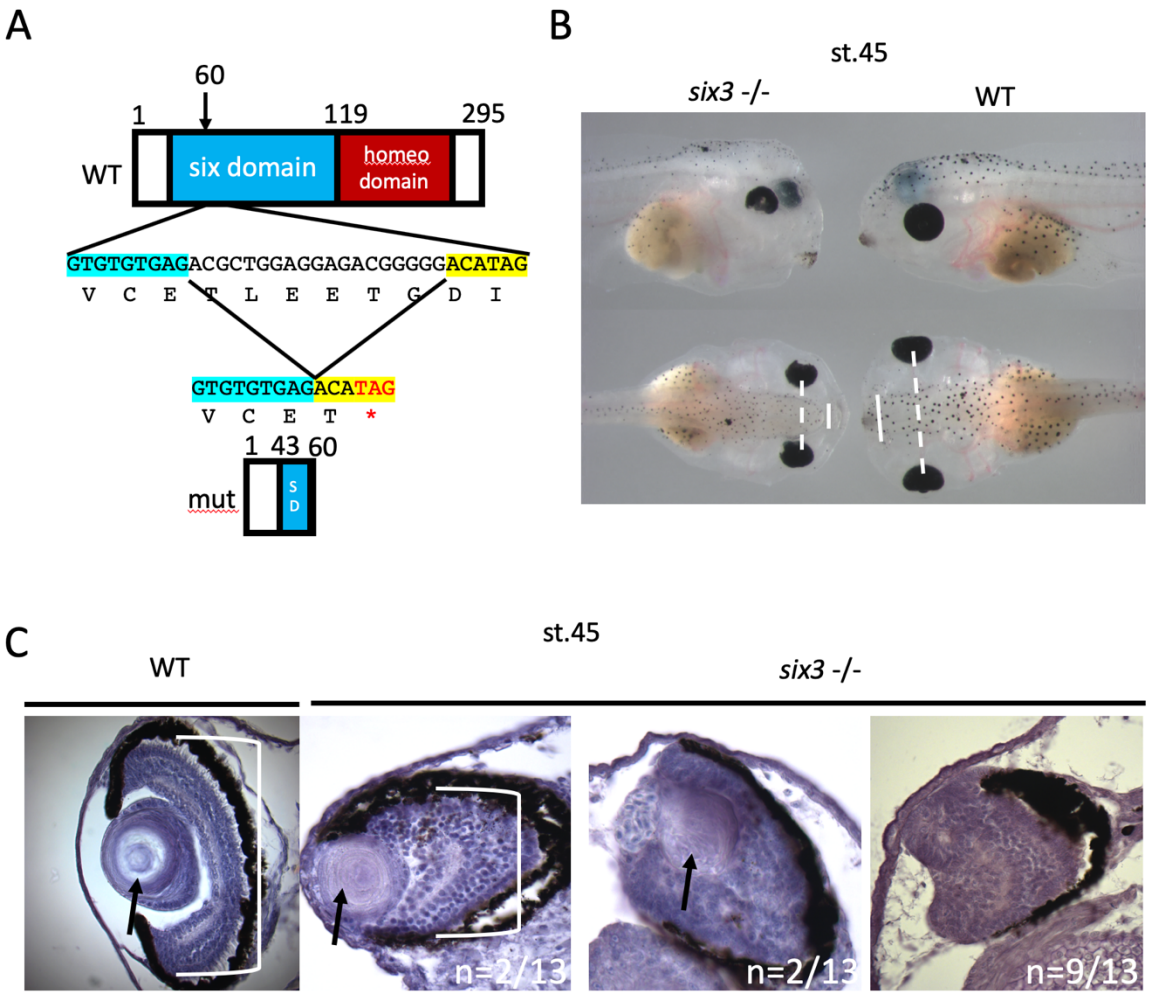

Figure. 2-2 CRISPR mediated mutation in *six3* locus results in malformed retina and loss of lens. A. Out of frame mutation is generated in the coding sequence at the start of the Six domain of *six3* resulting in generation of a putative null transcript. B. Whole mount phenotype shows a fused nose (solid white line, bottom panel) and loss of separation of the brain hemispheres (broken while line, bottom panel). C. 10µm paraffin sections through the eye show variable phenotypes with deformed retina and variable loss of lens in the *six3* mutant (compare wildtype in left panel with three mutant panels on the right) Number of cases of each phenotypic category shown below these panels.

six6* acts independently of and additively to *six3* activity in the eye of *Xenopus tropicalis

The *Xenopus six3* mutant shows a deformed eye and reduced or missing lens (Fig. 2-2B) while the mouse *Six3* mutant has a much stronger phenotype being essentially headless with complete loss of eye structures (Lagutin *et al.*, 2003). Species based differences could account for the milder phenotype especially since *Xenopus* retains at least a small amount of maternal *six3* transcripts until the mid-blastula transition (Zuber *et al.*, 2003). As mentioned previously, given the close relationships between the Six family of genes we hypothesized that one or more could complement the effect of the *six3* mutation and partially recover the phenotype due to the loss of *six3*. As previously mentioned another related member of the Six family, *Six6* is the most closely related (Fig. 2-1) and it is expressed in overlapping domain in the *Xenopus* retina (Ghanbari *et al.*, 2001). Due to these two factors we hypothesized that *six6* might act additively to *six3* in the developing *Xenopus* eye. Conditional removal of *Six3* in the mouse retina resulted in reduction in expression of *Six6* in the murine eye, implying that *Six6* is a target of *Six3* (Liu *et al.*, 2010). We show that in *Xenopus tropicalis,* unlike the mouse, *six6* expression is independent of *six3* activity (Fig. 2-3A). We also demonstrate that *six6* acts in a redundant fashion by generating a CRISPR mediated double knock out in a *six3* mutant background. *Six3* and *six6* double knock out animals tend to lose the eye completely with only rudiments of the RPE left (Fig. 2-3A). Activation of early lens induction is unchanged in the double knockouts as indicated by the normal expression of *pax6* in the neural tube (stage 18) embryos (Fig. 2-3B) which is known to be an early response to lens induction (Fujiwara *et al.*, 1994; Lang, 2004). Expression of *pax6* in the retina is unchanged in the *six3* mutant

and the double knockout (Fig. 2-3B). However, *mab21l2* expression which in the *six3* mutant is active in the eye field is diminished at stage 18 in the eye field of the *six3* and *six6* double knockout embryos (Fig. 2-3B). The latter expression data strengthens the hypothesis that *mab21* family genes play a key role in eye formation, mediated by *six3*, and further highlight the independence of *pax6* expression from *six* family members.

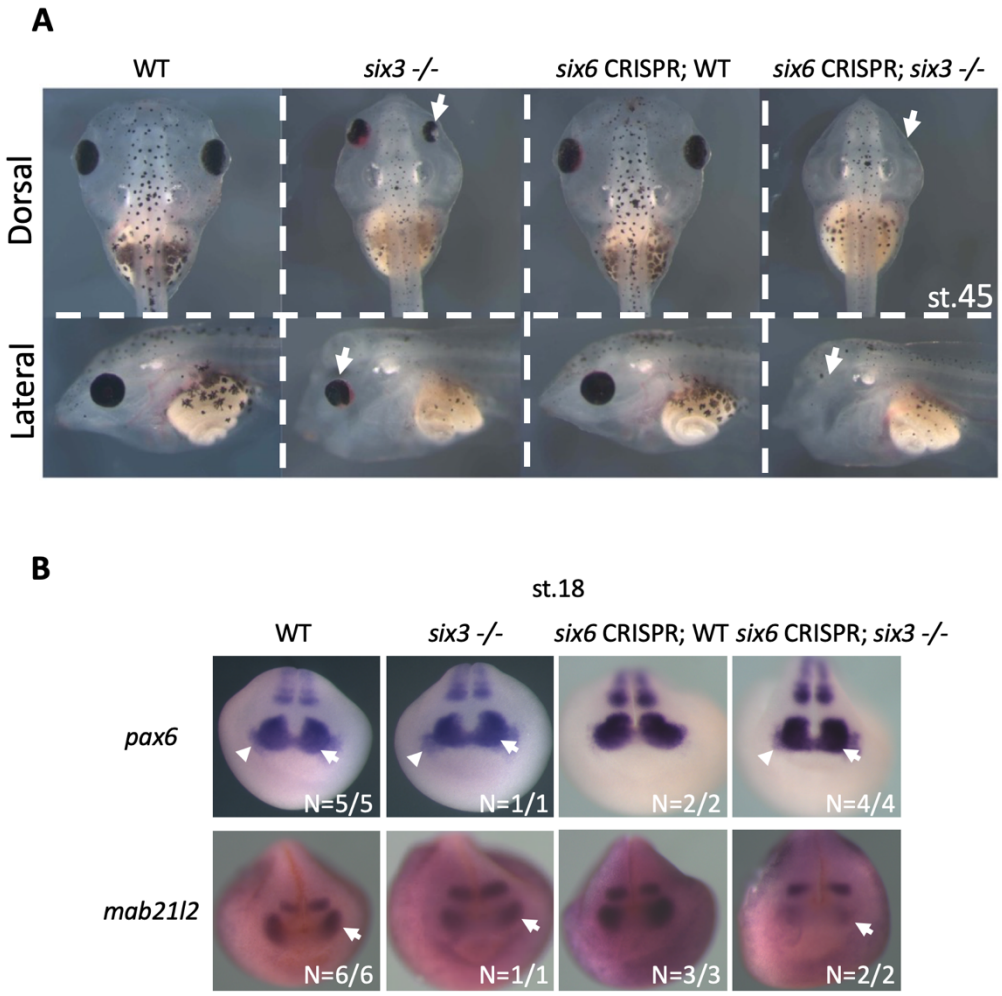

Figure. 2-3 *six3* acts independently of and additively to *six3* activity in *Xenopus.* A. *six3 -/-; six6* CRISPR injected (right most panel, white arrow) embryos show loss of eye features compared to the milder eye phenotype observed in the *six3*-/- embryos (left middle panel, white arrow). *six6* CRISPR injected embryos do not show any apparent phenotype (right middle panel). B, Expression of *pax6* is unchanged in *six3*-/-; *six6* CRISPR-injected embryos (right most panel, retina: white arrow and lens: white arrowhead) compared with *six3* -/- embryos (left middle panel, retina: white arrow and lens: white arrowhead). Expression of *mab21l2* is downregulated in the *six3*-/-; *six6* CRISPR-injected embryos (right most panel) compared with the *six3*-/- mutant (left middle panel).

Variable loss of lens phenotype observed in the *six3* mutant is correlated with loss of early and late lens genes

The variation observed in the eye phenotype, particularly related to the variable loss of the lens is also reflected by changes in gene expression seen *in situ* hybridization analysis of lens genes. We analyzed the expression of several lens genes in the *six3* mutant, categorized as early or late based on the observed onset of expression in the lens relative to key stages of lens development. Expression of *foxe3,* a gene that is expressed during lens specification (Fig 1-3) was severely reduced in the mutant with 86% of the embryos losing expression (Fig 2-4A, B) in lens ectoderm (gray, white and black boxes correspond to perturbed, no expression or normal expression as depicted in Fig. 2-4B). Expression of *foxe3* during lens specification and has been shown to be regulated by the co-ordinate inductive signals being mediated by *dll1* and *otx2* in the optic vesicle (Ogino et al., 2008). Expression of *otx2* (Fig. 2-5A) and Delta/Notch pathway components (Fig. 2-7A) are also reduced in the *six3* mutant eye indicating that this pathway might be a factor in the lens phenotype observed in the mutant. Expression of later lens marker such as the *maf* family of transcription factors *nrl* (old name: *l-maf*) and *mafb* show a marked reduction in expression although to a lesser extent than *foxe3* with loss of expression in 78% and 55% embryos respectively (Fig. 2-4B). Activation of *nrl* is thought to be important for the activation of late *crystallin* genes and is also downstream of *pax6* activity in mouse (Reza, Ogino and Yasuda, 2002). Just as in the case of *foxe3*, expression of *nrl* and *mafb* was either absent or severely reduced (Fig. 2-4A). Expression of lens epithelium marker *cryba1* is reduced in 50% of the mutant embryos (Fig. 2-4B) where as *cryg1* expression, a terminal differentiated lens fiber marker is lost in 77% of the mutant embryos (Fig. 2-4B).

Expression of both crystallins were either absent or severely reduced in the mutant (Fig. 2-4B) suggesting an effect on later lens formation with a higher loss of terminally differentiated lens fibers as indicated by the increased loss of *cryg1*. Both *mafb* and *cryba1* are normally activated at the time of lens determination, indicating that this process is significantly impacted in the *six3* mutant.

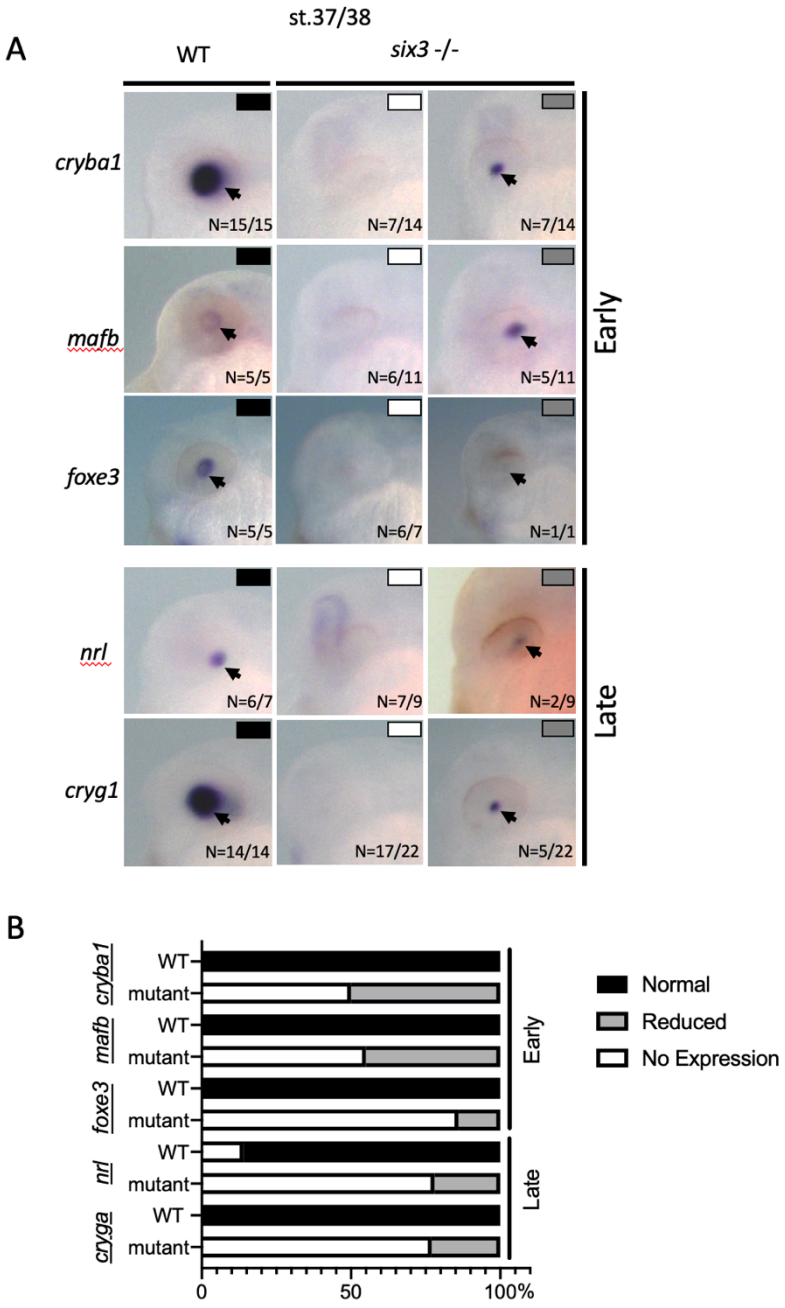

Figure. 2-4 Variable loss of lens phenotype is reflected in the expression of lens genes.

A, B. Expression of lens genes are reduced to different degrees in the *six3* mutant.

Early lens induction and early eye field transcription factors are largely unaffected in the *six3* mutant

Early retina and lens induction are not affected in the *six3* mutant. Experiments in rat and mouse have shown a requirement for *pax6* expression in the placodal region demarcating the future lens placode (Ashery-Padan *et al.*, 2000; Lang, 2004). However, conditional knockouts of *Six3* in the lens ectoderm in mouse suggested that expression of *Six3* is required for initial activation of *Pax6* expression in the presumptive lens ectoderm (Liu *et al.*, 2006). This in turn suggested that *Pax6* expression which is essential for lens formation is dependent on *Six3* expression in mouse. Although this result, as we later came to know, might be artifactual, it still led us to investigate this proposal in our *Xenopus* mutant where we then assayed the expression of *pax6* and other eye field transcription factors in neural plate stage (stage 15) embryos. Expression of *pax6* remains unchanged in the PLE of the *six3* mutant (Fig. 2-5A). This suggests that early lens induction remains unaffected due to loss of *six3*. Expression of other lens genes such as *mab21l1* and *mab21l2* are also unaffected in the *six3* mutant at this stage (Fig. 2-6A). Expression of *pax6* and *six6* in the retina and expression of *otx2* are also largely unaffected at the neural plate stage (Fig. 2-5A). Expression of other key eye field transcription factors like *rax*, *lhx2, vsx1* and *foxn4* shows a reduction of expression domain or loss of expression indicating early retina formation is affected likely impacting the eye size in the *six3* mutants (Fig. 2-5B). The expression of *otx2*, *rax* and *lhx2,* (Fig. 2-5A, B) show reduced expression domains. Expression of *vsx1* and *foxn4* (Fig. 2-6A) are lost at the optic vesicle stage (stage 21). At the neural plate stage *mab21l1* expression in the retina is moderately reduced (Fig 2-6A) whereas *mab21l2* expression to have only been activated at very low levels in the retinal

regions in the *six3* mutant at this stage (Fig 2-6A). Expression of both genes are retained in the presumptive lens ectoderm at the neural plate stage (Fig. 2-6A). However, at the optic vesicle stage expression of *mab21l2* is upregulated somewhat (Fig. 2-6A) while *mab21l1* expression is severely reduced in both the retina and lens indicating that *mab21l1* might play a particularly important role mediating *six3* activity in the eye (Fig 2-6A). Interestingly, it appears that essentially only one of the two genes are active at once during the stages from neural plate to the optic vesicle stage. It is important to also note that expression of *mab21l2* at stage 18 in the *six3* mutant (Fig. 2-2B) has started to become activated while in the *six3* mutant; *six6* CRISPant (Fig. 2-2B) its expression is reduced at stage 18 suggesting that the *mab21* gene family might be important for mediating the degree of the eye phenotypes in the two different mutant backgrounds. It is also interesting that *six6,* while not reduced at stage 15 is substantially reduced at stage 21, potentially implicating it do some degree in the smaller eye phenotype in the *six3* mutant.

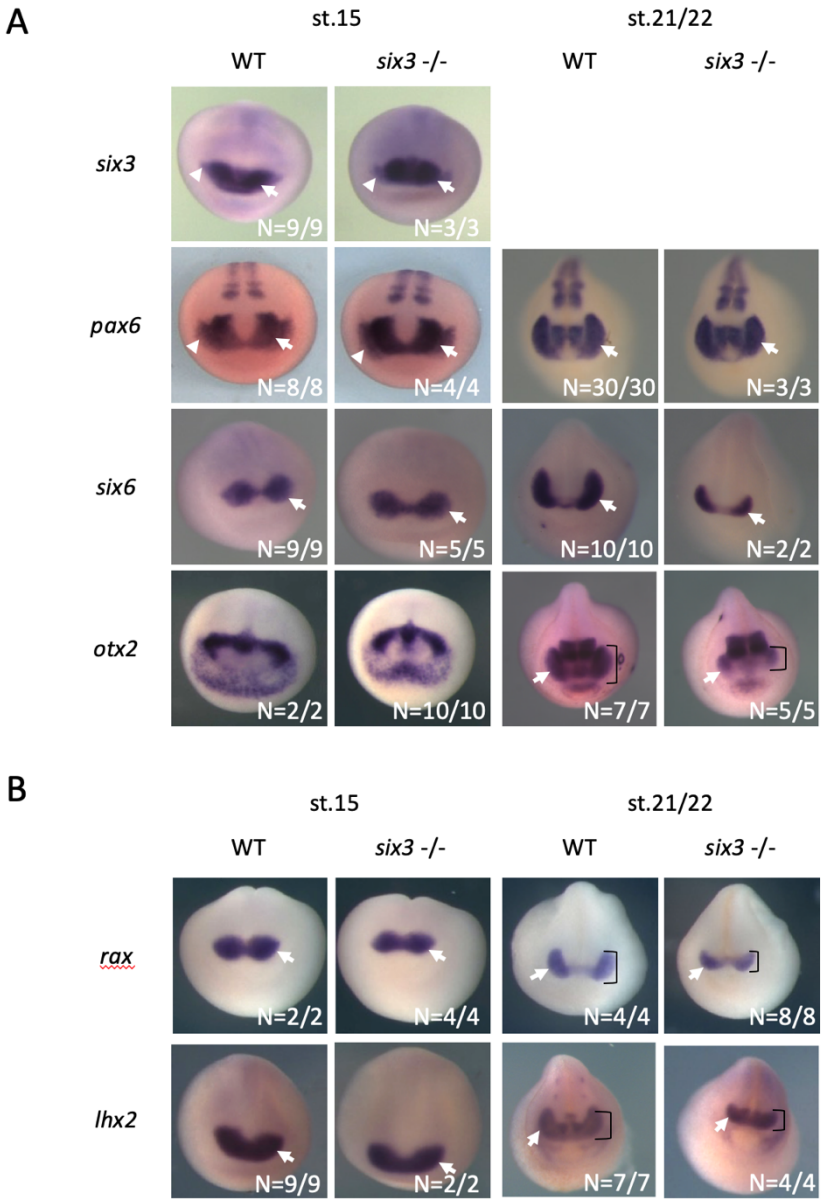

Figure. 2-5 Early lens and eye field transcription factors are largely unchanged in the *six3* mutant. A. Expression of *six3*, *pax6*, *six6* and *otx2* are not reduced at the neural plate stage in the regions where the retina (white arrows) or the lens (white arrowheads) will form; however, expression domains of some of these genes are reduced by optic vesicle stage (black brackets). B. Expression domains of *rax* and *lhx2* are changed at neural plate (white arrows) and optic vesicle stage (white arrows).

Lens formation is affected later during development and is regulated largely, but not exclusively, by inductive signals driven by *six3* in the retina

As shown earlier by the lack of early loss of expression of *pax6, mab21l1* and *mab21l2* in the PLE at stage 15 early lens induction appears to be normal in the *six3* mutant. Even by stage 24, expression of *pax6* is unchanged in both the lens ectoderm and optic vesicle (Fig. 2-8). The loss in expression of *mab21l1* or *mab21l2* even in the presence of *pax6* suggests that this is due to a major unrecognized role for *six3*, independent of *pax6* in lens and eye formation overall.

Expression of genes in the retina which have been previously shown to be important for lens formation like *bmp4* (Furuta and Hogan, 1998) and *dll1* (Ogino, Fisher and Grainger, 2008) are severely reduced in the *six3* mutant (Fig. 2-7A) suggesting that the effects on the lens in the *six3* mutant may be non-autonomous. A reduction in their downstream effectors of BMP signaling like *smad7* (expressed both in the lens and retina in mouse, (Zhang *et al.*, 2013)) and as shown earlier in Fig. 2-4 at st.37/38 *foxe3* (expressed in the lens) are also not activated in the *six3* mutant as early as stage 21 (Fig. 2-7A). In addition to the role of *bmp4* in lens formation, loss of *smad7* in mouse has also been implicated in defective lens formation (Zhang *et al.*, 2013). Conditional removal of *Bmp4* from the mouse optic vesicle inhibits formation of the lens by impinging the activity of several lens specification and terminal differentiation factors (Huang, Liu, Oltean, *et al.*, 2015) strengthening evidence for a non-autonomous role for BMP signaling in lens formation. *Bmp4* is expressed transiently in the lens ectoderm and optic vesicle early but becomes restricted to the dorsal optic vesicle later during development (Furuta and Hogan,

1998). In *Xenopus* delta/notch signaling pathway from the optic vesicle in conjunction with *otx2* is required for the expression of *foxe3,* a key requirement for lens specification also suggesting the involvement of a non-autonomous role in lens formation (Ogino, Fisher and Grainger, 2008).

Since we find that both BMP and Notch pathways are perturbed in the retina of the *six3* mutant, affecting the expression of components of each pathway, this result suggests the possibility of loss of *six3* in the optic vesicle is mediating lens formation in a non-autonomous fashion. To investigate this induction effect further, we performed transplant assays using wild type and mutant embryo PLE's from embryos injected with tracer dye that were dissected at the neural plate stage and transplanted into an unlabeled sibling host. The PLE at the neural plate stage has been partially induced, or biased, but still requires late inductive signals from the host to become determined (Fisher and Grainger, 2004). The embryos were assayed by *in situ* hybridization for the terminal lens differentiation gene *cryg1* at stage 37 - 40 (Fig. 2-9A). As expected, wild type donor transplanted to wild type hosts developed a normal looking expression domain for *cryg1* (Fig. 2-9A). Wild type PLE transplanted to mutant hosts failed to express *cryg1* in 58% of the transplants (Fig. 2-9A, inset) and in 41.6% of the transplants formed significantly smaller expression domains of *cryg1* (Fig. 2-9A). The wild type PLE transplant compared with the control side indicates the presence of an autonomous effect that is evidently stronger than the *cryg1* expression observed in the *six3* mutant alone (Fig. 2-4B). However, the mutant PLE transplanted to a wild type retina indicates that *six3* from the retina is able to overcome the loss of *six3* in the retina to generate a normal *cryg1* expression domain (Fig. 2-9B). Therefore, our evidence does not exclude an autonomous function for *six3* in PLE but suggests that the

inductive effects of the optic vesicle play a more predominant role in the formation of the lens. However, further analysis would have to be performed to determine any specific autonomous role for *six3* in lens induction as our and other evidence suggests and we have not pursued it further in this study (Liu *et al.*, 2006). Evidence for the role of *six3* in lens formation being non-autonomous is also supported by evidence from overexpression assays of *six3* in the fish medaka and in mouse embryonic cells resulting in expression of lens markers in neighboring cells in a non-autonomous fashion (Oliver *et al.*, 1996; Anchan *et al.*, 2014).

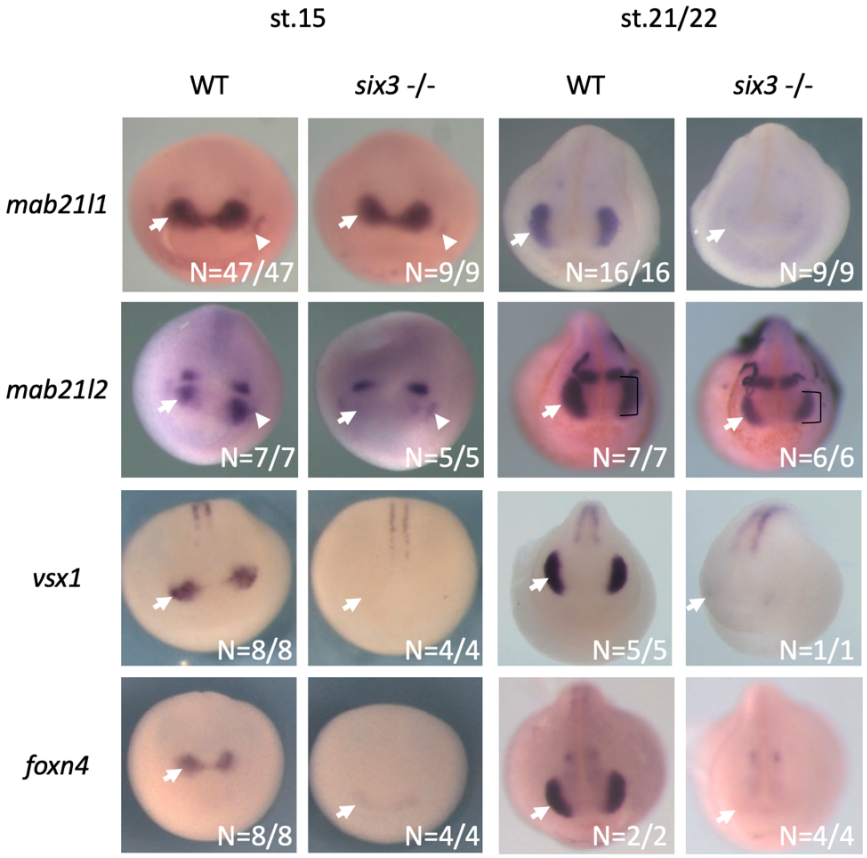

Figure. 2-6 Secondary retinal determination genes are reduced in the *six3* mutant; early expression of *mab21l1* and *mab21l2* in the PLE is unchanged. Expression of *mab21l1* and *mab21l2* show temporally regulated changes in expression. *mab21l2* is not activated in the retina at neural plate stage (left panels, white arrows). Expression of *mab21l1* is reduced at the neural tube closure/optic vesicle stage in the *six3* mutant (top right panels, white arrows). Expression of both *mab21l1* and *mab21l2* are retained in the PLE at the neural plate stage (top left panels). Expression of retinal markers *vsx1* and *foxn4* are not activated in the retina at both neural plate and neural tube closure (optic vesicle) stage in the *six3* mutant (bottom panels, white arrows).

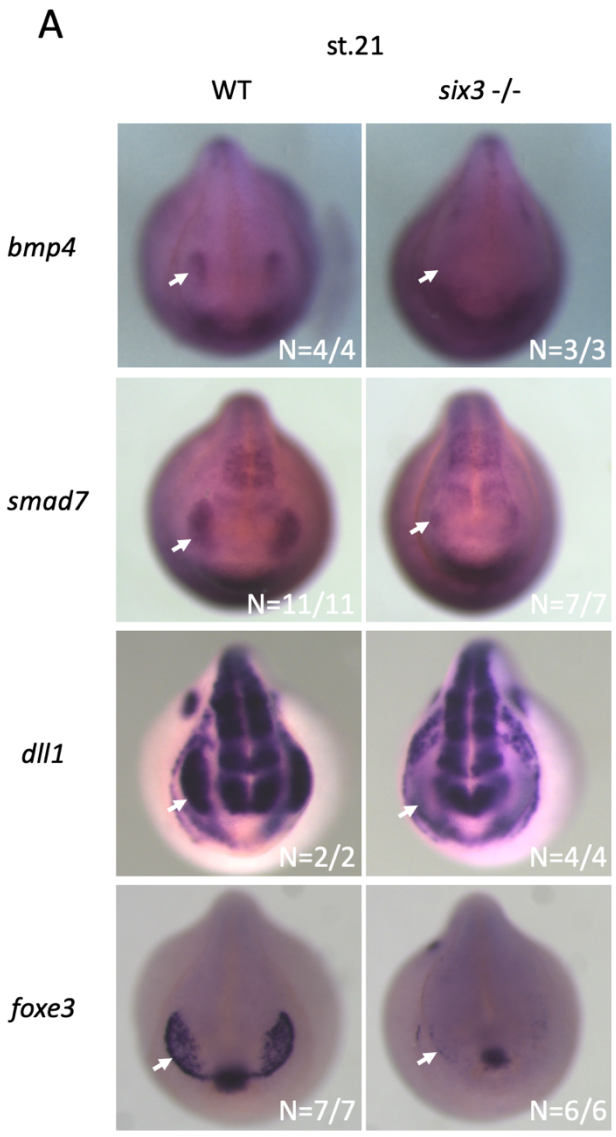

Figure. 2-7 Loss of *six3* affects late lens formation mediated by the loss of expression of components of the Delta/Notch and BMP signaling pathways. A. Expression of BMP signaling components – *bmp4* and *smad7* are not activated in the presumptive retina of the *six3* mutant (top panels, white arrows). Expression of *dll1, dlc, notch1* (*dlc, notch1*, shown in Chapter III) and *foxe3* (expressed during lens specification) are not activated in the presumptive retina and PLE respectively in the *six3* mutant at the optic vesicle stage (bottom panels, white arrows).

Lens induction signal from the retina is possibly being mediated by the regulation of BMP signaling by *mab21l1/l2* under the control of a *six3*-mediated gene regulatory hierarchy

With the transplant experiments arguing for an inductive role from the optic vesicle mediated by *six3* being important for lens formation, we set to determine a functional pathway for this mechanism. We have previously identified that expression of *mab21l1* is downregulated by stage 21 (Fig. 2-6A). *mab21l1* and *mab21l2* are highly conserved and have similar expression patterns in *Xenopus* as shown by the expression data presented in this study. Studies in mouse has indicated that loss of function of either *mab21l1* or *mab21l2* results in significant disruption of lens and retina formation (Yamada *et al.*, 2003, 2004). This suggests that both of these genes play an important role during eye formation downstream of key eye field transcription factors like *pax6* and now *six3* (Yamada *et al.*, 2003, our data)

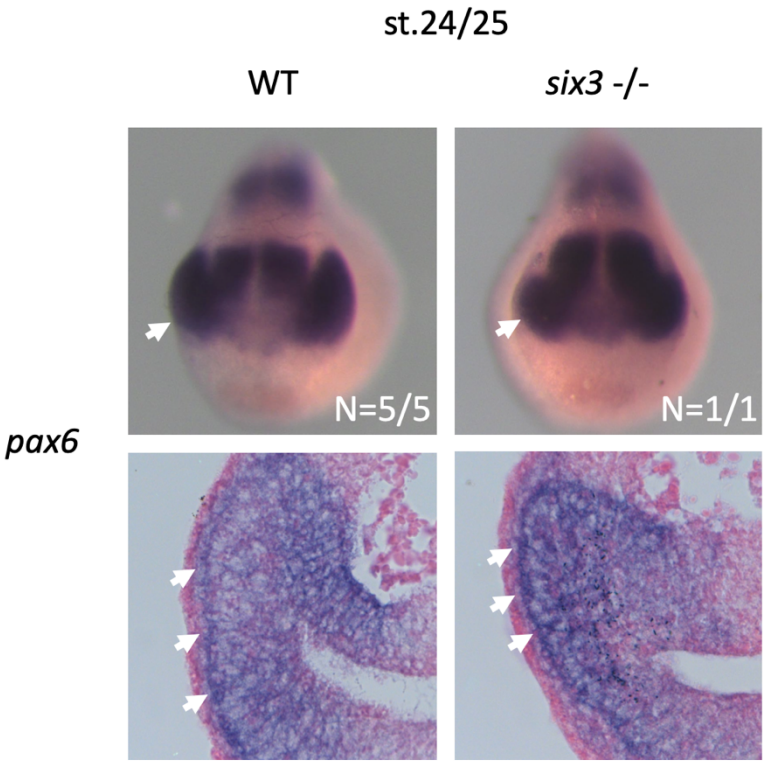

Figure. 2-8 Expression of *pax6* is not reduced in the optic vesicle and lens of the *six3* mutant. Top panel shows anterior view of whole mount expression of *pax6* in the eye field (white arrows). Bottom panel shows cross section through the optic vesicle region of the embryos shown above. White arrows indicate the apparently normal expression of *pax6* in the lens ectoderm.

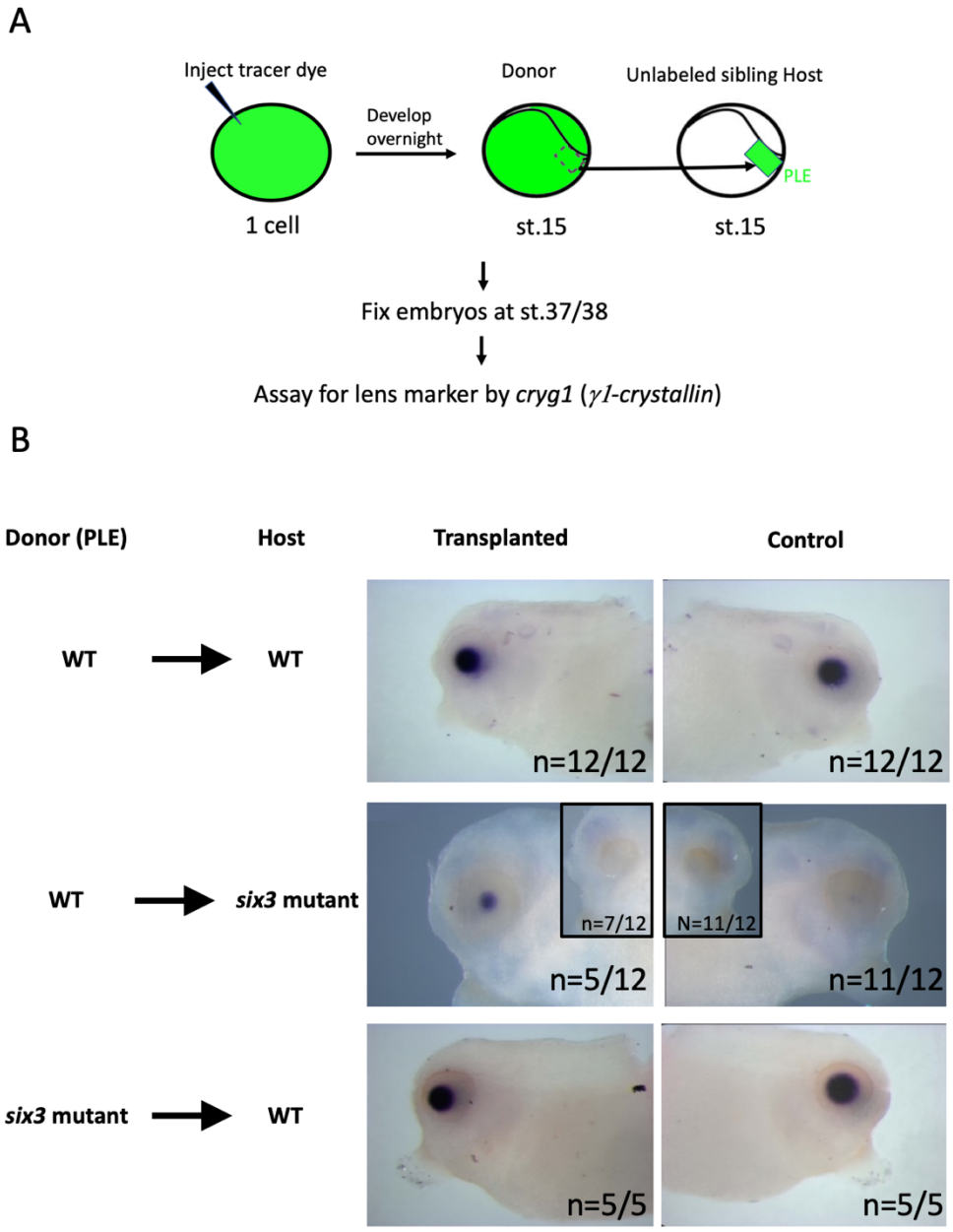

Figure. 2-9 *six3* regulates lens formation largely but not exclusively in a non-autonomous fashion mediated by the retina. A. Schematic describing the experimental plan to assay for the transplantation of PLE tissue from labeled donor to host embryo. B. Wild type PLE to wild type retina transplants yield normal *cryg1* expression profiles (compare transplanted with control side, top panels), Wild type PLE to mutant retina transplants do not express *cryg1* in the majority of the cases (inset panel); they do however they do show small

amounts of *cryg1* expression in some cases (middle panels). Mutant PLE to wild type retina transplants yield normal looking *cryg1* expression between the transplanted and control sides (bottom panels).

As mentioned in the previous section, loss of *mab21l1* and failure to activate then fully express *mab21l2* along with existing literature evidence suggested that these two genes might play a key downstream role for *six3* in lens and retina formation. Therefore, we investigated the possibility of rescue of the *six3* mutant phenotype by injecting 500pg of *mab21l1* mRNA into both of the dorsal blastomeres of 4/8 cell embryos and show that *mab21l1* was indeed able to significantly rescue expression of *cryg1* in the lens (Fig. 2-10A). In addition, of overall significance and particularly relevant to Chapter III, we note that partial rescue of the retinal patterning defects also occurs as assayed by *in-situ* hybridization analysis of *vsx1* and *pax6* in sections of stage 41/42 mature embryonic retina Fig. 2-10C).

Expression of *smad7* was also shown to be not activated in the *six3* mutant at stage 21 in the eye-field (Fig. 2-7A). Expression of *smad7* was rescued by injection of *mab21l1* mRNA suggesting that BMP signaling was recovered in the *six3* mutant upon injection of *mab21l1* mRNA (Fig. 2-10B). Loss of *Smad7* in mouse perturbs formation of lens in mouse (Zhang *et al.*, 2013). The rescue experiments suggest that *mab21l1* or perhaps *mab21l2* is an intermediary in *six3* signaling network mediating its activity by regulating the expression of *smad7* presumably by regulating BMP signaling.

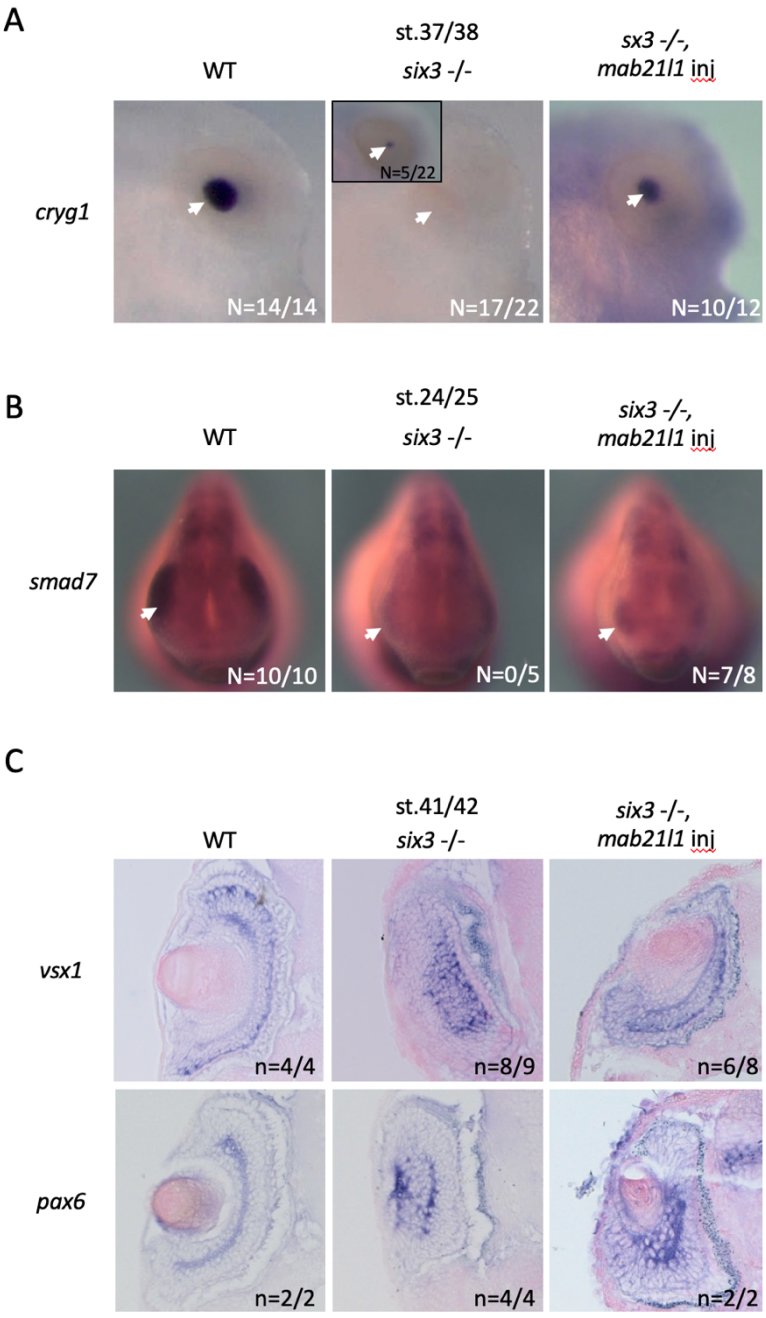

Figure. 2-10 *mab21l1* partially mediates *six3's* role in lens and retina formation. A. Expression of *cryg1* is recovered in the *six3* mutant injected with *mab21l1* (compare right panel with middle panel; inset shows low level of expression size of *cryg1* in some *six3* mutants, white arrows). B. Expression of *smad7* is recovered in *six3* mutant injected with *mab21l1* mRNA (compare right panel with middle panel, white arrows). C. Layering of

retina is partially rescued upon injection with *mab21l1* in the *six3* mutant background as shown by the rescue of patterning in *vsx1* and partially in the *pax6* case shown. (compare right panels with middle panels).

Discussion and future directions

In this study we have examined a *six3* mutant line in *Xenopus* that provides novel insights into the role of *six3* in vertebrate lens formation and leads to a new view of the eye gene regulatory network (GRN) with *six3* now positioned in a pivotal position within this GRN. Data presented in this study shows that *six3* and *six6* expression are independently regulated and additive to each other. We also show that *six3* affects the later stages of lens determination and its activity is independent of *pax6* as evidenced by the lens defects observed in the *six3* mutant in the absence of *pax6* expression. Further, we show that *six3* acts primarily by playing a non-autonomous role in lens formation by affecting inductive signals between the retina and PLE. These effects are mediated in significant part by *mab21l1*, and likely *mab21l2*, nuclear proteins whose functionality is not yet completely known but may be linked to BMP signaling (De Oliveira Mann et al., 2016).

At least at the neural plate stage, early eye field transcription factors are largely not changed in the *six3* mutant as shown by our *in situ* hybridization analysis. Of particular importance is the normal expression of *pax6* in the PLE. Expression of *pax6* in the PLE has been shown to be essential for lens formation acting from the earliest stages of lens formation (Fujiwara *et al.*, 1994). This and other data presented here suggests that *six3*, acts primarily at the later phases of determination. Lenses do fail to form in majority of the cases and even in the cases where a lens forms, it is typically small and poorly differentiated.

The presence of the defective retina and in some cases even a small lens, a milder phenotype than in the equivalent mouse mutant, led us to determine if there are any complementary genes active in *Xenopus* that may mitigate the phenotype. One of these that

is particularly relevant to the development of the eye is the expression of *six6* which has shown similar overlapping expression pattern in the optic vesicle with *six3* (Seo *et al.*, 1998). Our data presented here shows that loss of function of *six3* and CRISPR knockout of *six6* in a *six3* mutant background results in the complete ablation of eye formation suggesting an independent and complementary role for *six6* in eye formation. The *six3* mutants as shown by our data are not always completely missing lenses indicating that the phenotype is not completely penetrant. We hypothesized and then observed based on morphological evidence that in cases where retina defects were less severe, an incomplete lens-like structure could be formed even in the absence of *six3*. Although, evidence to determine the cause of incomplete penetrance is not available at this time. A speculative factor for the incomplete penetrance could be the maternal expression of *six3* (Zuber *et al.*, 2003) or the background variability in the *Xenopus* considering our population is closer to wild caught frogs than mouse lines which are much more inbred.

Our data suggests an independent and additive effect of *six6* as we lose the formation of the eye completely, including the retina and the lens in absence of *six3* and in concert with F0 mutations in *six6*. Expression of *six6* is not observed in the lens. Therefore, this suggests that loss of the lens is likely mediated by the complete loss of the retina in the double mutant. However, the double mutant also does not have any impact on the early induction of the lens, at least as assessed by the expression of *pax6* and therefore any effect on the lens is occurring later during its determination.

The establishment of the role of retina in lens formation in the *six3* mutant is revealed by the transplantation experiments which argue that *six3* from the optic vesicle plays a major role in mediating the formation of the lens. This is also consistent with the

loss of expression of *bmp4*, *smad7* and *dll1* in the optic vesicle of the *six3* mutant. These signaling factors have been previously implicated in the involvement of an optic vesicle signal being important for lens formation (Ogino, Fisher and Grainger, 2008; Huang, Liu, Oltean, *et al.*, 2015). The transplantation experiments however do not rule out an autonomous function for *six3* in lens formation as in some cases we do see the formation of a small *cryg1* expression domain in the wildtype PLE transplanted to a mutant retina whereas we do not observe *cryg1* expression on the unoperated mutant side (where mutant PLE is exposed to mutant optic vesicle). Comparing the transplanted side with the control suggests that there is sufficient activity of *six3* to make a lens-like structure (Fig. 2-9B). However, our data also shows that when a mutant PLE is transplanted onto a wildtype retina, they make normal *cryg1* expression pattern suggesting a primarily and perhaps strong non-autonomous function that can compensate for the loss of *six3*. A future direction to explore the autonomous effect would be to begin by sequencing the RNA of wildtype and mutant PLE at stages when the wildtype PLE is normally biased, specified and determined, which would shed light on the differentially regulated genes affected by the loss of *six3* in the PLE. Similar experiments in the retina would show us the differentially regulated genes that are in the retina in a manner that would be less biased than the selection of genes examined here, and we can identify confirm new targets by *in situ* hybridization and other functional analyses.

Another novel finding of our study is the identification of the *mab21* family of genes as being key mediators of *six3* function in the lens. *Pax6* is known to regulate mab2111 expression in both mouse and *Xenopus* (Yamada *et al.*, 2003; Nakayama *et al.*, 2015). Expression of *mab21l1* is not activated in the *six3* mutant even in the presence of

normal *pax6* expression suggesting the requirement of both these genes to regulate *mab21l1* and *mab21l2* expression. This highlights the important role of *six3* in a parallel pathway beside to *pax6* as one of the key early eye field transcription factors that regulate lens formation. The Grainger lab has *Xenopus pax6* mutant lines and although some analysis has been completed at this point of time, thorough analysis of this mutant would enable us to determine if there are other and its targets, paralleling what been done with the *six3* mutant would enable us to determine if there are other target genes that overlap or are different and determine if the mechanisms by which *pax6* and *six3* affect lens formation are similar or different.

The role of BMP signaling pathway and *mab21l1* in modulating *six3's* activity from the retina to the lens is evident from their loss in the *six3* mutant. We observe *mab21l1* and *smad7* along with *bmp4* are not activated in the retina of the *six3* mutant. Upon over expression of *mab21l1* mRNA in the *six3* mutant we observe a recovery of *cryg1* and *smad7* expression. This suggests a regulatory role of *mab2ll1* (or *mab21l2*) in rescuing the lens and in the recovery of component of the BMP target *smad7*. But the exact interaction of *mab21l1* and BMP signaling pathway is as yet unclear. Our next step would be to determine if *bmp4* expression is rescued by *mab21l1* in the *six3* mutant. We can also further expand on this to determine the downstream effectors of *mab21l1* and/or *mab21l2* by utilizing the *Xenopus mab21l1* and *mab21l2* mutant lines that have been established in the Grainger lab but not yet characterized and to determine whether interactions with the BMP pathway may be direct or indirect.

The functional relevance of BMP signaling in the eye of the *six3* mutant can be assessed by investigating the levels of phosphorylated Smad, indicative of active BMP

signaling (Massagué, Seoane and Wotton, 2005) using a commercially available antibody and comparing the staining between histological sections of wild type and *six3* mutant embryos. More definitively, we can also assay for rescue of the *six3* mutant lens phenotype by injecting a construct containing constitutively active form of *smad5* mRNA (Christian and Nakayama, 1999) to determine if it can rescue the lens phenotype in the *six3* mutant. If rescue is seen, it should be possible to establish whether this is due to an effect in the optic vesicle or the lens by doing transplants where the construct would be active in mutant retina and mutant (but untreated) PLE placed over it, or vice versa.

Overall, the current working model utilizing data that is generated in this study suggests that *six3* and *pax6* work in parallel pathways to regulate lens formation, highlighting a previously unrecognized role for *six3* in this process. The loss of components of this complex delta/notch and bmp signaling network due to the loss of *six3* results in the defective retina and lens (Fig. 2-11). Our working model suggests that the parallel components of *six3* and *pax6* have common targets like *mab21l1* and *mab21l2*. In the case of *six3* we show in this study that *mab21l1* can mediate lens formation via the BMP signaling pathway. However, the mechanism of *mab21l1*'s action in the BMP pathway is not yet determined. Further, *six3* also regulates expression of *dll1* and *dll2* and *otx2*, components of the Notch pathway which has previously been shown to be important for the activation of *foxe3*, a key gene expressed during lens specification (Ogino, Fisher and Grainger, 2008).

The *Xenopus six3* mutant reveals several key findings: 1) *six3* and *pax6* are independent of each other in the lens ectoderm and early lens induction is not affected by the loss of *six3* while later phases of lens induction are severely impacted in the mutant. 2)

six6 and *six3* are independent and additive to each other. 3) The missing and defective lenses in the *six3* mutant result primarily due to its non-autonomous function mediated by the optic vesicle. 4) Our data here suggests to an essential role for the *mab21* genes in regulating lens and eye formation overall.

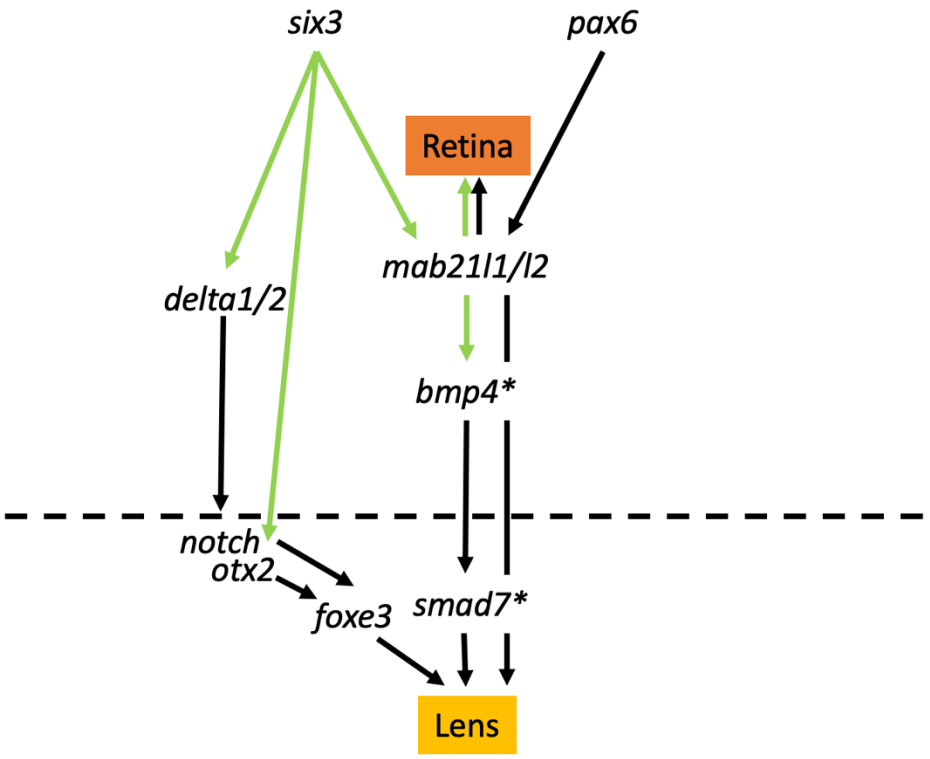

Figure. 2-11 Proposed model for the role of *six3* in lens formation. Green lines - Indicate connections that are proposed from this study. Black lines indicate data generated by previous studies (Nakayama *et al.*, 1998; Yamada *et al.*, 2003; Yoshimoto *et al.*, 2005; Ogino *et al.*, 2008; Nakayama *et al.*, 2015). The arrows do not necessarily indicate a direct relationship. * Indicates that the location of these components (in the retina or lens) is not determined.

Materials and Methods

Animal Handling and tissue collection and mRNA, CRISPR injections

Xenopus tropicalis were housed and cared for based on the guidelines set forth by the University of Virginia Animal Care and Use Committee. The *six3* mutant line was generated from CRISPR generated mutations (Nakayama *et al.*, 2013). F1 lines containing a 19bp deletion were raised by outcrossing with wild type frogs. Embryos were collected from either natural mating or in-vitro fertilization. Embryos for *in situ* hybridization were fixed in MEMFA (MOPS, EGTA, $MgSO_4$, formaldehyde - ('CSH Protocols', 2008)), gradually dehydrated and stored in -20°C. Capped mRNA's for *mab21l1* was prepared from linearized plasmid using a following protocol developed by the manufacturer (ThermoFisher, AM 1344). 500pg of mRNA was injected into both dorsal blastomeres at 4 or 8 cell stage. CRISPR injections were based on the protocol developed by (Nakayama *et al.*, 2013). An example of the injection protocol for mRNA and CRISPR is described in Appendix II.

In-situ hybridization, sectioning and imaging

The expression pattern for downstream targets of *six3* were determined by *in situ* hybridization assays for probes listed in Table 2-1. The protocol for *in situ* hybridization was adopted from (Harland, 1991) and modified by the Grainger lab to eliminate acetic anhydride treatment and the removal of the post fix step which interfere with genotyping assays. Embryos were embedded in paraffin and 10μm microtome sections prepared and stained with hematoxylin and eosin. An example of the protocol used is described in Appendix II.

For fluorescence and bright field imaging post *in situ* hybridization, we used a Zeiss SteREO Discovery V12 microscope and the images were captured on AxioVision software version 4.8.2. using the same settings for images for collecting images to be compared in a given experiment. Images were in some case modified in Adobe Photoshop to adjust for contrast and brightness, again with settings applied equally to all sets of sections being compared.

Gene Name	Species	Lab Clone Number	Vector	Source
dll1	*X. laevis*	1485	pCS2+	(Chitnis *et al.*, 1995)
notch1	*X. laevis*	1479	pCS2+	Chitnis *et al*, 1995
vsx1	*X. tropicalis*	1675	pCS108	Openbiosystems # 7614953
foxn4	*X. tropicalis*	1774		Openbiosystems # 7626378
bmp4	*X. tropicalis*	772	pSP64T	H. Brivanlou
smad7	*X. laevis*	1098	pCS2+	Nakayama *et al*, 1998
mab21l1	*X. tropicalis*	1137	pCS107	Sanger Center
mab21l2	*X. tropicalis*	1138	pCS107	Sanger Center
otx2	*X. laevis*	1250	pCS2+	(Blitz and Cho, 1995)
six6	*X. tropicalis*	1664		Openbiosystems # 7623919
six3	*X. laevis*	1469	pCS2+	Gestri *et al*, 2005
pax6	*X. tropicalis*	1569	pSP64T	Openbiosystems # 6992220
rax	*X. tropicalis*	1571		Openbiosystems # 9019330
lhx2	*X. tropicalis*	1698	pSP64T	Openbiosystems # 7657571
cryg1	*X. laevis*	512	bluescript	(Offield, Hirsch and Grainger, 2000)
foxe3	*X. laevis*	1337	pGEMTeasy	Ogino *et al*, 2008
nrl	*X. tropicalis*	854	pBSSK+	Grainger lab
mafb	*X. tropicalis*	1139	pSP64T	Openbiosystems # 7025366
cryba1	*X. tropicalis*	1761	pCS107	Openbiosystems # 9018035

Table 2-1 Information on the probes used for *in situ* hybridization

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Chapter III The role of homeobox transcription factor *six3* during retina formation in *Xenopus*

Summary

The role of *Six3* in patterning the retina has primarily been investigated in mouse models using conditional knockouts wherein early loss of S*ix3* results in ablation of neuroretina specification and later removal causes milder phenotypes (Liu *et al.*, 2010; Liu and Cvekl, 2017; Diacou *et al.*, 2018). The phenotype observed in the *Xenopus* mutant allows us to investigate the role of *six3* both early in RPC specification as well as its role in retinal patterning. Using *in situ* hybridization analysis we show that expression of several transcription factors *vsx1, foxn4* and nuclear proteins *mab21l1* and *mab21l2* are downregulated in the *six3* mutant. Expression of eye field transcription factors are largely unchanged indicating a independent parallel pathway in retina formation by *six3.* Further analysis of the mature embryonic data reveals that layering of the retina are disrupted. Injection of *mab21l1* mRNA rescued loss of patterning to a significant degree as shown by the expression of *vsx1* to a significant degree. Our data, though preliminary, provides novel insights that with further work will help to determine the position of *six3* in the retinal GRN and its role in retina formation.

Introduction

As discussed in the previous chapter, *six3* activity in the optic vesicle is largely responsible for the defective lens phenotype observed. Therefore, the activity of *six3* in retina formation becomes important to understand the regulatory role of *six3* in eye lens formation in addition to understanding the defects that are clearly occurring with regard to retinal patterning.

The initial formation of the retina is a complex process involving several different transcriptional and signaling networks, the integrated action of which leads to the generation of retinal neurons and glia from a common pool of retinal progenitor cells (RPC). In this study we focus on the regulatory role of *six3*, a homeobox domain containing transcription factor. It is expressed broadly early in the retina and becomes progressively restricted after the onset of differentiation (Ghanbari *et al.*, 2001). Loss of *SIX3*, along with other genes, is also associated with the human disease – holoprosencephaly (Wallis *et al.*, 1999). Murine *Six3* mutants lack the anterior forebrain and any eye structures (Lagutin *et al.*, 2003). Conditional knockouts therefore provide an alternative to study the role of *Six3* in the retina, but they do not deplete *Six3* from the time of its initial transcription *in vivo* because the lag associated with CRE-mediated gene inactivation. In contrast the *Xenopus* mutant shows a milder general phenotype with deformed retina and lens which allows us to investigate the role of *six3* in retina formation from the time of the zygotic onset of this gene. The mutant phenotype shows failure to form the definitive layers in the retina, and several genes are either not activated or expressed appropriately during early retinal development, e.g. transcription factors *vsx1* and *foxn4*, nuclear proteins *mab21l1*/*mab21l2* and ligands and receptors important for Delta/Notch and BMP signaling pathways. Both

BMP and Delta/Notch pathways are important for regulating retinal progenitor maintenance and neurogenesis. Genes associated with particular retinal layers at later stages, e.g. *pax6* and *vsx1*, are active in the mutant but expressed broadly throughout the disorganized mutant retina.

We were able to partially rescue the retina phenotype by injecting with *mab21l1* mRNA which indicated that these nuclear proteins are essential upstream regulators of retinal patterning. We have started to analyze the pathway involved in this rescue and our preliminary analysis suggests a model where in *six3* regulates retina patterning mediated by *mab21l1*/*mab21l2* impinging on either (or both) the BMP or the Delta/Notch pathways. Further analysis would involve following up on these initial determinations by investigating downstream effectors of BMP and Delta/Notch signaling pathway either by determining the functional activity of BMP signaling or by utilizing the constitutively active form of Notch to determine if these can recover the normal retinal patterning in the *six3* mutant.

The vertebrate retina and forebrain arise from distinct areas in the anterior edge of the neural plate to eventually form distinct tissues with clearly delineated regional differentiation. The mechanisms which control development of these tissues, although distinct also share some similar components. Both arise out of neural tissue and therefore I will in this chapter try to provide an overview of the early transcriptional and signaling networks that regulate the specific development of the retina.

Formation of the retina

The adult retina is a multi-layered sensorial tissue that originates from a pool of progenitor cells within the neural plate. It functions to transmit information received in the

form of light into electrical impulses to the brain via the optic nerve which then converts it into visual information. There are wide variations in the visual system through evolution ranging from light sensitive compounds in archaea to eyespots in euglena and planaria, compound eyes in invertebrates and the complex layered structure of vertebrate eyes (Schwab, 2018). The components of the eye or the retina can vary even amongst vertebrates which leads to the differences in visual acuity between different species (Hoon *et al.*, 2014). However, the basic mechanisms such as the role of opsins and *Pax6* which drive the generation of these visual systems are highly conserved throughout evolution (Cepko, 2014). The mature vertebrate retina is a multi-layered tissue consisting of ganglion cells, amacrine cells, bipolar cells, horizontal cells, rods and cone photoreceptors, Müller glia and other accessory cells (Marquardt, 2003). A schematic of the retinal layers and their orientation is shown in Fig. 3-1.

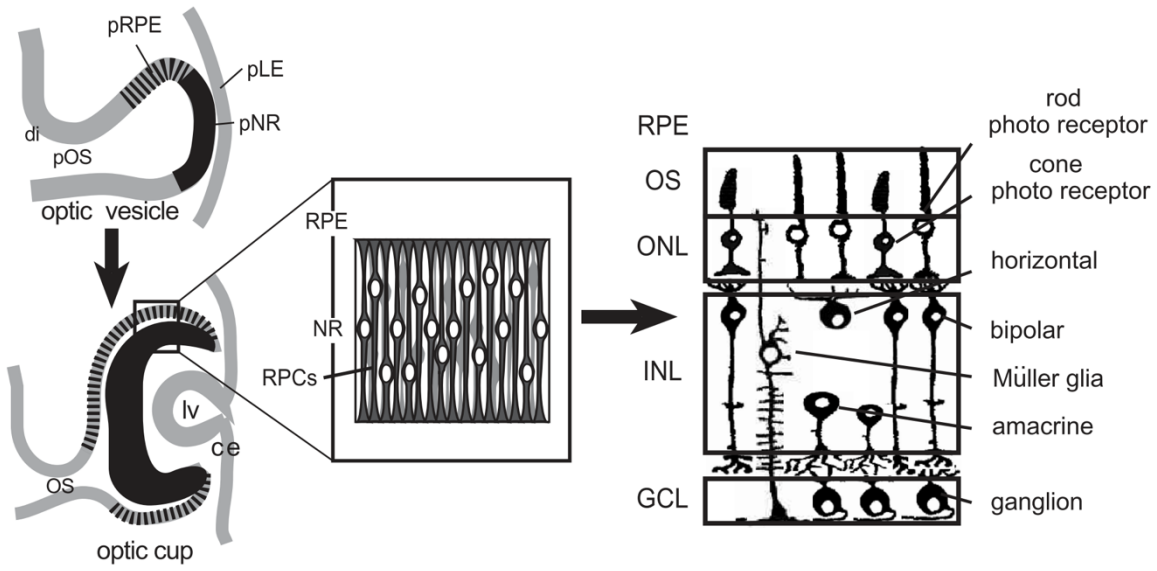

Figure. 3-1 Schematic of gross morphology and layering of the retina. Left: transformation of the optic vesicle into the mature retina forming a cup shaped tissue around the lens. Middle: cross-section of a region of the retina showing the common pool of retinal progenitor cells. Right: progenitors differentiate into the terminal neuronal layers shown. pRPE, RPE: retinal pigmented epithelium; pOS, OS: presumptive optic stalk, optic stalk; PLE: presumptive lens ectoderm; pNR: presumptive neuroretina; lv: lens vesicle; RPC: retinal progenitor cells, NR: neuroretina; GCL: Ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer. Reprinted with permission of Elsevier from (Marquardt, 2003)]; permission conveyed through Copyright Clearance Center, Inc.

The generation of these cell types from a single pool of RPC's involves both intrinsic and extrinsic signaling networks in a tightly regulated but overlapping developmental timeline (Cepko, 2014). Early studies using lineage tracing showed that a single clone can give rise to multiple different types of differentiated neurons (Holt *et al.*, 1988; Belecky-Adams, Cook and Adler, 1996). The generation of the differentiated retina from a common pool of cells which split into two retina derivatives mediated by Shh is conserved between frogs and mice (Chiang *et al.*, 1996; Jeong *et al.*, 2008). The generation of completely differentiated retina in the frog take approximately two days post fertilization whereas in mice it is much slower taking proximately two weeks to differentiate. The regulation of these RPC's is mediated by transcriptional factor networks that are influenced by signaling networks and the movement of cells within the retina which position them to differentiate into the various cell types. The birth order of the differentiated neurons is highly conserved but overlapping with the retinal ganglion cells born first followed by the horizontal, cone, amacrine, rod, bipolar and Müller glia being born last (Belecky-Adams, Cook and Adler, 1996; Bassett and Wallace, 2012; Cepko, 2014). The location of the progenitor also determines the birth order with central retina ganglion cells being born first and peripheral retinal ganglion cells born after central amacrine cells (Belecky-Adams, Cook and Adler, 1996; Agathocleous and Harris, 2009).

Several models have been proposed to describe the generation of the retinal cell types but without consensus at this point (Agathocleous and Harris, 2009; Bassett and Wallace, 2012; Cepko, 2014). From the late blastula stage up to neurogenesis, the retinal progenitors undergo lengthening of the cell cycle (Harris and Hartenstein, 1991; Alexiades and Cepko, 1996; Martinez-Morales, Cavodeassi and Bovolenta, 2017)

The transcription factors in early retinal progenitor cells are thought to promote proliferation in order to maintain number of competent cells to differentiate into the each of the different retinal neurons (Zuber *et al.*, 2003). Late in development, some of these factors become restricted to certain lineages and the timing of the loss of these early transcription factors determines the phenotypic outcome observed in conditional knockout mouse models (Marquardt *et al.*, 2001; Klimova and Kozmik, 2014). An important point that I have suggested previously is that although conditional knockouts provide valuable insights it is still limited by the lack of early deletion and possible side effects of the *Cre* recombinase system. Therefore, study of *six3* in the frog model with its milder phenotype would reveal novel mechanisms that might not be possible in the conditional knock out systems. In *Xenopus* and other model systems several eye field transcription factors are expressed and thought to be involved in cross regulating each other's expression in the neural plate stage such as *pax6, six3, six6*, *lhx2*, *rax* and *tbx3* (Zuber *et al.*, 2003). However, papers investigating the *Xenopus rax* and *pax6* mutants have suggested these to be independent of each other (Fish *et al.*, 2014; Nakayama *et al.*, 2015). Other transcription factors are also expressed in this and surrounding region whose roles in retinal patterning are relevant but is thought to be secondary to the establishment of the eye field compared to the eye field transcription factors described before. In this chapter, I will cover the literature about a few of the transcription factors that are relevant for this study due to the potential interactions with the *six3* gene.

One of the early factors expressed in the retinal region is the retina and anterior neural fold homeobox gene *Rax*. It is expressed throughout the retinal progenitor cells (RPC) and has been identified in numerous model systems. In *Xenopus*, the Grainger lab

has established *rax* as key factor that establishes the eye field and represses the diencephalon and telencephalon (Fish *et al.*, 2014). Given its role in defining the retinal tissue, it is expressed throughout the retinal progenitors but later becomes restricted to the photoreceptors and the ciliary margin zone in the mature embryonic retina, which in amphibians and fish is a source of retinal progenitor cells (Pan *et al.*, 2016). It is also known to directly regulate expression of arrestin and *otx2* via conserved regulatory elements PCE1 and EEPLOT respectively. Expression of *six3* and other eye field transcription factors are not changed in the *Xenopus rax* mutant at the neural plate stage but show reduction at stage 24, however given the role of *rax* in retinal formation this reduction could be an effect of change of fate of retinal tissue (Fish *et al.*, 2014). An autoregulatory network has also been recently identified in *Xenopus* (Pan *et al.*, 2016) similar to other major eye field transcription factors *Pax6* (Bhatia *et al.*, 2013) and *Six3* (Suh *et al.*, 2010), although in the case of *six3* the study only establishes this effect in *in vitro* and transient transgenic assays. This shows that *Rax*, like other transcriptional regulators, have multiple regulators and also have multiple regulatory roles during different developmental time points and can cross- and auto- regulate downstream gene expression.

Another transcription factor family that influences retina formation are the *Pax* family of paired homeobox genes of which *Pax2* and *Pax6* are expressed in the retina. Expression of *pax6* is much broader and occurs earlier than *Pax2* and is therefore thought to be higher in the gene hierarchy involved in the formation of the retina than *Pax2*. *Pax6* is expressed in the retinal progenitor cells and early knockdown of *Pax6* results in the loss of competency of the retinal progenitor cells in mouse (Klimova and Kozmik, 2014). However, later loss of *Pax6* resulted in activation of only amacrine cell population

indicating that the influence of *Pax6* is time dependent and becomes more restricted at later stages (Marquardt *et al.*, 2001). The early inactivation of *Pax6* suggests a key role in maintenance of multipotency of the RPC's possibly controlled by cell cycle regulation evidenced by premature cell cycle exit (accumulation of $P57^{kip2}$) and the decrease of *Cyclin D1* (Klimova and Kozmik, 2014). *Pax6* activity has been shown to be necessary for activation of *Atoh7* expression in the retina which is necessary for differentiation of the retinal ganglion cells (Brown *et al.*, 1998). Overexpression of *Pax6* does not affect initial eye formation but does result in defective retina and microphthalmia in mice suggesting that maintaining the appropriate level of expression during different developmental time is necessary for proper development of the retina (Schedl *et al.*, 1996; Manuel *et al.*, 2008). Reduction of *Pax6* expression in the Sey mouse does not impact the expression of *Six3* in the retina indicating they are independent in the retina (Goudreau *et al.*, 2002).

Lhx2, a member of the LIM homeobox transcription factor family, also overlaps in expression with *Pax6* and *Rax* in the retinal progenitor cells. Its expression later becomes progressively restricted to the Müller glia and a subset of amacrine cells. Germline knockout of *Lhx2* in mouse results in ablation of eye formation prior to retinal differentiation. Early loss of *Lhx2* results in the reduction of expression of *Rax*, *Six3*, *Pax6* and *Six6* (Tetreault, Bernier, 2009). Late conditional knockout of *Lhx2* results in a more moderate phenotype primarily affecting generation of Müller glia and expression of components of the Delta/Notch pathway (de Melo *et al.*, 2016). The Delta/Notch pathway is thought to inhibit differentiation and maintain proliferation of the retinal progenitor cells and as we show here in our study, its expression is also reduced in the *Xenopus six3* mutant (Perron and Harris, 2000, our data). Telencephalic activity of *six3* is mediated by the

zebrafish ortholog of *lhx2* (Ando *et al.*, 2005). Therefore, a potential upstream regulator of *six3* activity could be the expression of *lhx2.* This would warrant further study to determine its epistatic relationship with the other EFTF's.

Otx2, a homolog of the Drosophila *orthodenticle* gene is another anterior patterning factor that is important in early development for the formation of the anterior neural tissue including the eye (Martinez-Morales *et al.*, 2001). Germline loss of *otx2* is lethal due to the loss of development of the anterior regions of the forebrain, midbrain and hindbrains (Matsuo *et al.*, 1995). In the eye, *otx2* is thought to be important for the activation of the early eye field transcription factors and can induce ectopic eyes in a cocktail with other EFTF's outside neural tissues (Zuber *et al.*, 2003). Loss of *Otx2* results in reduction of expression of *Six3* although in this chimeric study the presence of wild type cells is thought to rescue *Six3* expression thereby leading to the proposal for a non-cell autonomous role for *Otx2* in regulating expression of *Six3* (Rhinn *et al.*, 1998, 1999). Deficiency of *Otx* genes in mouse did perturb retina formation, particularly the RPE formation, but the expression levels of *Pax6* and *Six3* were apparently normal (Martinez-Morales *et al.*, 2001). However the loss of *Six3* does not appear to adversely impact the expression of *Otx2* (Lagutin *et al.*, 2003). Later during development in the retinal progenitor cells, *Otx2*, along with *Mitf* specifies the retinal pigmented epithelium (Beby and Lamonerie, 2013). It is also involved in the formation of the photoreceptor layer along with *Otx5* (Nishida *et al.*, 2003). Like many of the transcription factors expressed in the retinal progenitors, *Otx2* also has temporal functions which differ based on the developmental time point (Beby and Lamonerie, 2013). Early neural plate expression of Xenopus *otx2* is not active in the retina but surrounds the prospective retinal region and it is thought to be repressed by the activity

of *hesx1* thereby allowing for the generation of the retinal field by *rax* (Ermakova *et al.*, 2007; Fish *et al.*, 2014).

Six3 or *Optx* is a member of the six family of homeodomain and six domain containing transcription factors that are expressed in the eye region. In mammals and frogs, *six3* and *six6* are expressed in overlapping regions in the eye and early anterior neural plate. Both factors are associated with human disease with loss of function or haploinsufficiency of *six3* leading to more severe disease manifestations. Mutations in *SIX3* are primarily associated with holoprosencephaly with some eye defects and mutations in *SIX6* are associated with anophthalmia and microphthalmia (Wallis *et al.*, 1999; Aldahmesh *et al.*, 2013). Overexpression of *six6* in *Xenopus* has been shown to generate giant eyes (Zuber *et al.*, 1999). Similarly, over-expression or mis-expression of *six3* also leads to the generation of giant and ectopic eyes in *Xenopus* and medaka fish respectively implying its role in regulating proliferation (Loosli, Winkler and Wittbrodt, 1999). Loss of function of *Six6* in mouse does not show any overt phenotypes with only mild pituitary and retinal defects being observed, milder than what has been observed in human patients (Li *et al.*, 2002). *Six3* plays a more major role in early development, at least in the mouse, as shown by the loss of *Six3* resulting in complete ablation of the anterior forebrain, eye and facial features (Lagutin *et al.*, 2003). A key factor suggested to be relevant to the functional role of *Six3* in patterning the anterior forebrain is its repressive activity of *Wnt1*, which is expanded anteriorly in the mutant (Lagutin *et al.*, 2003).

Subsequently, conditional knockouts in various tissues using the *Cre-lox* system have been generated which has further yielded evidence of the role of *six3* in patterning of the eye field (Liu *et al.*, 2006, 2010; Liu and Cvekl, 2017; Diacou *et al.*, 2018). Of interest

for the *six3* mutant phenotype in *Xenopus* is the conditional mouse knockout of *Six3* in the lens ectoderm using Le-Cre where it was shown that *Six3* was also crucial for lens formation and that *Six3* was required upstream of *Pax6* expression in the lens ectoderm (Liu *et al.*, 2006). Several other conditional mutants exist, resulting in depletion of *Six3* in regions of the retina and anterior forebrain (Liu *et al.*, 2010; Liu and Cvekl, 2017; Diacou *et al.*, 2018). Depletion of *Six3* by *Rax*-Cre results in truncation of neuroretina specification and that this process might be mediated by the expansion of *Wnt8b* and that *Six6* is downstream of *Six3* activity in the eye field based on the loss of *Six6* activity upon depletion of *Six3* (Lavado, Lagutin and Oliver, 2008; Liu *et al.*, 2010). However, two recent reports based on the conditional removal of *Six3* using *Six3*-Cre and α-Cre have reported that *Six6* is additive and complementary to *Six3* in retina formation and conditional removal of *Six3* and germline removal of *Six6* result in a more severe phenotype negatively affecting multipotency of retinal progenitor cells and resulting in the expansion of the ciliary margin zone into the peripheral regions of the retina (Liu and Cvekl, 2017; Diacou *et al.*, 2018). Although, the two papers suggest a potentially conflicting viewpoint about whether *Six3* is genetically linked to *Six6*, a possible explanation might be due to the early and broader loss of *Six3* in the in the Lavado *et al*., 2008 and Liu *et al*., 2010 paper results in failure to activate *Six6* expression in the eye while in the Liu *et al*., 2017 and Diacou *et al.*, 2018 papers *Six3* may be inactivated too late to prevent *Six6* expression or the loss of forebrain in the earlier mutants could account for the lack of expression of *Six6*. The studies mentioned above are data derived from loss of function studies which investigate the downstream regulatory mechanisms of *Six3*. However, a few studies have also suggested the presence of upstream regulators that drive the expression of *Six3* in medaka fish such

as *Sox2, Etv4/5,* Pax6 and *Tcf3* (Conte and Bovolenta, 2007; Beccari *et al.*, 2015). Taken together, the experimental evidence in mouse suggests that *Six3* functions to repress WNT expression in the anterior forebrain and eyes. It is also suggested that this gene plays a role in maintenance of multipotency of RPC's in conjunction with its closely related gene *Six6* (Lagutin *et al.*, 2003; Garcia *et al.*, 2011; Liu and Cvekl, 2017; Diacou *et al.*, 2018). However, our data presented here suggests that *Xenopus six3* and *six6* expression are independent and activity of *six3* affects layering of the retina and formation of the lens.

Other transcription factors such as *Vsx1* and *Foxn4* are also expressed in the retinal progenitors and these genes are involved in the specification of bipolar and horizontal cells. Among vertebrates *Vsx1* is shown to be more divergent than related but conserved *Vsx2* gene (D'Autilia *et al.*, 2006). Therefore, its expression pattern is different between *Xenopus* and other species (D'Autilla *et al.*, 2006). In *Xenopus*, *vsx1* is expressed early in the neural plate stage in the retinal progenitor cells with it being progressively restricted to retinal progenitors fated to have a bipolar fate (Ohtoshi *et al.*, 2004; D'Autilia *et al.*, 2006; Hellsten *et al.*, 2010). Although *vsx1* is expressed early in retinal progenitors, there might be temporal translational control regulating its activity (Decembrini *et al.*, 2006). *Foxn4* has similar expression pattern to *Vsx1* in the early retinal progenitor cells (Kelly, Nekkalapudi and El-Hodiri, 2007). However, it is thought to be involved in the differentiation of retinal progenitors fated to be amacrine and horizontal cells (Xiang and Li, 2013). In *Xenopus*, its expression in the mature embryonic retina is restricted to the ciliary margin zone which contains retinal stem cells capable of regenerating the amphibian retina (Kelly, Nekkalapudi and El-Hodiri, 2007).

There are other homeobox genes (*otx5*, *barhl2* and others) and bHLH genes (*neurod1*, *neurod4*, *ascl2* and others) that I am not covering in this chapter, but they do regulate both proliferation of the retinal progenitors and effect terminal differentiation of the retina.

Signaling pathways (some of which are mentioned in Chapter II in relation to lens formation) are also important for the development of the retina. The Delta/Notch intercellular pathway is involved in maintenance of the retinal progenitor proliferation upon release activate neurogenic bHLH factors that allows for differentiation of RPC's (Perron and Harris, 2000). In *Xenopus* morpholino based loss of function of *fzd5* which is expressed in the RPC reduces expression of *sox2* and subsequent impact on the Delta/Notch pathway by modulating the expression of *dll1* (Van Raay *et al.*, 2005). BMP and FGF signaling are important for neurogenesis and inductive signals from chick lens are thought to coordinate retina development (Agathocleous and Harris, 2009; Pandit *et al.*, 2015). WNT signaling plays an important role depending on developmental timepoint being both inhibitory as determined by the transgenic overexpression of *Wnt8b* in the mouse retina (Liu *et al.*, 2010) and permissive as shown by the morpholino inactivation of *fzd5* in *Xenopus* (Van Raay *et al.*, 2005). The involvement of these and other signaling and transcription factors result in the generation of complex neuronal circuitry from a common multipotent pool of progenitor cells in a conserved manner. The functions and role of these pathways in this process are only now being pieced together to make a regulatory network that drives the generation of the mature retina with a focus on defining the previously poorly characterized role of *six3* in retina differentiation.

The determination of retinal progenitor cells is thought to be mediated by both intrinsic and/or extrinsic events (Cepko, 2014). Much of this work was driven by lineage tracing and live imaging studies wherein the RPC's were thought to be a heterogenous mixture of cells (Cepko, 2014). Intrinsic factors are thought to be primarily driven by the expression of transcription factors, the homeodomain and bHLH domain containing families (Bassett and Wallace, 2012; Cepko, 2014). Although, the RPC are heterogenous they are limited by factors that prime them for certain fates indicating that intrinsic factors that might be differentially distributed and also influenced by extrinsic signals provide the roadmap for the RPC to differentiate (Trimarchi, Stadler and Cepko, 2008; Cepko, 2014; Lu *et al.*, 2019). This exemplifies a potential roadmap for the *six3* mutant would be a single cell RNA-seq analysis comparing the wild type with the *six3* mutant over the course of retinal development.

Results

Early eye field transcription factors are largely unaffected in the *six3* mutant

As described in the introductory section of this chapter, specification of the retina occurs at an early stage immediately after gastrulation and prior to the neural plate stage of the development (Saha & Grainger, 1992, Louie, Fisher and Grainger, unpublished). As described above the anterior edge of the neural plate is marked by the expression of several transcription factors that are collectively called the eye field transcription factors. These include *pax6, rax, six3, six6, lhx2* and *otx2* which are thought to be sufficient to generate ectopic eyes (Zuber *et al.*, 2003). Although *pax6* is often thought of as a master regulator of the eye, loss of *pax6* in mouse and *Xenopus* do produce a rudimentary deformed eye or form optic vesicles respectively (Hogan, Horsburgh and Cohen, 1986; Nakayama *et al.*, 2015). The Grainger lab and others have shown that *rax* may play a more fundamental role in defining the region of the neural plate that will make the retina as evidenced by the eyeless phenotypes of the zebrafish, mouse and *Xenopus* mutants (Mathers *et al.*, 1997; Loosli *et al.*, 2003; Fish *et al.*, 2014). To understand the role of *six3* in a proposed gene hierarchy, we note, as shown in Chapter II, that the mutants are not eyeless, although the mouse mutants have a stronger phenotype: they are headless and therefore have no eye structures (Lagutin *et al.*, 2003). The moderate phenotype in the *Xenopus* mutant could be attributed to the additive expression of *six6* unlike what is observed in the mouse or other factors that are not tested in this study (Fig. 2-3A). Expression of the eye field transcription factors such as *pax6*, *six3*, six6, *lhx2* and *otx2* are normal at the neural plate stage in the retinal progenitors (Fig. 3-2). Expression of *rax* does show some modest reduction during this stage (Fig. 3-2). This indicates that early establishment of the retina is largely occurring

normally in the *six3* mutant with respect to the expression of the above-mentioned eye field transcription factors. This also establishes *six3* to be largely independent of the activity of these eye field transcription factors. Looking at effects of these transcription factors on *six3* expression is also not changed at the neural plate stage in the *Xenopus pax6* mutants (data not shown, unpublished data). However, *six3* expression although present is reduced in later stages of development in the *Xenopus rax* mutant (Fish *et al.*, 2014). This however it likely due to the fact that the retina is itself greatly reduced in size. Taken together this suggests that during the neural plate stage *six3* expression is largely independent and early eye field specification is not changed.

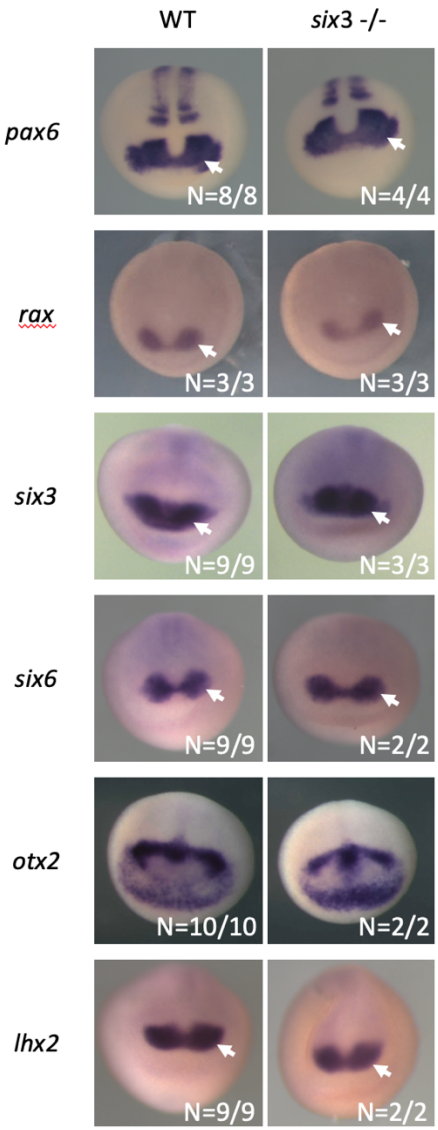

Figure. 3-2 Expression of early eye field transcription factors are largely unaffected in the *six3* mutant. Left panels show mutant expression in WT embryos; right panel: *six3* mutant embryos. White arrows indicate location of the retina. N = number of embryo showing normal expression out of total / number of WT or mutant embryos scored.

Retina formation is impacted due to the loss of secondary retinal progenitor factors

As mentioned in the previous paragraph, early specification of the retina is not strongly affected in the *six3* mutant. However, formation of the retina is still severely perturbed in the *six3* mutant. Therefore, we assayed via *in situ* hybridization other key factors expressed in the retina because of their role downstream of other eye field transcription factors such *rax* and *pax6* (Fish *et al.*, 2014; Nakayama *et al.*, 2015). However, less is known about these targets in the *pax6* mutant at present. Expression of *vsx1* was significantly and specifically reduced in the retinal progenitors at the neural plate stage (Fig. 3-3). Although the role of *vsx1* at this early stage remains poorly understood and there is evidence to suggest that there might be translational control over its activity at these early stages (Decembrini *et al.*, 2006), and its expression is important for the generation of bipolar cells (Ohtoshi *et al.*, 2004). The expression of *vsx1* is broad in the progenitors and becomes progressively restricted to the bipolar cells (D'Autilia *et al.*, 2006). Similarly, expression of *foxn4* is also lost in the *six3* mutant (Fig. 3-3). Predifferentiation activity of *foxn4* involves the regulation of Notch signaling pathway and therefore might play a role in initiating differentiation of the retina (Luo *et al.*, 2012). The loss of notch activity, if being mediated by *Foxn4* activity is thought to be vital for the retinal differentiation and in the generation of the amacrine and horizontal cells in the mature retina (Nelson *et al.*, 2006; Luo *et al.*, 2012).

The *mab21* family of genes, of which two are expressed in the eye field (*mab21l1* and *mab21l2*) show similar but different expression patterns in the mouse and *Xenopus*. Expression of *mab21l1* is stronger in the lens in mouse whereas expression of *mab21l2* is stronger in the retina (Yamada *et al.*, 2003, 2004). Wildtype expression of both *mab21l1*

and *mab21l2* at the early neural plate in *Xenopus* is observed in both the retina and the PLE (Fig 3-3). Expression of *mab21l1* is not changed at this stage in the *six3* mutant (Fig 3-3). However, expression of *mab21l2* is not activated specifically in the retina of the *six3* mutant (Fig 3-3). It is also important to note the result from Chapter II where loss of both *six3* and CRISPR *six6* results in reduced activation of *mab21l2* at stage 18 suggesting that this pathway might be mediated by both *six3* and *six6* in an complementary and additive manner as indicated by the complete ablation of eye formation in the double mutant (Fig. 2-3A, B).

The *mab21* family of genes were first identified in *C. elegans* where mutations in the gene caused defective fusion of the sensory rays in the posterior of the male worm (Chow, Hall and Emmons, 1995). They are known to interact and regulate activity of BMP signaling (Morita, Chow and Ueno, 1999). *mab21l2* is shown to interact with *smad1* to transduce BMP signaling (Baldessari *et al.*, 2004). Expression of another member of the BMP signaling pathway *tbx3* is also downregulated in the *six3* mutant (Fig. 3-3). Early post gastrulation activity of *tbx3* is thought to be important for the establishment of the eye field along with *pax6* by inhibiting BMP4 activity (Motahari *et al.*, 2016). However, later expression of *Tbx3* in the optic vesicle is thought to come under the regulation of BMP4 but is not affected by its loss indicating that low levels of BMP4 might be sufficient to regulate *Tbx3* or that there might be other factors that might also regulate its expression (Behesti, Holt and Sowden, 2006). Studies in heart and limb development has shown that BMP substrate *Smads* regulate *Tbx3* expression (Yang *et al.*, 2006). In summary, our data here leads us to hypothesize that the loss of *vsx1*, *foxn4*, *mab21* and *tbx3* in the *six3* mutant may be important in eliciting retinal defects seen in the *six3* mutant.

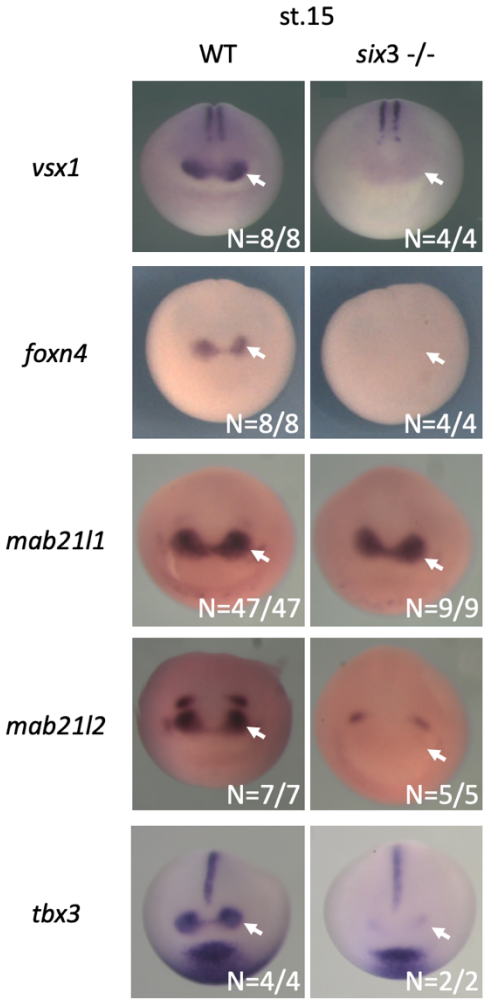

Figure. 3-3 Secondary transcription factors expressed in the retinal progenitors are variably reduced in the *six3* mutant. Expression of *vsx1, foxn4* and *tbx3* fail to be activated in the *six3* mutant. Expression of *mab21* family are also impacted with *mab21l1* showing normal expression pattern but *mab21l2* is initially not activated in the retina. White arrows indicate location of the retina. N = number of embryo showing normal expression out of total / number of WT or mutant embryos scored.

Optic vesicle stage expression patterns show reduction in eye size and loss of separation of the two retinal regions

Early pattern of expression of eye genes show variable expression. However, by the optic vesicle stage expression domains of many of these genes are either fail to be activated, normal or have their expression domain reduced. Expression domains of *pax6*, *rax*, *six6*, *lhx2* are reduced in the optic vesicle stage of the *six3* mutant (Fig. 3-4A) indicative of failure to proliferate, a morphogenetic defect or a result of cell death (these three observations are yet to be tested in the *Xenopus six3* mutant). The two retinal domains are closer to the midline suggesting that although some separation had occurred in the *six3* mutant, it is reduced perhaps mediated by the *six3* role in regulating *shh* activity and reduction of the development of the anterior brain regions (Chiang *et al.*, 1996; Jeong *et al.*, 2008; Nakayama *et al.*, 2013). Expression of factors that are not activated earlier at the neural plate stage continue to be inactive such as *vsx1*, *foxn4*, *tbx3* (Fig 3-4B). However, expression levels of the *mab21* genes – *mab21l1* and *mab21l2* are interchanged in the *six3* mutant with dramatic reduction in expression of *mab21l1* and expression of *mab21l2* being activated (Fig. 3-4B). The reasons for this change are unknown at this point and previous studies have suggested that given the high levels of homology between *mab21l1* and *mab21l2* protein sequence that they might function redundantly (Huang *et al.*, 2016). However, human mutations in *mab21l1* result in cerebellar, ocular, craniofacial and genital (COFG) syndrome suggesting that they might not be fully redundant in humans (Rad *et al.*, 2019) and some of the phenotype observed in the *six3* mutant could be due to the reduced levels of one or more of the other genes that fail to activate. Expression of *dll1*, a ligand in the Delta/Notch pathway shows a specific and dramatic failure to be expressed in

the eye field at this stage (Fig. 3-4B). As discussed earlier previous studies have shown that the Delta/Notch pathway is important in maintenance of retinal progenitors (Ahmad, Dooley and Polk, 1997) and that loss of Notch signaling resulted in increased generation of retinal ganglion and other neural cells (Austin *et al.*, 1995; Perron and Harris, 2000). Our preliminary data suggests that this might not be the result of the loss of Delta/Notch components in the *six3* mutant as we see a potential failure to differentiate and not premature differentiation. This might be attributable to the loss of other factors in addition to the loss of the Delta/Notch pathway alone. However, a conclusive analysis using terminal retinal markers would have to be done to confirm this finding.

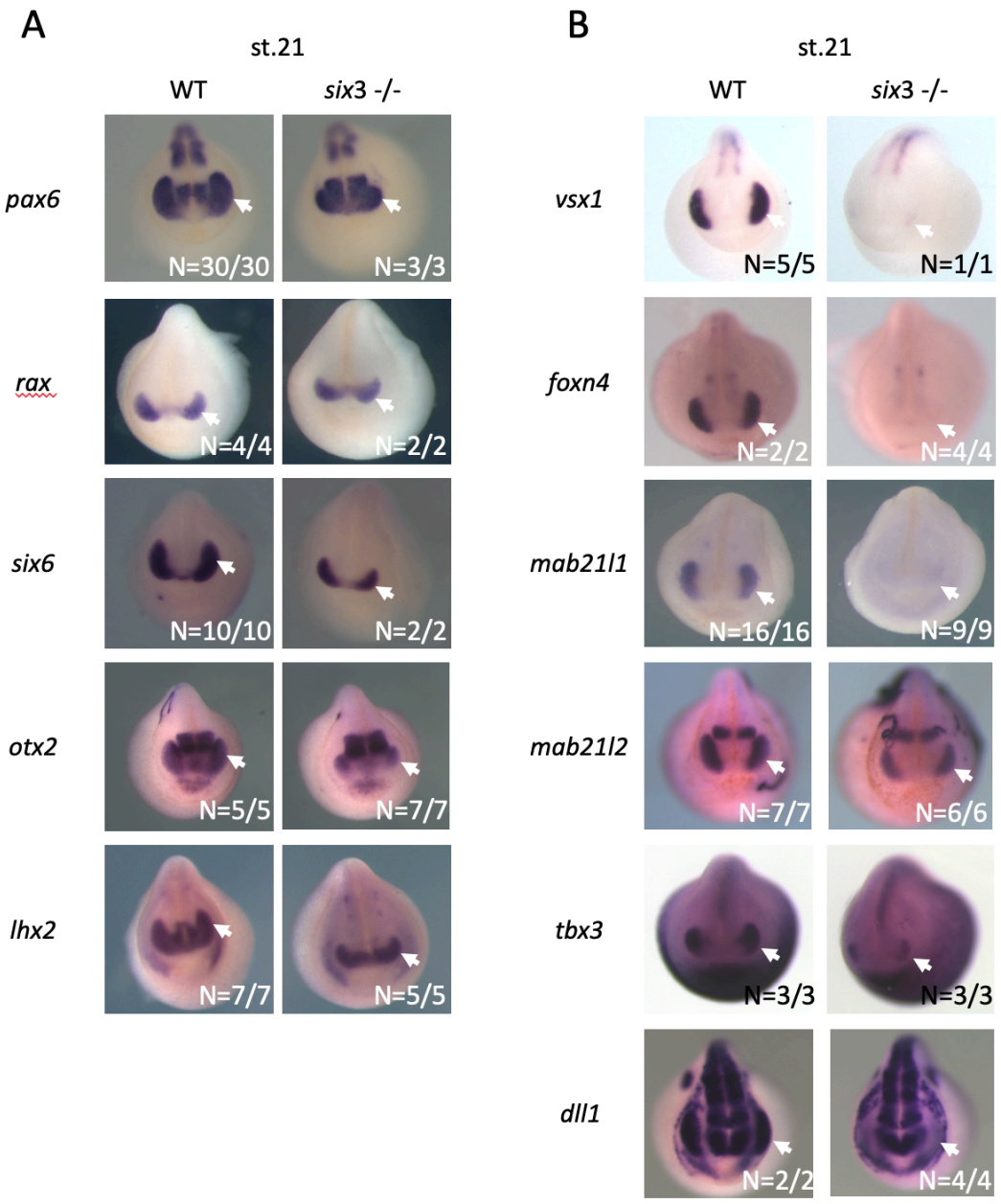

Figure. 3-4 Effect of loss of *six3* becomes apparent during the optic vesicle stage; expression patterns suggest reduction in separation of the retinal fields. A. Expression of *pax6*, *rax*, *six6* and *lhx2* all have a reduced expression domain. Expression of *otx2* shows specific overall reduction in the retina. B. Expression of *vsx1*, *foxn4* and *tbx3* show continued reduction in the retina. Expression levels of *mab21l1* and *mab21l1* switch from the neural plate stage with expression of *mab21l1* severely reduced in the retina. White

arrows indicate location of the retina. N = number of embryo showing normal expression out of total / number of WT or mutant embryos scored.

BMP, Notch and WNT signaling are perturbed in the *six3* mutant

Perturbation of the signaling networks is evidenced by failure to express genes such as *dll1* and *tbx3*. Perturbation of the BMP signaling network is shown by failure to express *bmp4* and *smad7*. In addition we notice the moderate expansion of BMP antagonist *nog2* (Fig. 5A) into the eye field, which may further reduce BMP activity in the eyes, and we also note a potentially lower level of *nog2* in the brain region, which may be then implicated in the reduced brain phenotype seen in the *six3* mutant (McMahon *et al.*, 1998). Studies in mouse have shown that *Bmp4* expression is necessary for both lens and retina formation (Furuta and Hogan, 1998; Huang *et al.*, 2015). Conditional loss of BMP receptors in the murine retina reveals abnormal retina formation affecting both retinal progenitor survival and loss of retinal neurogenesis as evidenced by the loss of retinal ganglion cell markers – *Atoh7* and *Brn3b* (Murali *et al.*, 2005). *smad7*, which is an inhibitory *smad* that acts as a substrate for BMP4 activity is activated at a low level in the *six3* mutant optic vesicle (Fig. 3-5A). This is indicative that BMP signaling networks are negatively affected due to the loss of *six3*. Further analysis will have to be performed to verify the functional relevance of impinging of BMP signaling in the retina by assaying P-smad activity for functionality of the BMP pathway in the retina and lens of the *six3* mutant (Massagué, Seoane and Wotton, 2005).

Perturbation of Notch signaling pathway is shown by failure to activate *dll1*, *dlc* and *notch1* expression at the optic vesicle stage (Fig. 3-5B). The Notch signaling pathway and its downstream targets – bHLH factors such as *Hes1*, *Hes4* and *Hes5* are known to regulate maintenance of the retinal progenitor cells (Austin *et al.*, 1995; Ahmad, Dooley and Polk, 1997; Perron and Harris, 2000; El Yakoubi *et al.*, 2012). Loss of function of *Hes1*

results in a similar effect as observed with the constitutively active form of Notch which results in the retinal progenitors maintaining a neuroepithelial phenotype (Ahmad, Dooley and Polk, 1997; Perron and Harris, 2000). Further, Delta/Notch also regulates specification of the lens by mediating the activation of *foxe3* in the lens ectoderm in *Xenopus* further indicating that cross talk between the retina and lens is important for their formation (Ogino, Fisher and Grainger, 2008). The loss of Delta/Notch would have to be further investigated to be more conclusive with *in situ* hybridization analysis of *Hes* family and other downstream effectors of the Notch signaling pathway.

Expression of *vsx1*, *foxn4* and *mab21l1* which were not active during the neural plate and/or optic vesicle stages respectively are now detectable in the retina of the *six3* mutant embryo at st.24/25 (Fig. 3-6). The developmental timepoint is relevant since it is during this stage that the onset of neurogenesis in *Xenopus* (Holt *et al.*, 1988). Investigations in mouse has suggested that reduction in Notch signaling drives differentiation and retinal ganglion cells are thought to be the first born during retinal neurogenesis (Nelson *et al.*, 2006). Expression of these genes become active at a stage when their dependence on notch signaling is not required. At this point of time, we have not assayed for downstream targets of Notch activity nor assayed for the effect of impinging Notch activity in the *six3* mutant.

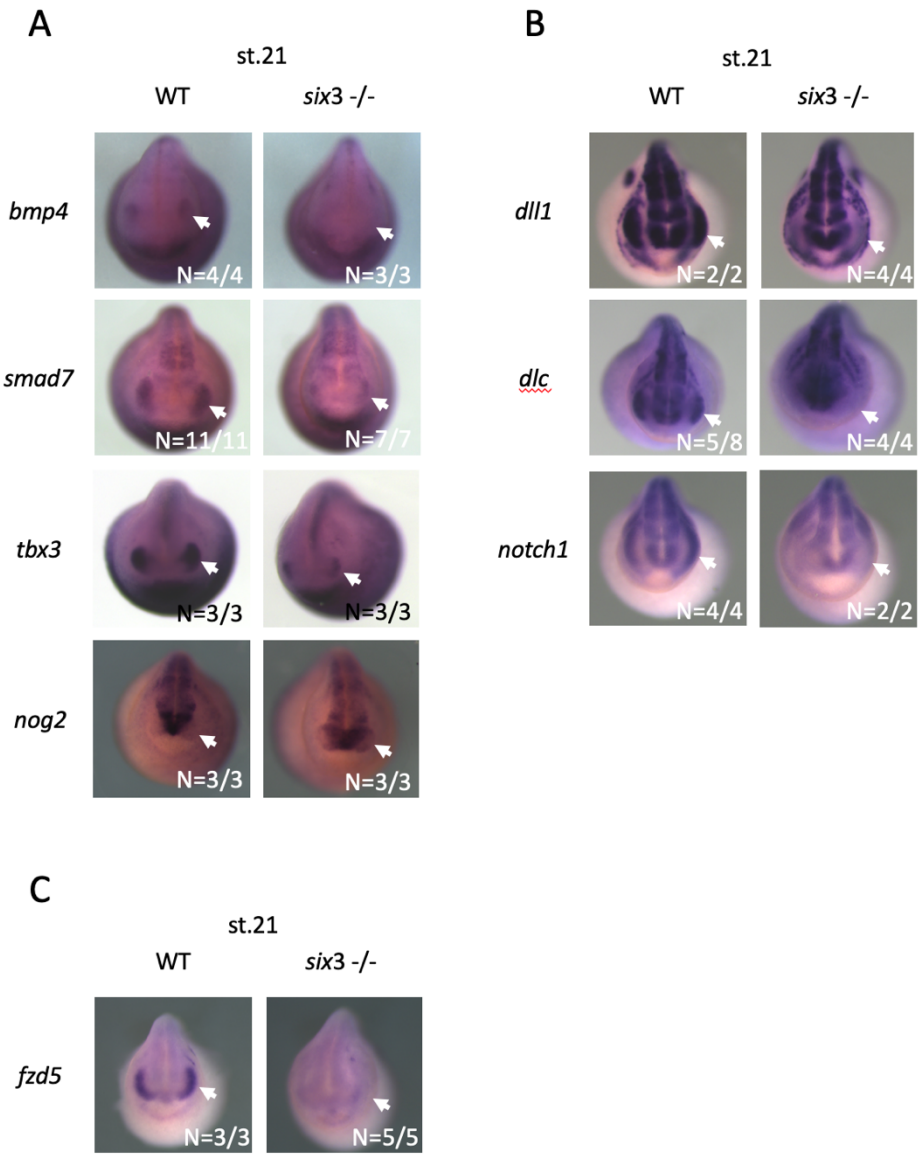

Figure. 3-5 Major signaling networks are perturbed in the *six3* mutant. A. Components of the BMP signaling network – *bmp4*, *smad7* and *tbx3* are not activated or in the case of *tbx3* not fully activated in the *six3* mutant (top 3 panels, white arrow). BMP antagonist – *noggin* (*nog2*) is expanded into the retinal field suggesting an ectopic inhibitory action in the retina of BMP activity (bottom panels, white arrow). B. Components of the Delta/Notch pathway are also perturbed as indicated by the failure of activation of *dll1*, *dlc* and *notch1* in the retina. C. Canonical Wnt signals are also thought to be perturbed as shown by the loss of

fzd5 receptor expression in the retina (compare white arrows). White arrows indicate location of the retina. N = number of embryo showing normal expression out of total / number of WT or mutant embryos scored.

Expansion of WNT signaling in mouse has been indicated to be an important early function of *Six3* in the mouse in the regulation of the anterior neural plate. Loss of *Six3* results in anterior expansion of *Wnt1* and *Wnt8b* and this expansion is thought to inhibit anterior forebrain and eye formation in the respective *six3* mouse mutants (Lagutin *et al.*, 2003; Liu *et al.*, 2010). Ectopic activation of WNT signaling in the murine eye field results in phenocopying the *six3* mutant retinal phenotype (Liu *et al.*, 2010). As described previously Wnt signaling pathway mechanisms can act in both as an inhibitory or permissive fashion during the formation of the eye (Agathocleous and Harris, 2009). In *Xenopus*, literature suggests a role of Wnt signaling in retinal proliferation as evidenced by the expression of Wnt receptor *fzd5* is observed in the optic vesicle region after the neural plate stage (Van Raay *et al.*, 2005). Its expression is broadly located in the retinal progenitors and becomes limited to the ciliary margin cells upon formation of the mature embryonic retina (Van Raay *et al.*, 2005). Loss of function of *fzd5* in *Xenopus* results in loss of *sox2*, *dll1*, *notch1* and reduced expression of retinal ganglion cell markers *atoh7* and *brn3d* (Van Raay *et al.*, 2005). However, this phenotype does not seem to be conserved in mouse. Although, *fzd5* germline mutants are embryonic lethal and they do not survive past optic vesicle stage and show a reduction in expression of *Mab21l1* and *Foxe3* (Burns *et al.*, 2008). However, conditional knockouts in the retina are apparently normal suggesting a different mechanism or redundancy might be involved in the mouse retina compared to the frog (Burns *et al.*, 2008). Based on the importance of Wnt signaling and previous evidence of the importance of *fzd5* in eye formation (Van Raay *et al.*, 2005), we decided to investigate this signaling network further in the *six3* mutant. We see a significant reduction in *fzd5* expression in the *six3* mutant (Fig. 3-5C). To test we generated a *Xenopus*

CRISPR mutant targeting *fzd5* coding region. CRISPR injected embryos were raised until stage 42. The embryos developed normally, and injected embryos were apparently normal up until stage 42 (Fig. 3-7A). We assayed for functional generation of the mutation by sequencing the locus which showed multiple peaks at the target region indicating detectable levels (Fig. 3-7B) of the mutation (Nakayama *et al.*, 2014). However, the percentage of cells that were mutant and the type of mutation was not established in these experiments. The mosaic loss of function mutants in *fzd5* did not show change in expression of either *mab21l1* or *dll1* expression (Fig. 3-7C). Further, we also generated and injected *fzd5* mRNA into *six3* mutant embryos. The phenotypes of the injected embryos were not different than that of the *six3* mutant (data not shown). We assayed for downstream targets – *dll1* and observed no change in the injected sides of the embryos (Fig. 3-7D). However, we did notice reduction in size of the *dll1* expression region in the injected side of wildtype embryos indicating a potential artifact of injection and that more refinement of the amount of mRNA being injected may need to be performed before deriving a conclusive result. As described earlier, Wnt signaling plays a role in retina formation and further analysis of Wnt signaling pathway effectors in the *six3* mutant has to be performed to be able to determine its role more conclusively.

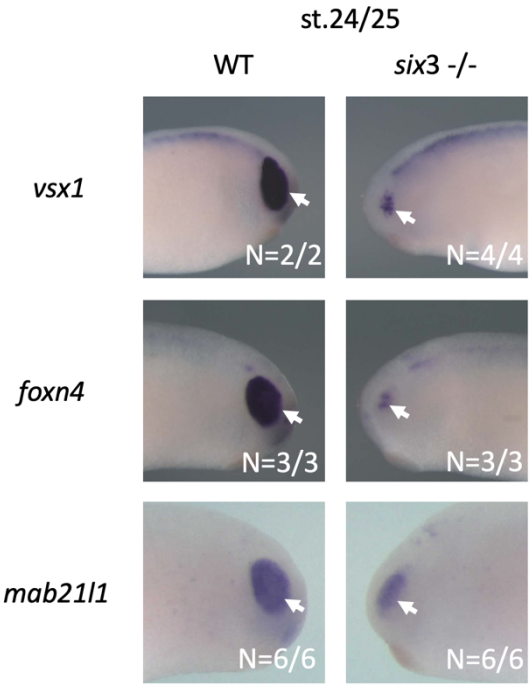

Figure. 3-6 Onset of retinal differentiation results in activation of early targets under regulatory control by *six3*. Expression of *vsx1* and *foxn4* are activated; expression of *mab21l1* is reactivated compared to earlier developmental stages suggesting that regulatory control by *six3* is temporally limited. White arrows indicate location of the retina. N = number of embryo showing normal expression out of total / number of WT or mutant embryos scored.

Differentiated embryonic retina in the *six3* mutant shows severely disrupted patterning with loss of typical retinal layers

In the *six3* mutant we observed that morphologically that the layering of the retina appeared to be perturbed in the mutant embryos (Fig. 2-2C). Here we have initiated studies to determine the basis for this loss of organization. So far, we have investigated the early activity of *six3* in the RPC's and how they might affect differentiation. Here we show the loss of retinal patterning and the possible mechanism by which this is effected in the *six3* mutant. In the mature embryonic retina at stage 41/42, broadly expressed factors in the RPC and other neurogenic factors become restricted to specific differentiated neurons. *vsx1*, whose expression we previously identified as being downstream of other retinal determinants, *rax* and *pax6*, is downregulated during the neural plate stage is re-activated by the start of retinal differentiation (Fig. 3-6). However, in the wild type mature embryonic retina, its expression is limited to the bipolar cells. However, in the *six3* mutant, its expression is seen broadly throughout the

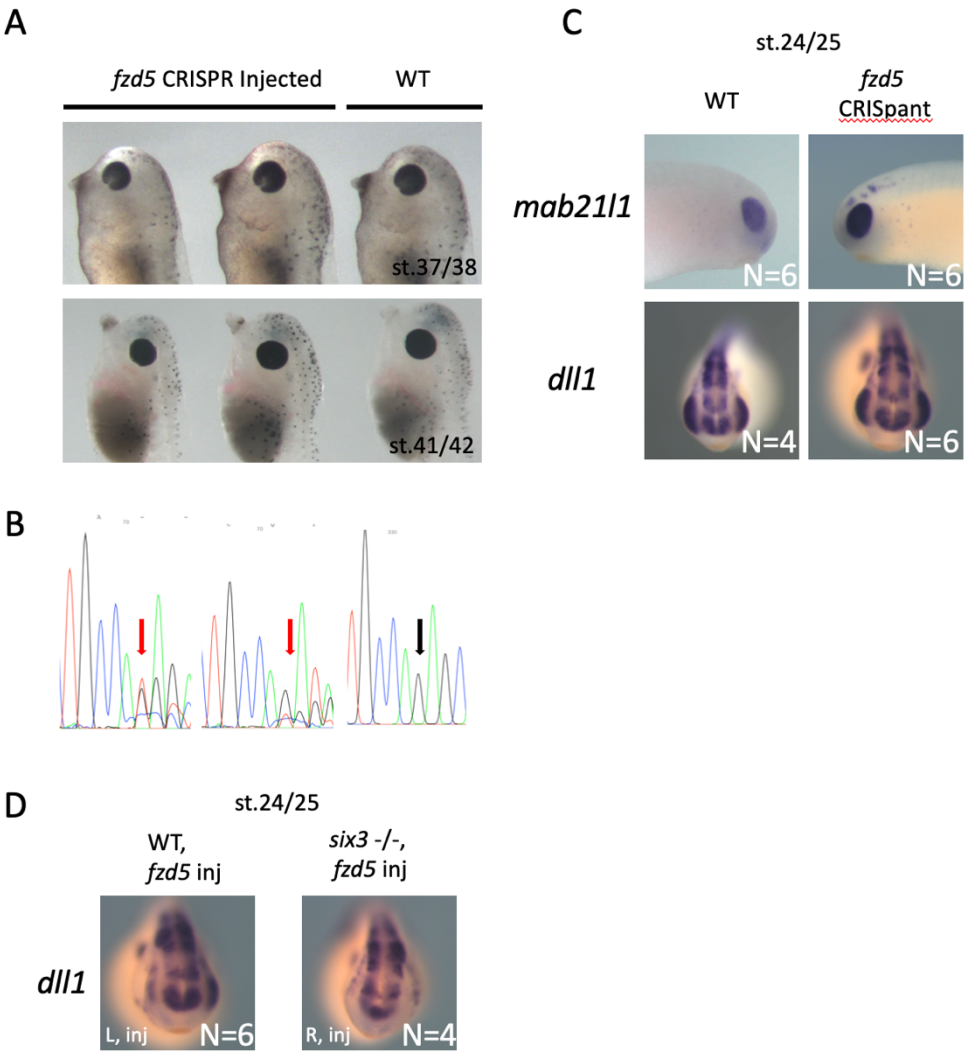

Figure. 3-7 Loss of *fzd5* does not show any apparent phenotype; no rescue of *six3* phenotype is observed. A. Whole mount embryo images of CRISPR F0 tadpoles targeting *fzd5* show no apparent phenotype (2 injected embryos placed left of the WT un-injected sibling embryo). B. Sequencing trace of the embryos in A shows that the injected embryos have characteristic multiple peak near the PAM region (red arrows) indicating successful cleavage of the DNA compared to the single peak of the WT un-injected sibling (black arrow). C. WISH analysis of *fzd5* CRISPR injected embryos show no apparent change in expression patterns of *mab21l1* and *dll1*. (Fig. 7D) Rescue of six3 mutant embryos did not show any apparent phenotype (data not shown) and expression of *dll1* is not changed in

the *fzd5* mRNA injected *six3* mutant (right panel) compared to the *six3* mutant. The injected sides (L, left, R, right) are indicated in the bottom left of the images. However, *dll1* expression reduced on the injected side of WT embryos (left panel). N = number of embryos assessed by *in situ* hybridization.

retina without any clear layering (Fig. 3-8A). Similarly, expression of *pax6*, which is seen in the amacrine and ganglion cell layer is similarly disrupted in the *six3* mutant (Fig. 3-8A). Although, there might be some patterning that might be visible in some cases, it is n general not conclusively clear and as shown in Fig. 2-2C there is a range of phenotypes and ones with a weaker phenotype may show a modest amount of layering. Similarly, we see disruption of *brn3b* expression pattern which is normally activated in the ganglion cell layer (Fig. 3-8A) (Badea *et al.*, 2009). We also examined expression of *otx5* which in the wildtype embryos is expressed in the photoreceptor layer and is shown to control the regulation of gene expression in the photoreceptor cells (Hennig, Peng and Chen, 2008). In the *six3* mutant, the expression of the layering seems to be intact although we see a broader expression of *otx5* in other parts of the retina (Fig. 3-8A). The patterning defect observed here could be mediated due to the perturbation of the Delta/Notch pathway in the *six3* mutant. Expression of the *Notch1* receptor is necessary to inhibit the premature formation of the photoreceptor cells in murine retina (Jadhav, Mason and Cepko, 2006). Although, at this point of time we have a hypothesis, we have not tested it. A test for this hypothesis would be to assay for expression of *opsin* to determine if it is expanded outside the photoreceptor layer. The broader expression and partially recovered photoreceptor layering as evidenced by the expression of *otx5* suggests that loss of Notch signaling could push the retinal program towards premature differentiation and/or predominant photoreceptor fates which can be tested further by injection of a temporally controlled constitutively active notch receptor.

As part of our analysis of the role of *six3* in lens formation described in Chapter II, we identified *mab21l1* as a potential candidate to mediate *six3* activity in both the retina

and the lens. We show here that injection of 500ng *mab21l1* mRNA into the dual dorsal blastomeres at 4 or 8 cell stage of *six3* mutant embryos resulted in the partial rescue of retinal layering as evidenced by the expression pattern of *vsx1* in the injected embryo. Similar, but to a lesser extent, we see similar layering rescue in the expression of *pax6* in the injected embryo. However, this result presented here is preliminary since we need further cases and more genes have to be analyzed to determine the overall effectiveness of the rescue. Further, we have evidence presented in Chapter II that *mab21l1* mRNA injection rescues the lens as indicated by the increased fraction of cases expressing *crgy1* and higher levels of expression when compared to untreated *six3* mutants.

Our data, although not complete at this point of time leads us to hypothesize that *six3* may mediate retinal patterning early via the control of at least three key signaling pathways, BMP, Notch and WNT signaling pathway. To summarize our data, we see failure to activate homeobox transcription factors at the neural plate stage like *vsx1* and *foxn4* and altered regulation of nuclear factors such as *mab21l1* and *mab21l2*. Subsequently, during the neural tube or optic vesicle stage expression of *bmp4*, *dll1*, *dlc*, *notch1* are also not activated in the eye field of the *six3* mutant. We also show (from Chapter II) that BMP signals play an important role in mediating the effect of *six3* on lens formation. BMP signals could also potentially regulate retina formation. We show recovery of *smad7* and *cryg1* (Chapter II) by *mab21l1* injection and discuss its involvement in modulating BMP signals both in the retina and the lens. However, our data at present does not show any functional change of the BMP signaling network in the mutant or in the rescue experiments. Future directions would involve investigating the P-smad activity by immunostaining in the *six3* mutant retina and lens in addition to attempting to perform the

rescue of *six3* mutant by injecting constitutively active *smad5* mRNA in the event that we confirm that BMP activity is reduced in the *six3* mutant. Additionally, we also see clear perturbation of the Delta/Notch signaling pathway which could affect formation of both the retina and the lens as described in previously in this chapter and in chapter II.

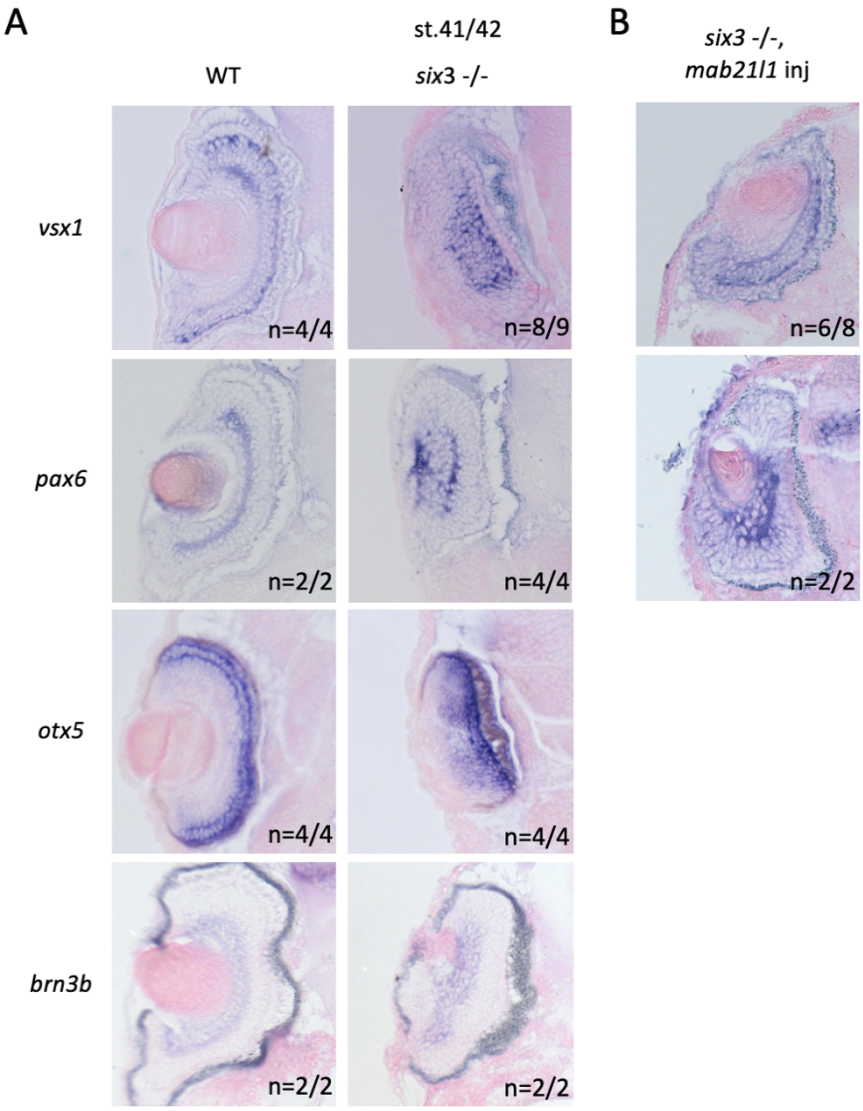

Figure. 3-8 Sections of mature embryonic retina after *in situ* hybridization shows loss of retinal patterning in the *six3* mutant and partial rescue by *mab21l1* injection in the *six3* mutant. A. Expression of bipolar (*vsx1*), amacrine/ganglion (*pax6*) and ganglion cell (*brn3b*) markers show disorganization and expansion to regions outside of their normal expression pattern. Expression of photoreceptor (*otx5*) marker shows some retinal patterning but also shows expanded expression domain in the six3 mutant. B. Rescue of the retinal phenotype is observed in sections of embryos for *vsx1* and *pax6*. 10µm Paraffin sections; n = number of eyes.

Discussion and future directions

The *Xenopus* mutant demonstrates novel roles of *six3* in the formation of the retina. Although previous studies in Drosophila suggested a prominent role in retina formation, studies in mouse have only suggested a role in neuroretina specification and maintenance of the RPC, its role in mediating patterning has not been clear. Here, the *Xenopus* mutant has started to provide initial evidence to determine its role in retinal patterning.

Compared to the mouse mutant (Lagutin *et al.*, 2003) the *Xenopus* mutant shows a milder phenotype. We show that this phenotype is partially related to the redundant action of *six6* (Fig. 2-3). Other factors are likely to be involved in the utilizing similar genes but in a different regulatory context between the mouse and frog. Our preliminary evidence suggests that this might at least in part be related to the lack of anterior expansion of *wnt1* and *wnt8b* in the *Xenopus* mutant (data not shown). Loss of function in the mouse results in anterior expansion of WNT signaling thereby posteriorizing the anterior forebrain (Liu et al, 2008). Part of continuing this study would involve further investigation of the role of *six3* in anterior forebrain formation. Since the retina is bordered by the telencephalon and diencephalon, it is possible that mal regulation in these regions due to loss of *six3* might play a role in the formation of the retina. For example, the Grainger lab has demonstrated that activity of *Xenopus rax* establishes the retinal boundary and that loss of *rax* results in the expansion of the telencephalic and diencephalic regions into the eye field (Fish *et al.*, 2014).

A future direction that our lab could consider is to analyze in detail the downstream targets of *six3*, *pax6* and *rax* mutants to determine the independence, cross talk and differential targets of all three transcription factors during retina development since all of

these have been made in the Grainger lab and in *Xenopus* are readily amenable to studies during key early stages leading eye formation. The work that we have started already shows common targets and pathways but also other independent mechanism that might regulate each of these three genes in regulating retina formation.

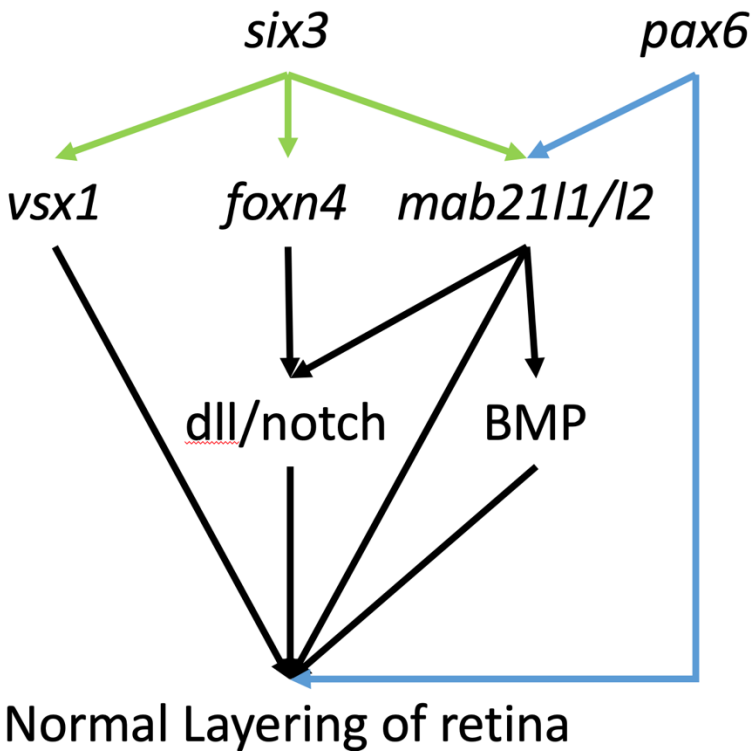

Figure. 3-9 Proposed model for the role of *six3* in regulating retina formation. Green lines - Indicate data that is generated from this study. Black and blue lines - Indicates data generated from previous studies - (Ahmad, Dooley and Polk, 1997; Luo *et al.*, 2012; Klimova and Kozmik, 2014; Huang *et al.*, 2015). Lines with arrows do not imply direct relationship.

Our data also shows that early retina specification is largely not affected in the *six3* mutant with the relatively normal expression of the eye field transcription factors. However, there is change in expression of secondary factors which are important for retina formation. Both *vsx1* and *foxn4* are downregulated in the *six3* mutant. *Vsx1* is thought to be transcriptionally regulated at this early stage in *Xenopus* (Decembrini *et al.*, 2006). Further, its roles in *Xenopus* and mouse might be interchanged since *vsx1* and not *vsx2* is more important for bipolar fates (Wang and Harris, 2005). Currently, we have not assayed for change in expression of *vsx2* by *in situ* hybridization

In general, it is clear a more systematic approach is required here, specifically a strong case for RNA-seq analysis to determine in a high throughput fashion differentially expressed genes in the developing retina in wildtype and *six3* mutants, as well as in the *rax* and *pax6* mutants available in the Grainger lab.

Foxn4 is another factor that is important for amacrine and horizontal cell differentiation (Xiang and Li, 2013) that may be important in the retinal patterning process under study here. In mouse, *Foxn4* activates the Notch signaling pathway during neurogenesis suppressing photoreceptor fates (Luo *et al.*, 2012). Although I have putatively identified a *Xenopus* homolog of the mouse *dll4* through which *foxn4* is thought to mediate this activity, we have not studied its activity or expression in *Xenopus*. Preliminary CRISPR knockout of *foxn4*, however shows no apparent phenotype and potential for *foxn2* and *foxn3* redundancies meant that this direction may require multiple knockouts in addition to more complete study of the *foxn4* mutants, verifying the efficiency of CRISPR mutagenesis as well for a clear interpretation.

The data presented here also focuses on the roles of *mab21* family of genes in retina formation. Our rescue experiments suggest that *mab21l1* (or *mab21l2*, given the high similarity in amino acid sequences, (Huang *et al.*, 2016)) acts intermediary to mediate the action of *six3* on the patterning of the retina. The mechanisms by which this action is produced in the retina is suggestive of being mediated by BMP signaling pathway but it is still unclear and functional assays are currently underway to investigate the role of BMP signaling by assaying for the levels of P-Smad in the retina and lens of the *six3* mutant. Additional assay is through the use of constitutively active form of *smad5* to rescue the *six3* mutant retina and lens phenotype.

As described previously both BMP and Delta/Notch signaling networks have previously been shown to regulate retina formation (Austin *et al.*, 1995; Ahmad, Dooley and Polk, 1997; Murali *et al.*, 2005; Huang *et al.*, 2015; Pandit *et al.*, 2015). Conditional loss of *Bmp4* in the retina ablates retina and lens formation (Huang *et al.*, 2015) and expression of *Bmp4* in cultured mouse RPC led them to differentiate into neural cells (Du, Xiao and Yip, 2010). Notch activity is required to maintain the generation of progenitor cells and its loss leads to premature differentiation and increased photoreceptor cell production (Austin et al, 1995, Jadhav et al, 2006). However, our results indicate that there might not be any premature differentiation, a possible explanation for this could be that the loss of BMP and other signaling mechanisms that are required for differentiation are also perturbed in the *six3* mutant. Therefore, a possible future exploration of this would be the analysis of downstream effectors of BMP signaling such as the *Id* family of transcription factors in the *six3* mutant (Du, Xiao and Yip, 2010).

Co-expression of *pax6* and *Notch* in *Xenopus* results in the formation of ectopic eyes and injection of activated Notch resulted in generation of duplication of the eyes and activation of the eye program (Onuma *et al.*, 2002). Our data in *Xenopus* suggests *six3* regulates Notch components and correlates with other data that suggests *Six3* mediation of Notch signaling is important for the maintenance of retinal progenitor cells (Diacou *et al.*, 2018). The mouse conditional double knockout provides a template to determine the role of *six3* in retinal patterning. Given that the conditional knockout results in depletion of *Six3* only in the peripheral regions of the retina, their observation concluded that *Six3* inhibits the domain of the ciliary margin and loss of its activity along with loss of *Six6* results in an expansion of the ciliary margin zone at the expense of the neuroretina. Their observation of decrease in expression of *Notch1* and *Otx2* (Diacou *et al.*, 2018) is similarly observed in the *Xenopus six3* mutant (our data). Another example, an upstream regulator of Notch signaling – *foxn4* is also lost in the mouse (Diacou *et al.*, 2018) and *Xenopus six3* mutant. An interesting observation of our study was the activation of transcripts of *vsx1*, *foxn4*, *mab21l1*, *mab21l2* which were all not activated at earlier stages prior to the onset of retinal expression of *dll1* suggesting that they are either upstream or independent of Delta/Notch activity. Studies in mouse has suggested that *Foxn4* could be an activator of *Dll4/Notch* signaling (Luo *et al.*, 2012) which we have not assessed in the *six3* mutant at present. A future direction, therefore, would be to assay for the expression of *hes1* and *hes4*, substrates for notch signaling which has not been investigated in the *six3* mutant.

We have however tried rescue of *six3* phenotype by injecting *dll1* mRNA. However, the results were inconclusive with no apparent rescue of the retina or the lens and in some cases did result in the loss of eye from the injected side of the embryo even in

a wild type background. It would be difficult to control the amount of *dll1* due to the nature of ligand-receptor binding and their regulation in neighboring cells. Therefore, as mentioned in the previous paragraph we have to investigate downstream effectors of Notch signaling which are mediated by the *Hes* family of transcription factors (Hatakeyama and Kageyama, 2004; El Yakoubi *et al.*, 2012). The genetic ablation of *Hes1* leads to formation of small eyes with deformed retinas and in extreme cases do not form any retina (Tomita *et al.*, 1996).Other than overexpressing components of Notch signaling, injection of constitutively active form of Notch (Onuma *et al.*, 2002) or potentially more valuable, an inducible version of *notch1*, which has been previously used in *Xenopus,* (Rones *et al.*, 2000) would provide a more regulated way of studying the role of notch signaling in the *six3* mutant background.

Taken together our data presents a picture where *six3* is at the top of the hierarchy regulating Notch, BMP and Wnt signaling networks in the retina (Fig. 3-9). We propose that *six3* is in an independent albeit parallel pathway along with *pax6*. Activity of *mab21l1* mediated by *smad7* could play a key role in mediating both retina and lens formation. We investigated functional relevance of *mab21l1* and determined that it could partially rescue the retina in the *six3* mutant. Activities of other targets such as *foxn4*, which is reduced in the *six3* mutant, could also play a role in mediating the role of *six3* in retina and lens formation. The disruption of the *six3* pathway leads to perturbation of retinal patterning perhaps retaining a progenitor state even in the older stage 41/42 mature embryonic retina (Fig. 3-8B). To determine if the older mutant retinal cells are in an undifferentiated state, we could assay for overlapping expression of early genes such as vsx1, foxn4 and pax6 to determine if they overlap in the mature retina by using new multiplex *in situ* hybridization

techniques (Choi *et al.*, 2018). An additional test would be to investigate if there is any change in the expression pattern of terminal differentiation markers for the retina to see if retinal cells, though disorganized based on early retinal patterning genes, are nonetheless differentiated on schedule, even if disorganized, or if the differentiation program is impacted in the program due to failure of early patterning genes to segregate appropriately. This data would help guide us in tying the nature of the defects here, and their rescue by *mab21l1*, into the existing frameworks (e.g. (Cepko, 2014)) for how retinal patterning occurs, adding in the new information about the role of *six3* in this process from the insights from our new observations about patterning in our mutant. Future work would continue the development of a robust *six3* gene regulatory network which would combine with similar GRN's being developed in the Grainger lab for other mutants that are important for the formation of the eye field.

Materials and Methods

Animal Handling and tissue collection and mRNA, CRISPR injections

Xenopus tropicalis were housed and cared for based on the guidelines set forth by the University of Virginia Animal Care and Use Committee. The *six3* mutant line was generated from CRISPR generated mutations (Nakayama *et al.*, 2013). F1 lines containing a 19bp deletion were raised by outcrossing with wild type frogs. Embryos were collected from either natural mating or in-vitro fertilization. Embryos for *in situ* hybridization were fixed in MEMFA (MOPS, EGTA, $MgSO_4$, formaldehyde - ('CSH Protocols', 2008)), gradually dehydrated and stored in -20˚C. Capped mRNA's for *mab21l1* was prepared from linearized plasmid using a following protocol developed by the manufacturer (ThermoFisher, AM 1344). 500pg of mRNA was injected into both dorsal blastomeres at 4 or 8 cell stage. CRISPR injections were based on protocol developed by (Nakayama *et al.*, 2013). An example of the injection protocol for mRNA and CRISPR is described in Appendix II.

In situ hybridization, sectioning and imaging

The expression pattern for downstream targets of *six3* were determined by *in situ* hybridization assays for probes listed in Table 1. The protocol for *in situ* hybridization was adopted from (Harland, 1991) and modified by the Grainger lab to eliminate acetic anhydride treatment and the removal of the post fix step which interfere with genotyping assays. Embryos were embedded in paraffin and 10μm microtome sections prepared and stained with hematoxylin and eosin. An example of the protocol used is described in Appendix II.

For fluorescence and bright field imaging post *in situ* hybridization, we used a Zeiss SteREO Discovery V12 microscope and the images were captured on AxioVision software version 4.8.2. using the same settings for collecting images to be compared in a given experiment. Images were in some cases modified in Adobe Photoshop to adjust for contrast and brightness, again with settings applied equally to all sets of sections being compared.

Gene Name	Species	Lab Clone Number	Vector	Source
dll1	*X. laevis*	1485	pCS2+	Chitnis et al, 1995
dlc	*X. tropicalis*	1654		Openbiosystems # 7603863
notch1	*X. laevis*	1479	pCS2+	Chitnis et al, 1995
vsx1	*X. tropicalis*	1675	pCS108	Openbiosystems # 7614953
foxn4	*X. tropicalis*	1774		Openbiosystems # 7626378
bmp4	*X. tropicalis*	772	pSP64T	H. Brivanlou
smad7	*X. laevis*	1098	pCS2+	Nakayama Cristian 1999
nog2	*X. tropicalis*	1186	pCS107	Sanger Center
mab21l1	*X. tropicalis*	1137	pCS107	Sanger Center
mab21l2	*X. tropicalis*	1138	pCS107	Sanger Center
otx2	*X. laevis*	1250	pCS2+	Blitz & Cho, 1995
six6	*X. tropicalis*	1664		Openbiosystems # 7623919
six3	*X. laevis*	1469	pCS2+	Gestri et al, 2005
pax6	*X. tropicalis*	1569	pSP64T	Openbiosystems # 6992220
rax	*X. tropicalis*	1571		Openbiosystems # 9019330
lhx2	*X. tropicalis*	1698	pSP64T	Openbiosystems # 7657571
fzd5	*X. laevis*	1665		
brn3b	*X. laevis*	1113	pBSIISK+	N. Hirsch
otx5	*X. tropicalis*	1602		Openbiosystems # 7029264

Table. 3-1 Probes used for *in situ* hybridization

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Chapter IV Dynamic change in chromatin accessibility during the lens commitment process

Introduction

Lens development is a good model for the study of determination, a process by which a tissue gets committed to a certain fate. Although, studies have shown the importance of several transcription factors and their role in lens formation, the networks that regulate the expression of these transcription factors are poorly elucidated. Several key genes have been identified as being important for lens formation such as *pax6*, *six3*, *sox2*, *sox3*, *foxe3*, *mab21l1*/*l2* amongst others. A low throughput way determining downstream targets of a gene of interest would be via generating mutants, morpholino or si-RNA inhibition and performing *in situ* hybridization assays or immunohistochemical staining. These techniques are biased, slow, tedious and importantly do not identify the upstream regulators of the genes of interest. High throughput techniques like RNA-seq improve upon the low throughput techniques by providing faster, less biased datasets but are still limited to the observation of downstream targets of a certain mutant or transcription factor being immunoprecipitated. In order to identify upstream regulators of these key transcription factors, we have to investigate the regulatory regions of these key transcription factors of interest to identify upstream factors relevant for their activation supplementing the downstream studies using mutants or RNA-seq analysis of these mutants. Insulators, promoters and enhancers are three key regulatory regions on the genome which play an important role in mediating the expression of a specific target gene. They control its expression in a spatiotemporal manner by providing the necessary local environment and in the case of promoters are necessary to provide transcriptional machinery to the transcription start site. Identifying the activity of these regulatory regions during the course of commitment will help to determine the spatiotemporal activity of their target genes.

These regions contain the DNA binding sites for multiple regulatory proteins. In order for these regions to be able to accommodate the binding of these regulatory proteins, they need to have an open confirmation to provide accessibility to the DNA binding proteins. The open or closedness of these regions determine whether these regions are sites of active regulation or are inactive. Several high throughput techniques such as ChIP-seq, DNAse-seq, ATAC-seq and more recently chromosomal structure capture techniques have provided significant tools to advance the study of regulation of the commitment process.

Enhancers can be located either upstream, downstream or within intronic spaces of the genes they control (Calo and Wysocka, 2013). In addition to being located within introns of their target genes, they can also be located in the intronic regions of neighboring genes (Harmston and Lenhard, 2013). In many cases these *cis*-regulatory elements or enhancers are located in an extremely wide range of 10 to a 100 Kb (Calo and Wysocka, 2013). Occasionally, they can also be found to be 1000 Kb or more away from the genes they regulate (Calo and Wysocka, 2013). A large number of enhancers have been identified in human and mouse cell lines with good annotation providing for the location of the enhancers and the genes under their control (Calo and Wysocka, 2013; Nord *et al.*, 2013). Similarly, promoters are typically located upstream of the transcription start site (TSS) and multiple promoters can be present to regulate expression of the target genes in same or different tissues. For example, *pax6* is expressed in multiple tissues during early development and therefore its expression is highly regulated by multiple enhancers and promoters (Kleinjan *et al.*, 2006). Likewise, *hox* genes are also highly regulated given their crucial role in axis formation in early development (Montavon and Duboule, 2013). Identification of transcription factor binding sites in the regulatory regions has been done

by localized ChIP and/or ChIP-seq analysis. Several databases such CIS-BP, TRANSFAC and JASPAR exist which provide computational and literature-based databases of transcription factors and their respective DNA binding sites. However, several individual studies have also identified transcription factor binding sites which are functionally verified by *in vitro* and *in vivo* techniques (Suzuki-Yagawa, Kawakami and Nagano, 1992; Epstein *et al.*, 1994; Jeong *et al.*, 2008; Suh *et al.*, 2010)

Our knowledge of the role of enhancers has dramatically improved over the years in terms of the number of enhancer elements identified, however very little has been established about the spatio-temporal activity of enhancers (Calo and Wysocka, 2013). Characterizing this is a crucial step towards understanding the progression of a tissue from an un-determined to a determined state (Nord *et al.*, 2013). In terms of establishing cell fates, activity of enhancers is one of the factors that play an important role since the tissues contain the same genome, but gene expression is regulated in a precise differential fashion (Herz, Hu and Shilatifard, 2014). Deletions in enhancer sites with no modification in respective coding sequences has been linked to human diseases (Spitz, 2016).

Sequence conservation in enhancers has been linked to conservation of transcription factor binding sites. Enhancer sites of developmentally crucial genes tend to be conserved amongst evolutionarily divergent species (Weirauch and Hughes, 2010). However recent evidence has moved towards increased recognition of non-conserved enhancers (Fisher *et al.*, 2006; Weirauch and Hughes, 2010; Chatterjee, Bourque and Lufkin, 2011). In some cases, a minority of the enhancers identified in a study was conserved while the majority turned out to be non-conserved (Friedli *et al.*, 2010). The

aspect of enhancer sequence conservation has been crucial in its identification across different species. Removing the conservation bias present in traditional identification methods and identifying non-conserved enhancers has been a challenging process. Being non-conserved, traditional enhancer determination techniques, such as comparative genomics are not able to identify non-conserved enhancers. However, with the advent of high throughput sequencing techniques such as ATAC-seq, DNase-Seq and ChIP-seq, identification of non-conserved enhancers has improved significantly (Chatterjee, Bourque and Lufkin, 2011).

Xenopus tropicalis is a strong candidate to study the presence of non-conserved enhancers due to the presence of the closely related species – *Xenopus laevis*. These two species split from a common ancestor about 50 million years ago (Hellsten *et al.*, 2007). Their shared but diverged genomes provide an ideal environment to study "non-conserved broadly but conserved within *Xenopus"* enhancer sites in which transcription factor binding sites might be more closely related than with other vertebrate and mammalian species.

Transcription factors called pioneer factors are thought to be at the top of the hierarchy of transcriptional factors (Rieck and Wright, 2014). These factors might act independently or bring in other secondary factors that drive gene expression (Herz, Hu and Shilatifard, 2014). Since these regions need to be accessible to transcription factors, either the enhancer chromatin states should be able to accommodate the binding of these factors or the factors possess the ability to bind DNA when it is bound to histones. Previous work has shown that enhancer regions are nucleosome depleted when they are active and are

sensitive to DNase I treatment (Bulger and Groudine, 2011; Calo and Wysocka, 2013). In my work the question arises about what, if any, chromatin states precede the activity of enhancers of lens determination genes? If so, then what factors are responsible for generating these marks as well as to the priming of these enhancers for quick activation? It has been suggested that the presence of enhancer-specific chromatin marks (see details in next paragraph) as well as the accessibility of the region plays a role in the generation of cell specific memory for gene expression (Ong and Corces, 2011). We initially planned to assay for histone marks, but our preliminary assessment indicated that with the current technical limitations that would require large amount of tissue to be collected. In order to overcome this hurdle, we decided to assay for accessibility of chromatin regions using a relatively recent technique called ATAC-seq (assay for transposase accessible sequencing) (Buenrostro *et al.*, 2013; Bright and Veenstra, 2019). The technique provides us with very useful insights into the dynamic configuration of chromatin in the regulatory elements and their variability during development. Although the presence of accessible chromatin suggests that there might be enhancer, promoter or transcriptional activity, it could also indicate sites of insulator activity. Therefore, a combinatorial dataset generated from ATAC-seq, ChIP-seq and RNA-seq provides valuable datasets towards building a dynamic gene regulatory network (GRN). Furthermore, doing this in a tissue specific manner would allow us to identify regulatory sites that are highly enriched in that particular tissue rather than being masked in whole embryo analysis.

The chromatin landscape around enhancer sites are known to be diverse with specific signatures marking active, primed or poised enhancers (Calo and Wysocka, 2013). Embryonic stem cells have a more open chromatin and repressive factors are generally

linked to differentiation (Pekowska *et al.*, 2011). Enhancers have also been shown to express a similar pattern of differential histone marks based on their differentiation status (Smith and Shilatifard, 2014). Several histone modifications have been demonstrated to correlate to enhancer sites on the genome (Hon, Hawkins and Ren, 2009; Weirauch and Hughes, 2010). H3K4me1 and H3K4me2 are associated primarily with enhancer sites (Magnani, Eeckhoute and Lupien, 2011). The presence of H3K27ac has been shown to mark active (has both H3K4me1 and H3K27ac) as opposed to primed (H3K4me1) enhancers (Creyghton *et al.*, 2010). H3K4me3 is a mark indicative of active promoters (Calo and Wysocka, 2013). H3K27me3 is generally associated with repressed sites on the genome (Magnani, Eeckhoute and Lupien, 2011). Other factors such as EP300 have been shown to associate with tissue specific enhancers (Cotney *et al.*, 2012). Since EP300 is one of the HATs responsible for depositing the H3K27ac mark (Calo and Wysocka, 2013), it might duplicate the pattern established by H3K27ac. RNA Pol II enrichment is observed at promoter regions (Pekowska *et al.*, 2011). However, histone signatures such as H3K4me1 are often found to be larger than the core enhancer region making it difficult to identify the core of the enhancer region based on a single mark (Pekowska *et al.*, 2011). A combinatorial approach using H3K4me1/2/3, H3K27ac/me3 and EP300 can be used as markers to identify enhancers (Calo and Wysocka, 2013). Activating histone marks such, as H3K4me1 and H3K27ac are associated with either primed or active enhancers. Primed enhancers have been characterized to harbor H3K4me1 as well as potentially bound pioneer factors in comparison with poised enhancers that have only been characterized in human and mouse embryonic stem cells (Calo and Wysocka, 2013). Poised enhancers have more transcription factors bound in addition to being marked by both H3K4me1 and

H3K27me3 (Calo and Wysocka, 2013). Since enhancer elements can be identified and characterized as either active, primed or poised based on their specific histone modification, the timing of change in chromatin state can be utilized to identify potential pioneering factors.

In this chapter, we investigate and functionally analyze the sites of open chromatin for the presence of enhancer activity in the presumptive lens ectoderm. We have identified several putative novel regulatory sites for genes that are involved in the lens commitment process. Further, we have also identified several of these regions that are broadly non-conserved between *Xenopus tropicalis* and other vertebrates and mammals. However, we have identified that they do show conservation with closely related species the closely related species *Xenopus laevis* (Fig. 4-3). Percent identity plot (PIP) analysis shows that these regions are conserved in an identifiable way when compared to the *X. laevis* genome. Using this conservation, we have been able to identify several putative transcription factor binding sites that may provide very useful leverage for identifying factors involved in controlling key developmental events. Future goals for this project would involve functional analysis of these transcription factor binding sites that are located in these enhancer regions.

Results

Genome wide analysis of open chromatin regions in *Xenopus* reveal novel enhancers for key lens regulatory genes

ATAC-seq visualization reveals location of several novel enhancer sites that are located in the *Xenopus* genome by focusing on several key eye regulatory genes where we have been able to identify both several known and novel enhancer sites of interest. For example, Fig. 4-1 shows peaks around *pax6* through in stage 12 whole embryo and present in stages 11, 15, 18 and 21 of the PLE covering the key developmental timepoints during the lens commitment process (discussed in Chapter I). Open regions of chromatin are observed in locations of gene coding regions that are active in the tissues at that point of development. We can also identify peaks located in the region of proximal promoters of these genes. Further, we also observe several peaks that are spread out in the genomic region that is viewed in Fig.4-1. Although we have not performed a systematic analysis of the peaks, our anecdotal evidence indicates open chromatin profiles surrounding genes that are expressed in the lens region. In the example given in Fig. 4-1 known enhancers for *pax6* identified from previous literature suggests that many enhancers do not show any activity in the presumptive lens region. However, we have identified one conserved site HS2/3 (Kleinjan *et al.*, 2006) (Fig. 4-2) that is thought to regulate pax6 activity in the eye. Another conserved enhancer, SIMO – implicated in human aniridia (Bhatia *et al.*, 2013) does not show any open chromatin profile in our analysis even though it is linked to *pax6* expression in the eye – perhaps suggesting that it might play a role later in development. Surprisingly, during our analysis of the peaks surrounding lens genes we identified several novel non-conserved enhancers (called OCE – open chromatin elements) during our

screening process. These non-conserved enhancers are of interest since few have been identified in other tissues (Chatterjee, Bourque and Lufkin, 2011).

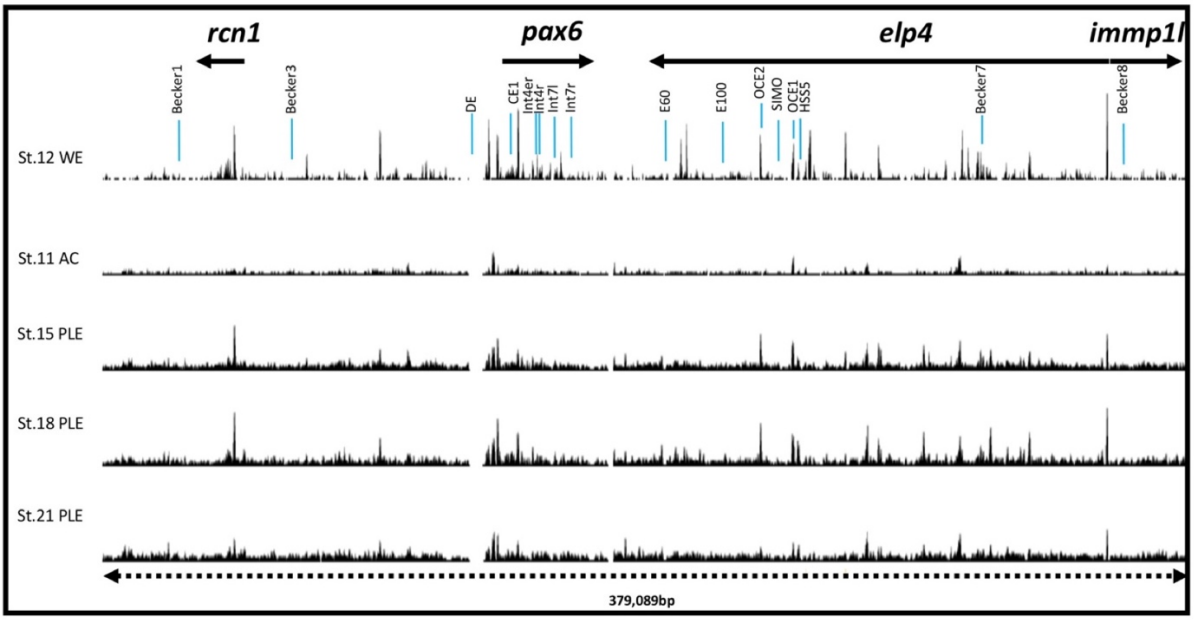

Figure. 4-1 ATAC-seq tracks around *pax6* genomic locus. Stage 12 whole embryo (WE), stage 11 animal cap (AC), stage 15, stage 18 and stage 21 PLE tracks for ATAC-seq shows peaks of open chromatin surrounding expression of *pax6*. Previously identified enhancers are shown in with blue lines indicating their position (see text for references). The arrows indicate direction of transcription and the gene names are indicated above them.

foxe3

Forkhead box or *foxe3* expression in the PLE is thought to be essential for lens specification and is activated by the coordinated action of *otx2* and Delta/Notch signals originating in the optic vesicle (Ogino, Fisher and Grainger, 2008). Along with influence from the Notch signaling pathway, it is also thought to be regulated by BMP signaling and our known data presented in Chapter II (Yoshimoto *et al.*, 2005). Its expression is observed in the pre-placodal ectoderm and becomes specific to the PLE by the time its specified. The Grainger lab has demonstrated that it plays an important role in lens specification (Ogino, Fisher and Grainger, 2008). The coordinated action that regulates *foxe3* expression is thought to occur through a conserved enhancer, named here as CE1 (conserved element 1). This enhancer is evolutionarily conserved between vertebrates and mammals. Our data shows the presence of peaks, indicating open configuration on this enhancer (Fig. 4-2). However, our data also shows peaks on another putative enhancer (OCE1, Fig. 4-2) downstream proximally of *foxe3*-CE1 but do not show any conservation with other mammals. Transgenic expression pattern of this enhancer – OCE1 element suggest that it is expressed in the eye and neural tissue. This expression pattern covers the endogenous expression pattern of *foxe3* which is observed in the lens. The neural expression would indicate exogenous expression possibly due to the transgene insertion region in the genome. OCE1 is also identifiably conserved in *X. laevis* (Fig. 4-3). The second enhancer that we identified, the OCE2 element has not been tested yet but does not show conservation with *X. laevis* (Fig. 4-3). By utilizing the conservation of sequence between *X. tropicalis* and *X. laevis* where regions of enhancer element show sufficient conservation to identify several conserved transcription factor binding sites. Of interest are the putative conserved sites for

otx, *sip1* and *smad* (Fig. 4-4) which have previously been demonstrated to regulate *foxe3* expression (Yoshimoto *et al.*, 2005; Ogino, Fisher and Grainger, 2008).

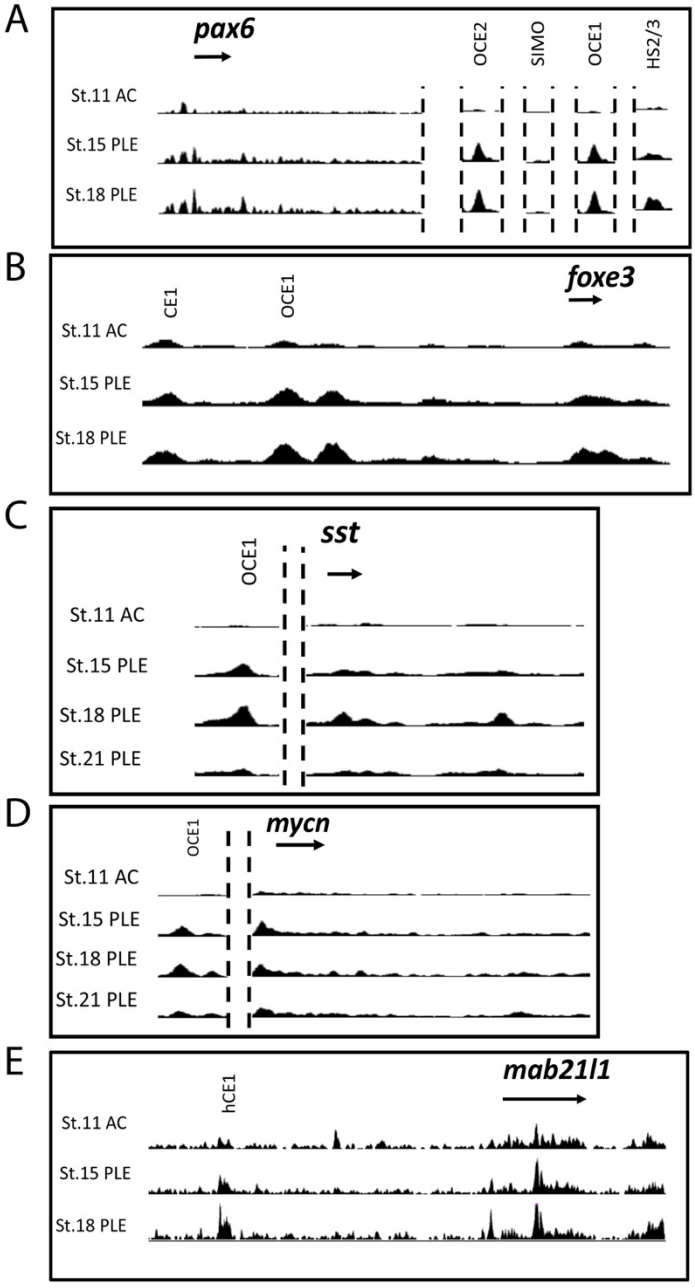

Figure. 4-2 Open chromatin regions of interest surrounding key eye genes. A. Two conserved and putative novel non-conserved long-range downstream enhancers identified for *pax6* – SIMO does not show an open chromatin configuration, HS2/3 and the non-conserved sites show open configuration increasing through the lens commitment process. B. Conserved (CE1) and putative novel non-conserved enhancer for lens specification

factor foxe3. C, D. Putative non-conserved enhancer sites observed for gene expressed in the lens with minimal understanding of their role in early lens commitment process *sst* and *mycn*. (Fig. 2E) Shows peaks around hCE1 (a conserved element) in the neighborhood of *mab21l1*.

pax6

Paired box 6 or *pax6* is a transcription factor that is described as the master regulator of eye development. Its expression in the presumptive lens ectoderm is necessary for lens formation. Early expression in the PLE distinguishes that groups of cells to form lens compared to others in the placodal region which also express any overlapping variety of transcription factors. It is expressed in regions outside of the eye and so plays a crucial role in early development. Due to its importance in formation of multiple tissues, the expression of *pax6* is controlled by several different enhancers and multiple promoters. Currently, literature evidence suggests that there are up to 11 or more different enhancers for *pax6* etc. regulating its expression in various tissues and different time points. Out of these conserved enhancers, the ectodermal enhancer (EE) (Dimanlig *et al.*, 2001), lens enhancer (LE) , HS2/3 (Kleinjan *et al.*, 2006) and SIMO (Bhatia *et al.*, 2013; Antosova *et al.*, 2016) have been shown to be important for lens and eye formation. However, the putative expression pattern of these elements being assayed (meaning their ATAC-seq pattern) might be relevant for later lens formation and do not account for early expression of *pax6* in the PLE. Mutations in these regulatory element regions have been implicated in a small number of aniridic patients with no coding region mutations implicating the importance of identification and characterization of these regulatory elements for human disease modeling (Plaisancié *et al.*, 2018). Out of the conserved enhancers identified in Fig. 4-1, only HS2/3 (Kleinjan *et al.*, 2006) element showed a peak indicating an open configuration in that region during early lens development. Surprisingly, SIMO did not show an open configuration (Fig. 4-2). Two new novel non conserved enhancers were identified OCE1 and OCE2 (Fig. 4-2). Preliminary transgenic analysis suggests that OCE1 is expressed in

the eye (data not shown) further analysis would have to be performed to show conclusive evidence. The *pax6*-OCE2 construct has not yet been tested. Phylogenetic footprint analysis of both *pax6*-OCE1 and OCE2 has revealed the presence of several candidate binding sites for transcription factors such as *meis* (Antosova *et al.*, 2016) and multiple *smad* binding sites (Fig. 4-5) (indicative of BMP signaling - (Wawersik *et al.*, 1999)).

mab21l1

mab21 like 1 or *mab21l1* and its closely related gene – *mab21l2* are homologs of *C.elegans mab21*. Both of these genes are expressed in the developing eye region. Their molecular function is largely unknown, though it is thought to function as a nucleotidyl transferases (De Oliveira Mann *et al.*, 2016). These paralogous genes are nested in the intronic regions of *lrba* (*mab21l1*) and *nbea* (*mab21l2*). Previous studies have shown that regulatory elements might be conserved between these two closely related genes and transgenic analysis of these conserved enhancers partially recapitulate gene expression of *mab21l1* and *mab21l2* (Tsang *et al.*, 2009). Loss of function of both genes results in malformation of the eye(Yamada *et al.*, 2003, 2004). We identified a peak ~11.5kb of transcription start site of *mab21l1*, labeled hCE1 (Fig. 4-2E). This region was highly conserved in *X. laevis* and upon closer analysis it was conserved in mouse and human genomes. We were able to identify this region shares homology with the human hs1333 element as described in Vista Enhancer Browser (Visel *et al.*, 2007). Transgene experiments in mice suggest that this particular enhancer shows strong expression in the brain and olfactory regions. Of the 11 embryos assayed, at least one embryo shows expression in the eye region (VISTA ENHANCER BROWSER – hs1333) (Visel *et al.*, 2007). Although, due to its presence in the intronic region further analysis would be required to determine its target gene.

Computational analysis indicates that *mab21l1* could be a potential target gene for this enhancer (Dimitrieva and Bucher, 2013). Our transgenic analysis shows strong expression in the eye and neural regions (Fig. 4-7B). Phylogenetic footprint analysis has also shown that this enhancer could contain potential transcription factor binding sites for *Pax6*, *Six3* amongst others which are expressed in the eye region (Fig. 4-6). Further analysis would have to be performed to characterize this enhancer.

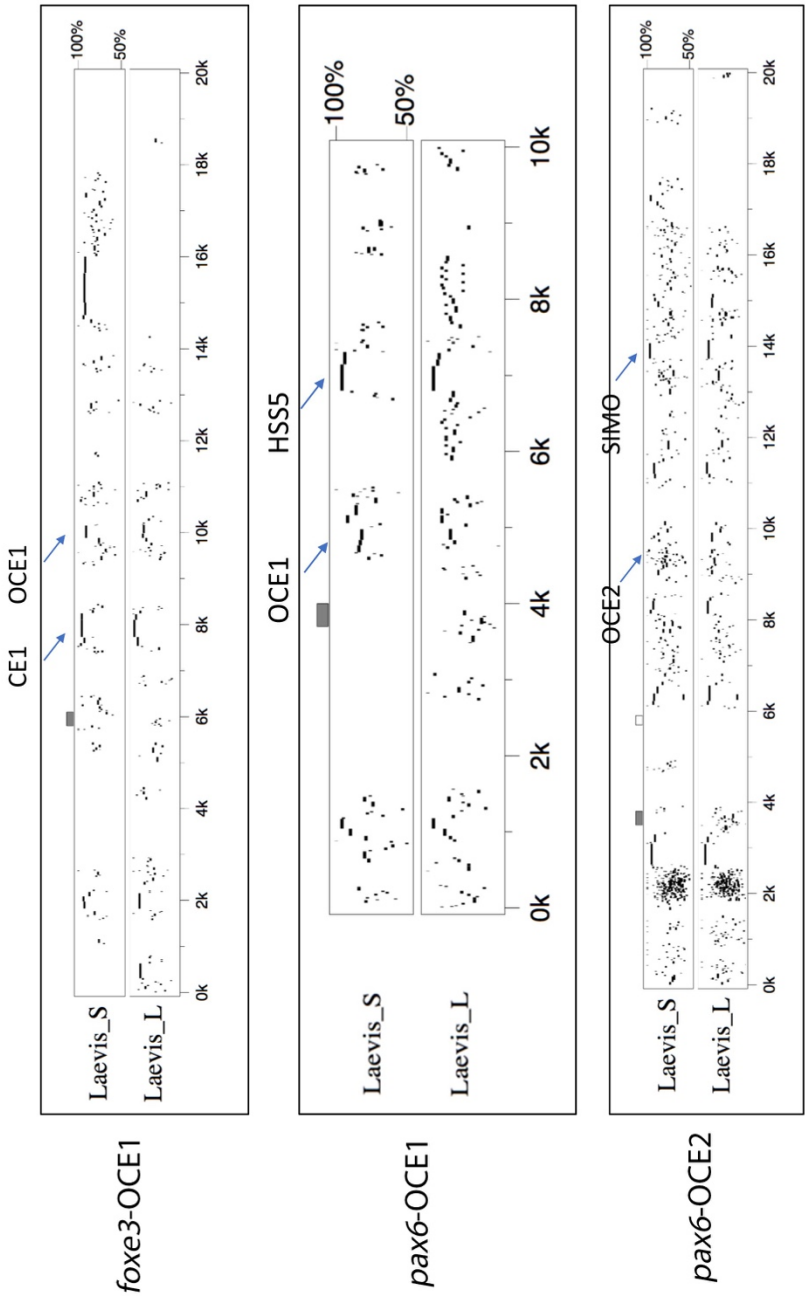

Figure. 4-3 Percent identity plots (PIP) of regions surrounding open chromatin elements foxe3-OCE1, *pax6*-OCE1 and *pax6*-OCE2 comparing *Xenopus laevis* and *Xenopus tropicalis.*

sst and *mycn*

The ATAC-seq data also allows us to identify novel genes that are involved in lens development. For example, somatostatin (*sst)* and *mycn* have been implicated in lens formation, their roles and expression patterns are unclear. *sst* is thought to regulate *pax6* expression from the mesoderm in the chick (Lleras-Forero *et al.*, 2013). However, the Grainger lab has identified its expression in the PLE in *Xenopus* (unpublished, data not shown). Likewise, *mycn* and *myc* are thought to be relevant for lens development later during differentiation (Cavalheiro *et al.*, 2017). However, their early activities in lens induction are unclear. The Grainger lab has shown that *myc* is downstream of *pax6* in PLE of *Xenopus* (Nakayama *et al.*, 2015). Open configuration in the coding region and potential regulatory elements in the genomic neighborhood suggests potential role for these genes in lens commitment autonomously (in the PLE) but requires further analysis to elucidates their roles in lens commitment (Fig. 4-2C, D).

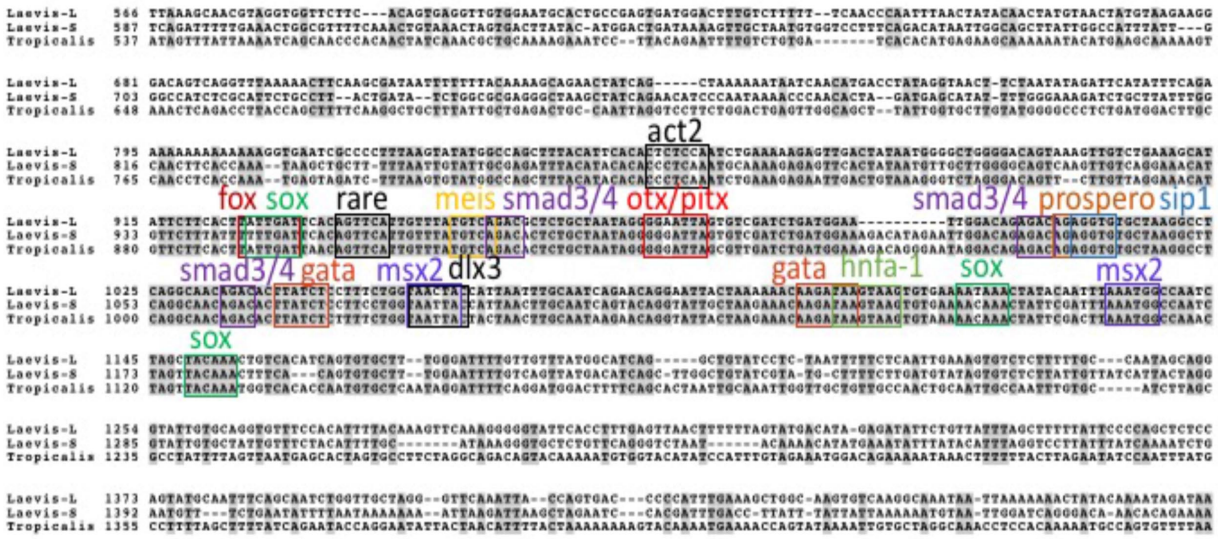

Figure. 4-4 ClustalW alignment of the region surrounding the *foxe3*-OCE1 putative open chromatin element region in *X. tropicalis* with *X.laevis S* and *L* genomes.

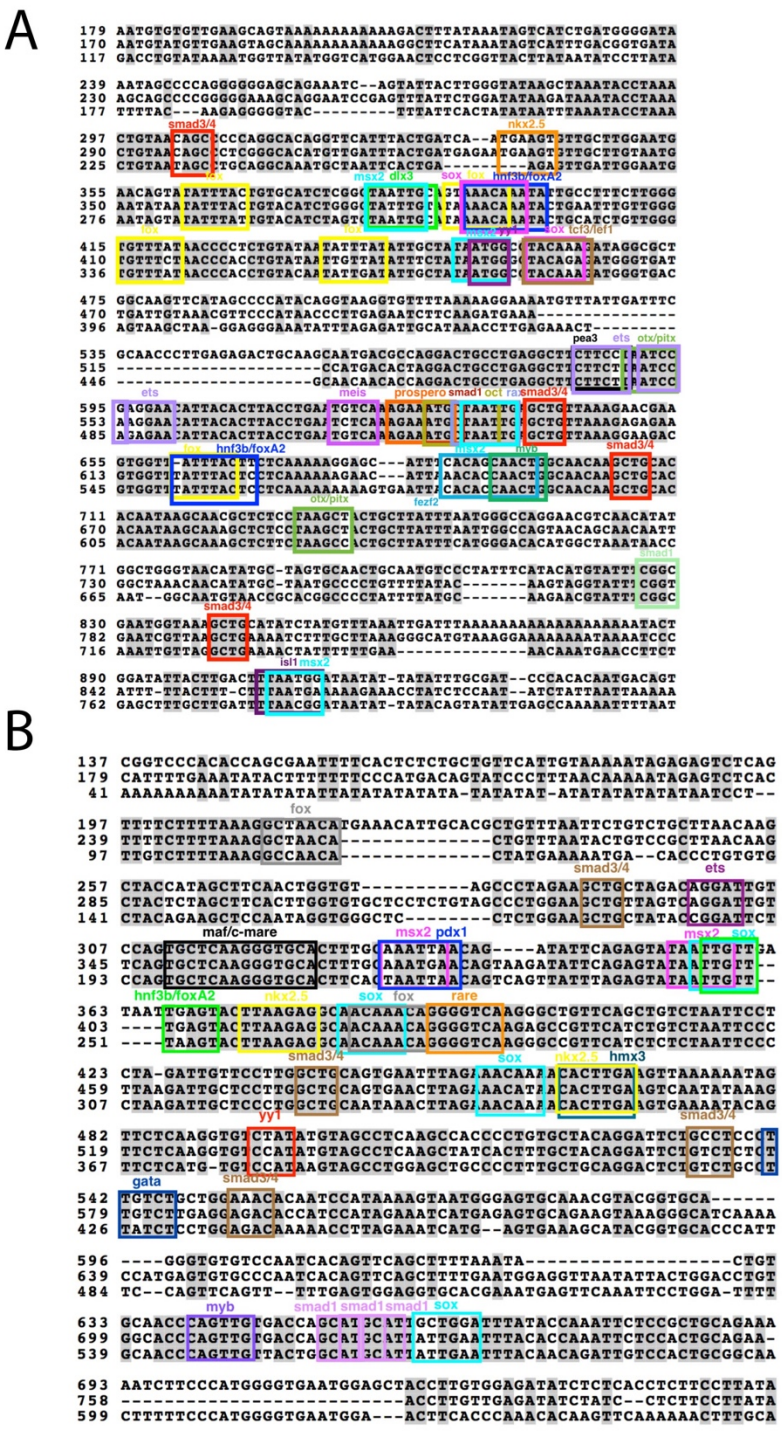

Figure. 4-5 ClustalW alignment of the region surrounding the A. *pax6*-OCE1 and B. *pax6*-OCE2 putative open chromatin element region in *X.tropicalis* with *X.laevis S* and *L* genomes.

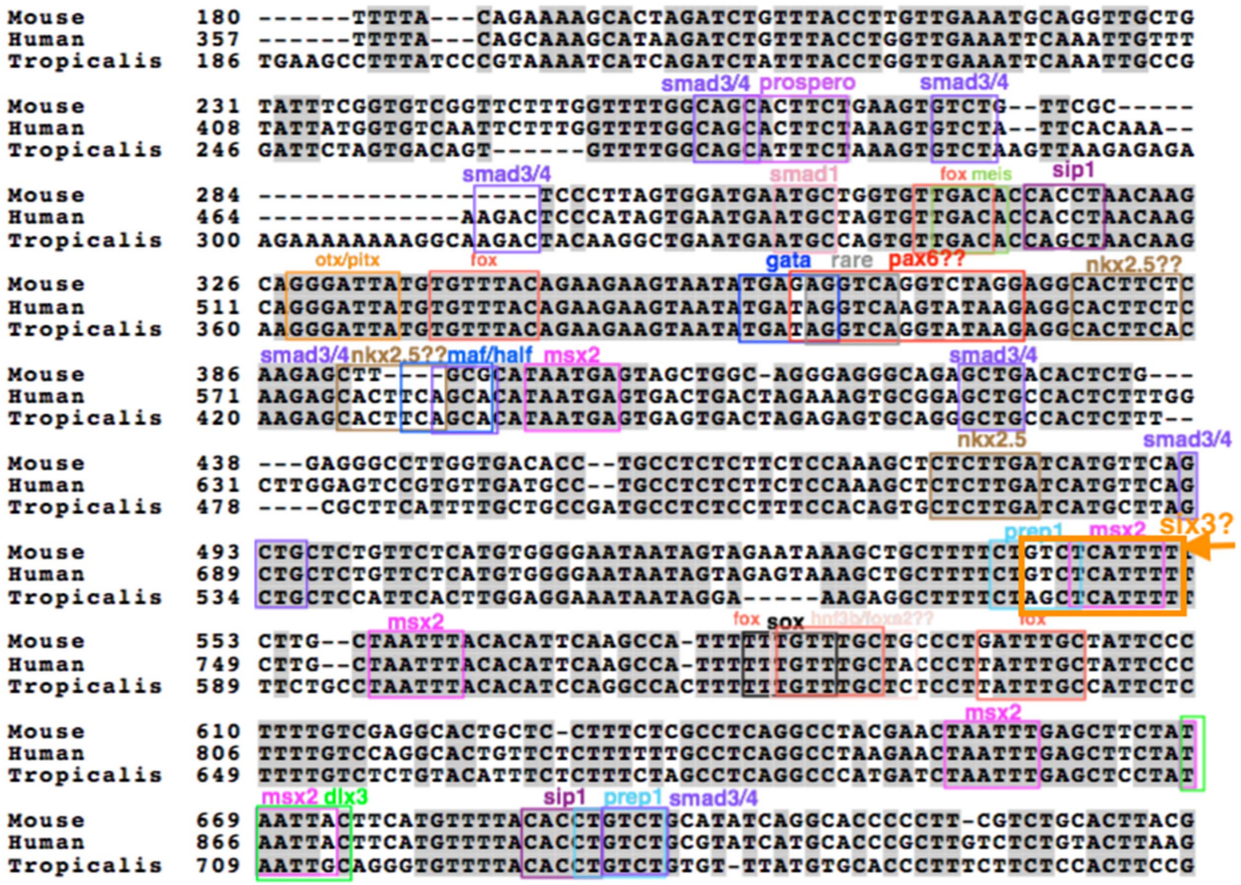

Figure. 4-66 ClustalW alignment of the region surrounding *mab21l1*-hCE putative open chromatin conserved element region in *X.tropicalis* with human and mouse sequence.

Discussion and future directions

The data presented here shows a preliminary importance of finding novel enhancer elements in an unbiased fashion, the functional analysis by transgene expression, identification of novel minimally conserved enhancers and the ability to computationally identify transcription factor binding sites. Taken together, combining high through put analysis with functional assays, both transgenic and in-vivo allows us to investigate the dynamic regulatory and chromatin landscape during lens commitment. This is the first time where ATAC-seq has been performed in *Xenopus tropicalis* in a tissue specific manner, although a more recent pre-publication has examined isolated explant tissues for ATAC-seq (Esmaeili *et al.*, 2019). However, our current analysis of the chromatin data is targeted to a few high priority genes, and largely descriptive, and the next step would be to take a more systematic approach with the whole genome datasets and undertake more extensive functional studies.

Due to the larger abundance of open and/or closed chromatin regions, we were able to dissect and collect enough tissue to successfully sequence the samples. Other techniques like ChIP-seq (in particular for transcription factors) would have required a larger number of samples to be dissected. Therefore, with ATAC-seq we were able to gather at least a subset of relevant information. ChIP-seq with other transcription factors and histone markers would provide a more specific insights regarding the precise state of the chromatin during lens commitment. These large datasets coupled with RNA-seq would provide a further enhancement wherein we can computationally link active enhancer sites with active transcription leading to the development of a gene regulatory network. *Xenopus* offers the

ideal model system to qualify the GRN by generating transgenics, localized ChIP experiments and CRISPR mutant lines.

The functional analysis of the transgenic constructs assists in determining if the element in question can function as an enhancer in a pseudo-*in vivo* context. We and others have shown that *Xenopus* transgenic system to identify both conserved and non-conserved enhancers. Using the conservation with *X. laevis* we can identify minimally conserved transcription factor binding sites as well as phylogenetic comparisons with other vertebrates and mammals. We would be able generate mutant transgenic constructs to identify a functional transcription factor binding sites. Alternatively, CRISPRi (Larson *et al.*, 2013; Dominguez, Lim and Qi, 2016) is also useful under knockout conditions to reveal in-vivo activity of an enhancer, although the presence of multiple enhancers and shadow enhancers provides redundancies and may mitigate the effects of a knockout. We have generated several CRISPRi constructs by making a fusion of deactivated cas9 (dcas9) with either an activator (VP64) or a repressor (Engrailed) element and preliminarily tested its activity to knockdown an enhancer for *six3* which shows modest reduction in *six3* expression levels. Multiplexing of CRISPR mutants could also yield the ability to knockout activity of two or more enhancers. Studies in mouse have already shown the utility of doing this by the double knockout of *Pax6* SIMO and EE enhancers (Antosova *et al.*, 2016). In *Xenopus*, this can be achieved by either making mutant lines or F0 CRISPR multiplexing to delete multiple enhancers at a time much as was done to study *six6* activity in our *six3* mutant (Chapter II).

Our data shown in this chapter presents a preview of how powerful combinatorial analysis utilizing both high throughput and in-vivo analysis in *Xenopus* could be used to

determine both the chromatin changes and gene regulatory networks that drive key developmental timepoints during the lens commitment process.

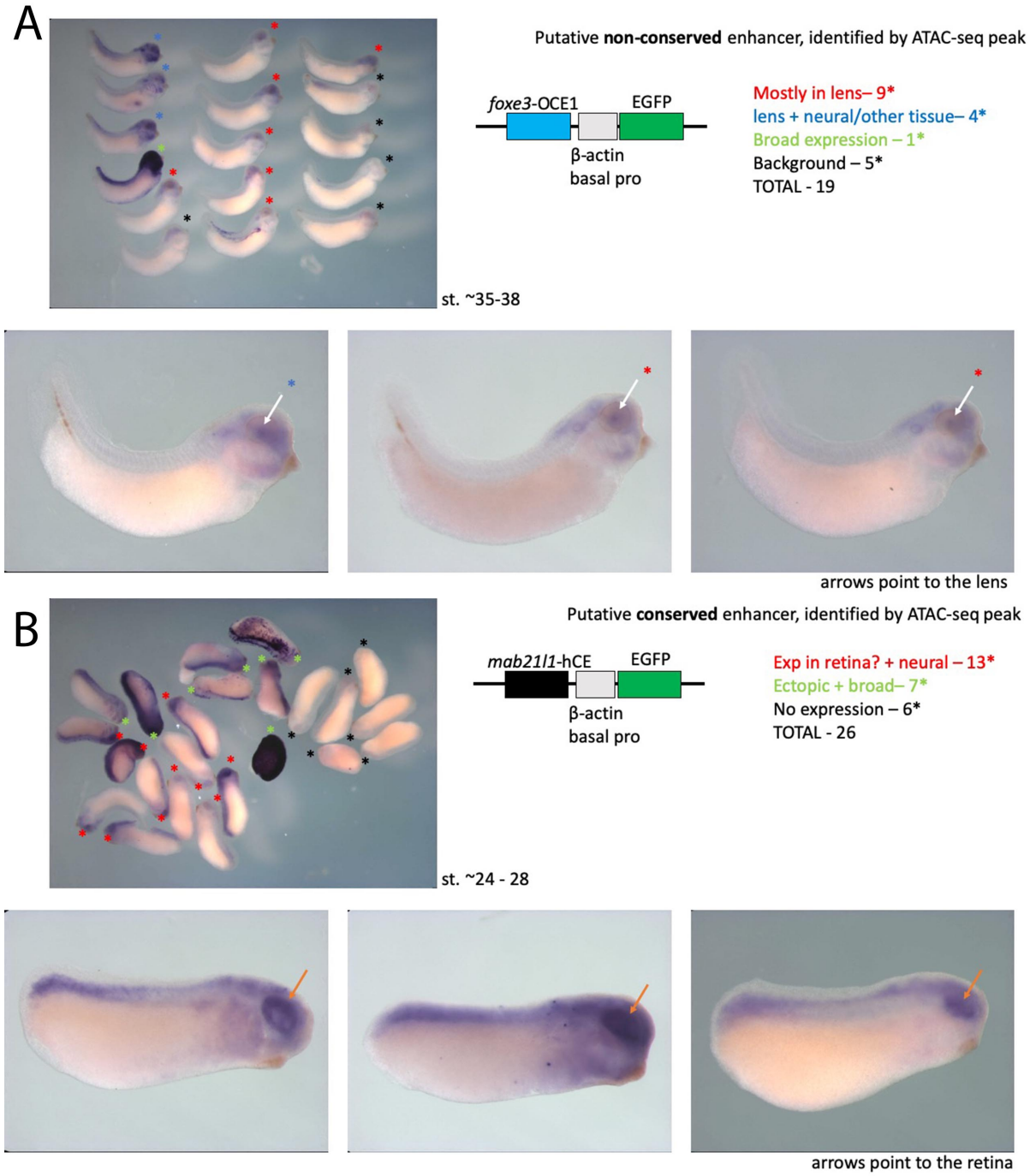

Figure. 4-7 Transgenic expression of (A) *foxe3*-OCE1 and (B) *mab21l1*-hCE fragment in *X. tropicalis* (*foxe3*-OCE1) and *X. laevis* (*mab21l1*-hCE). Expression pattern overlaps with region of endogenous expression of *foxe3* and *mab21l1* respectively. Some ectopic expression is observed in the neural tissue in both transgenic injections.

Materials and Methods

Animal Handling and tissue collection

Xenopus tropicalis and *Xenopus laevis* were housed and cared for based on the guidelines set forth by the University of Virginia Animal Care and Use Committee. Embryos were collected from either natural mating or in-vitro fertilization. The Jelly coats were removed enzymatically using either 2 or 3% cysteine in 0.1X MBS solution. Embryos were raised in 22C or 25C until ready for operations. Vitellin membranes were mechanically removed from the embryos carefully using sharp forceps without damaging the site of dissection. The embryos were raised to the appropriate stage and animal caps were dissected from stage 11 embryos, presumptive lens ectoderm (PLE) were dissected from stage 15, stage 18 embryos. 20 PLE was collected per replicate and 2 replicate experiments were performed. The tissues were pooled per replicate in Eppendorf tubes and flash frozen in liquid nitrogen and stored in -80C. Once all tissues were collected, the samples were shipped overnight to the Veenstra lab (Nijmegen University, Nijmegen, NL) for extraction, processing, sequencing and data validation (Bright and Veenstra, 2019).

Data Visualization

The Veenstra lab generated UCSC Genome browser compatible bigwig files originally assembled for *Xenopus tropicalis* genome assembly version 7 (UCSC genome assembly ID: xenTro7). Later, the data tracks were made compatible with assembly version 9.0. Data was visualized using UCSC Genome Browser and/or UCSC Genome Browser in a Box (James Kent *et al.*, 2002). Phylogenetic alignments and conservation analysis were performed by using MultiPIP and PIP Helper (Schwartz *et al.*, 2000).

Generation of plasmids and microinjections

All identified enhancer constructs were ligated into plasmids containing either a minimal chick β-actin (Ogino and Yasuda, 1998; Ogino, Fisher and Grainger, 2008), minimal human β-globin (Ochi *et al.*, 2012) or a zebrafish GATA2 (Meng *et al.*, 1997; Navratilova *et al.*, 2009) promoter constructs driving EGFP. The putative enhancer fragments were amplified from *Xenopus tropicalis* genomic DNA using primers tagged with 5' notI and 3' pstI or sbfI restriction enzyme sites. List of primers used are shown in Table 1. The plasmids were linearized and injected by either a modified version of restriction mediated microinjection (REMI) (Kroll and Amaya, 1996) or non-linearized ISce-I mega nuclease mediated injection (Ogino, McConnell and Grainger, 2006). A typical meganuclease injection protocol is shown below

Xt*foxe3*-OCE1-NotI5Pr	TTGCGGCCGCACCCACAACTATCAAACGCTG
Xt*foxe3*-OCE1-PstI3Pr	AGGCTGCAGGCCCAGTGTATTTCAGCACA
Xt*pax6*-OCE1-NotI5Pr	TTGCGGCCGCTGCCAATTTCATAGCAAAATCCT
Xt*pax6*-OCE1-PstI3Pr	AGGCTGCAGGGTCCCCAGTTTCCGGATAA
Xt*pax6*-OCE2-NotI5Pr	TTGCGGCCGCTCCCTTATCCGGAAAACCCC
Xt*pax6*-OCE2-PstI3Pr	AGGCTGCAGACACAGCACAGAAACCCCTA
mab21l1-hCE-5NotI	TTGCGGCCGCTTTCCACAATAACAAAGAGGGA
mab21l1-hCE-3SbfI	TCACCTGCAGGAGGGAAAACAAACCAACAGCAA

Table 4-1: Restriction enzyme tagged primers used in generation of transgenic constructs

Frogs

Females were isolated and placed in 6-liter buckets in appropriate amount of water. The males were also isolated and placed in a bucket two days prior to injection. Females were primed with 10U HCG 48hrs prior to injection and boosted with 100U HCG approximately 3 to 4 hrs prior to injection.

Buffers and Media required for injection

0.1X MBS with Gentamycin

6% Ficoll in 0.1X MBS, pH to 7.5 and filter sterilize

2 – 3% Cysteine in 0.1X MBS, pH to 7.9

Agarose injected and coated dishes to raise embryos overnight

Injection needles

Pulled glass capillary and calibrated with 60 – 80 sec continuous flow of 1µl sterile water.

Injection Mixture (Ogino, McConnell and Grainger, 2006)

10x I-SceI buffer 0.5 µl
Plasmid: 1 µl
nuclease free water: 3.5 µl
TOTAL: 5 µl

Injection mixture was prepared just prior to injecting. Injections were completed within 40 minutes post fertilization. Embryos were then sorted 40 min post injection and healthy embryos placed into agarose-coated dishes. The embryos were raised in a 22 $^{\circ}$C incubator overnight and moved to 25 $^{\circ}$C incubator the next day.

The modified variant of REMI used in these experiments involved primarily in reducing the size of the injection needle to a size similar to the meganuclease injection (60 - 80 sec calibrated needle). This did not appear to adversely affect the successful injection

of the sperm nuclei but did mitigate the damage caused due to the large bore needle in our hands and improved survivability of the embryos. The injected embryos were raised until st.37/38 in 0.1X MBS incubated initially in 22°C and later in 25°C incubator. The embryos were either fixed in MEMFA or processed for live in-situ fluorescence microscopic imaging. We discontinued using of the GATA2 and β-globin promoter due to the presence of basal activity in the control plasmids. Additionally, we also tested adding insulator fragments (Sekkali *et al.*, 2008) flanking the enhancer, promoter and EGFP sequence. However, this technique did not yield sufficient benefits. The ectopic expression of the transgene expression was minimized but endogenous expression patterns were of lower intensity. It also made generation of the insulator plasmids difficult due to the presence of homologous sequences leading to difficulties in ligation and transformation. Plasmids used for injection were purified using phenol chloroform and quantified using a nanodrop spectrophotometer. REMI injections yielded best results when transgene constructs were isolated from the plasmid and injected with I-SceI meganuclease.

***In-situ* hybridization and imaging**

The expression pattern for the enhancers were determined by *in situ* hybridization assays for EGFP. The protocol for *in situ* hybridization was adopted from (Harland, 1991) and modified by the Grainger lab to eliminate acetic anhydride treatment and the removal of the post fix step which interfere with genotyping assays. A detailed example of the *in situ* hybridization process is described in Appendix II.

For fluorescence and bright field imaging post *in situ* hybridization, we used a Zeiss SteREO Discovery V12 microscope and the images were captured on AxioVision software version 4.8.2. using the same settings for images being compared in a given experiment.

Images were in some case modified in Adobe Photoshop to adjust for contrast and brightness, again with settings applied equally to all sets of sections being compared.

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Chapter V Future directions

Future directions

Role of *six3* in lens and retina formation

The work presented in Chapter II and Chapter III of this dissertation presents a beginning in the broader goal of developing a *six3* gene regulatory network. In this chapter I will try to concisely put forward future directions for both the retina and lens projects. The Discussion and future directions sections of each of those chapters contain more detailed information.

The analysis of the *Xenopus* mutant shows a milder phenotype when compared to the mouse mutants. There are several ways that this can be assessed. We are currently awaiting the generation of a *Xenopus* specific *six3* antibody. We could assay for the functionality of the *six3* mutant by generating mutant constructs and injecting into *six3* to assay if its capable of rescuing the mutant phenotype in comparison with a full-length wildtype construct. A similar assay has been performed in zebrafish previously (Domené *et al.*, 2008).

The data shows that *six3* acts largely in a non-autonomous fashion in lens formation mediated by the activity of *mab21l1*. Components of the same mechanism might also play the formation of the retina. However, the mechanism by which *mab21l1* regulates both lens and retina formation is unclear from our studies. We present preliminary evidence to suggest that two signaling pathways – BMP and Delta/Notch (without ruling out Wnt) might be relevant.

We know from previous literature that BMP signaling network plays an important role in lens and retina formation (Furuta and Hogan, 1998; Yoshimoto *et al.*, 2005; Huang *et al.*, 2015). To answer the question of the involvement of BMP signaling we can

undertake immunohistochemical staining of phosphorylated smad (antibody commercially available), indicative of active BMP signaling (Massagué, Seoane and Wotton, 2005) or by utilizing constitutively active *smad5* (Green *et al.*, 2016) to rescue the loss of function phenotype in the lens and retina of the *six3* mutant.

Although the above experiments would help determine BMP's role, the detailed epistatic relationship with *mab21l1* would still remain unclear. To investigate this, I propose an immediate step would be to determine if *bmp4* expression is rescued by injection of *mab21l1* in the *six3* mutant. We know that *smad7* is rescued but the interaction between *mab21l1* and BMP signaling pathway is still unclear. Secondarily, we can readily design hormone inducible construct of *mab21l1* and restrict protein synthesis by cycloheximide treatment to determine which components of the BMP signaling pathway are changed upon *mab21l1* activation. This can also be further expanded to determine the downstream effectors of *mab21l1* and/or *mab21l2* by utilizing the *Xenopus mab21l1* and *mab21l2* mutant lines that have been established in the Grainger lab but not yet characterized.

The impact of the Delta/Notch pathway components is clear from our data and previous evidence in literature suggests that Notch signaling plays an important role in both retina differentiation and lens specification (Austin *et al.*, 1995; Ahmad, Dooley and Polk, 1997; Perron and Harris, 2000; Ogino, Fisher and Grainger, 2008; Luo *et al.*, 2012). What remains unclear is the mechanism of Delta/Notch action in the retina and the relationship (if any) between *mab21l1* and the Delta/Notch pathway. A first step in this process would be investigate the expression of Notch substrates – the *Hes* family of transcription factors (El Yakoubi *et al.*, 2012). This would be relatively simple assay with established *in situ*

hybridization protocols readily available in the Grainger lab. Similar to our proposed experiment to determine the epistatic relationship between *mab21l1* and components of the BMP signaling pathway, we could similarly investigate its relationship with the Delta/Notch pathway. Reactivating the notch signaling pathway could be achieved by the expression of hormone inducible constitutively active *notch1* (Rones *et al.*, 2000) in the *six3* mutant background to determine if this can rescue the loss of function retina and/or lens phenotype.

The data we present here also shows aberrant retina patterning with loss of layering. A similar loss of retina morphology is observed when *Pax6* is conditionally removed from the mouse retina (Klimova and Kozmik, 2014). Although the retina is completely abolished in that mutant and therefore any effect on lens formation might be a result of the loss of retina rather than activity of *Pax6.* That study however shows RPC's undergo premature cell cycle exit due to loss of *Pax6* leading to loss of retinal layering. In our study we have not yet analyzed *six3* role in regulating cell cycle in the RPC's although it is thought to regulate cell cycle in the neural plate and the retina in the mouse (Gestri *et al.*, 2005; Diacou *et al.*, 2018). It would therefore be an important step to investigate impingement of the cell cycle in the RPC of the *six3* mutant in *Xenopus*. In addition, the *Xenopus six3* mutant retinas do maintain some retinal morphology in some cases and therefore, it appears that the expression of some early RPC genes are present or are activated later which would be useful to determine if their expression remains broad i.e., keeping them in an undifferentiated state utilizing new *in situ* hybridization methods as well as investigating the expression of terminal retinal markers.

The *Xenopus six3* mutant also shows defects in formation of the anterior forebrain. Previous work in the Grainger lab has demonstrated that expression of *rax* defines the eye field and in its absence, anterior forebrain tissue expands into the eye field (Fish *et al.*, 2014). Therefore, it would be very relevant (even though *rax* is only modestly reduced in the *six3* mutant) to investigate the factors that are regulated by *six3* in the anterior forebrain making it relevant not only for forebrain formation but also for the eye field.

Finally, the Grainger lab has many benefits like the concurrent mutants available in *pax6, rax, mab21l1* and *mab21l2.* Of these only *rax* has been fairly thoroughly investigated (Fish *et al.*, 2014). Combining the gene regulatory networks from all of these mutants would be invaluable to generating a functional gene regulatory network in the eye. Additionally, tissue specific RNA-seq would remove some of the biases due to *apriori* information required for *in situ* hybridization. This would also be a high throughput process to generate novel targets of *six3* in the lens and retina. We have already collected tissues at multiple stages during neurulation for both PLE and anterior neural plate (presumptive retina) in both the *pax6* and *six3* mutants. Processing and sequencing these would help in closing the gaps in our understanding of the lens and retina gene regulatory network.

Changing chromatin dynamic during lens commitment

The ATAC-seq dataset has already yielded several novel conserved and non-conserved regulatory elements. A few of these have been functionally validated. The next step in this process would be building on the bioinformatically-identified putative transcription factor binding site identifications by mutational analysis to determine if transgene activity of putative enhancers can be reduced or terminated. These regulatory

networks can then be assayed by traditional mutational techniques to determine their role in the lens commitment process.

Although, ATAC-seq marks enhancer regions, it also marks locations of active insulators, promoters and coding sequence. To narrow down the focus of the study, a combination of tissue specific histone and transcription factor ChIP-Seq and RNA-seq during the key stages of commitment would yield more specific results.

Multiplexing enhancer deletions

We have already generated two lines carrying enhancer deletions for *pax6* SIMO and *six3* CE3. These mutant frogs do not show any apparent phenotype either during tadpole or adult stage suggesting redundancies in enhancer function. Although human patients have been identified to have aniridia with the loss of function of SIMO (Bhatia *et al.*, 2013), both mouse (Antosova *et al.*, 2016) and *Xenopus* (our data) show no phenotype. Mice do show a strong eye phenotype when two enhancers of *pax6* in the lens – EE and SIMO are deleted (Antosova *et al.*, 2016). Therefore, a multiplex analysis would reveal the importance of these enhancers and help define their relative importance to either *pax6* or *six3*'s expression in different tissues.

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Appendix I Loss of *pax6* SIMO and *six3* CE3 show no apparent phenotype; role of shadow enhancers and redundancies in regulatory networks

Introduction

The reduction in cost and improvements in sequencing have resulted in improved annotation of the human, mouse, *Xenopus* and other genomes (Hellsten *et al.*, 2010). However, the characterization of non-coding regulatory regions has lagged behind (Woolfe *et al.*, 2005). Comparative genomics have identified presence of regulatory elements that are either located proximally or distal to the gene that they regulate. In the context of this chapter, I refer to enhancers and not insulators or promoters which are also regulatory regions in the genome. Many genes involved in early development have complex regulatory mechanisms that intricately control the spatiotemporal nature of the expression of these genes (Long, Prescott and Wysocka, 2016). In the past couple of decade clinical research has highlighted the importance of mutations in these non-coding regions in human disease (Plaisancié *et al.*, 2018). As mentioned in Chapter IV, *Pax6* has several conserved enhancer regions that are located both upstream and downstream of its location in the genome (Dimanlig *et al.*, 2001; Griffin *et al.*, 2002; Kleinjan *et al.*, 2006; Navratilova *et al.*, 2009). Some of the regulatory elements are spread out into the intronic regions of neighboring gene such as *elp4* (Kleinjan *et al.*, 2006). Further, as described in Chapter IV our preliminary evidence in *Xenopus* suggests that even more enhancers could be identified that are non-conserved and therefore could provide a more complex regulatory pattern for its expression in particular organisms. Whole genome studies provide a key method by which to identify and specify activity of an enhancer. However, low throughput functional studies are critical to better understand their function. The VISTA enhancer browser is an example of a resource that bioinformatically identified conserved enhancer fragments (Visel *et al.*, 2007). However, these identify putative enhancers in whole genome data and

the expected expression pattern but does not functionally assay the relevance of transcription binding sites located within the enhancer site for activity.

Therefore, in our study we proposed to functionally analyze the activity of conserved enhancer fragments *in-vivo* in *Xenopus.* We had previously identified two enhancer regions for *pax6* and *six3* that would be important for their activity in the retina and lens for *pax6* SIMO and *six3* CE3.

***pax6*-SIMO enhancer**

Haploinsufficiency of *pax6* has been one of the causes of the human disease – Aniridia (Kleinjan *et al.*, 2001; Bhatia *et al.*, 2013). Analysis of patients identified several point mutations and premature termination as causative for loss of function of *PAX6* (Kleinjan *et al.*, 2001). However, early clinical investigations revealed loss of the SIMO element was identified initially back in the 1980's in human patients with Aniridia (Simola *et al.*, 1983; Bhatia *et al.*, 2013). This was later identified to be a highly conserved region located downstream of *PAX6* coding sequence located in the intronic region of neighboring gene, *elp4* (Plaza *et al.*, 1995). Transgenic assays have implicated the importance of these regulatory regions in human disease. Transgenic assays in homozygous transgenic mice using yeast artificial chromosomes showed under WT conditions, the YAC was able to rescue the mutant phenotypes but YAC's carrying the human patient mutations did not (Kleinjan *et al.*, 2001). More recently, another report has suggested that a single base mutation in SIMO located in the putative *Pax6* binding site results in inhibition of *Pax6* autoregulation and causes Aniridia in a human patients with no exonic mutations (Bhatia *et al.*, 2013).

Therefore, we wanted to use *Xenopus* to model and functionally assay the loss of SIMO *in vivo* rather than in transgenic assays described previously. To generate this mutant, we utilized multiplex CRISPR to create 2 separate target sites upstream and downstream of the SIMO core region to generate a deletion of this enhancer. A generalized schematic is shown in (Fig. A-1A). Putative transcription factors and their binding sites in the SIMO element are shown in Fig. A-1B. We raised F0 frogs to maturity and identified the extent of the deletion as shown in Fig. A-1C. The deletion spanned 1.15kb with a 4-base insertion. Initial analysis was stymied due to lack of mature animals and so we generated a compound heterozygous animal by crossing a *pax6*-SIMO +/- line with a *pax6*-ex7, ex9 +/- line, anticipating that the null allel on one chromosome (which alone has no phenotype) would increase the chance of seeing a phenotype due to the SIMO deletion on the other. The offspring of this mating did not, however, present any apparent phenotype during tadpole development (data not shown) and maybe a mild post metamorphosis phenotype that we have not categorized at present (Fig. A-2).

Generation of true *pax6*-SIMO -/- animals later yielded similar results with no apparent phenotype during tadpole stages (Fig. 1D, upper panels). Cross sections through the eye revealed no aberrant phenotypes (Fig. 1D, lower panels). Concurrently, a recent publication revealed that *pax6*-SIMO -/- in mice did not yield any apparent phenotype identifiable at present (Antosova *et al.*, 2016). However, when compound heterozygote was generated between *Pax6* small eye mice and *Pax6* SIMO, lens formation was ablated (Antosova *et al.*, 2016). Heterozygote *Pax6* small eye mice do tend to show eye defects but they always formed a lens. This group further revealed that a homozygous loss of *Pax6* SIMO and another enhancer for lens – EE resulted in failure of lens formation suggesting

redundancy in the regulation of *Pax6* (Antosova *et al.*, 2016). Therefore, our next course of action would be to generate mutations in EE under the SIMO null background. This would either confirm the importance of the redundancy of SIMO + EE in frogs or reveal if there are different enhancers that can function to achieve functional redundancy in vertebrates when compared to mammals.

***six3*-CE3 enhancer**

Much like *Pax6*, regulation of *Six3* is a complex process involving multiple putative enhancer sites (Hellsten *et al.*, 2010; Beccari *et al.*, 2015). However, their characterization has been poor to date. The Grainger lab first identified several putative conserved elements located upstream of the *six3* coding region (Hellsten *et al.*, 2010). Transgenic assays in Xenopus revealed the importance of 2 of these sites captured expression of *six3* in the eye – CNS3 and CNS5 (later renamed CE3 and CE5 respectively). Another group working in medaka and cell culture identified several putative proximal sites surrounding *six3*.2 and characterized their activity in cell culture (Beccari *et al.*, 2015). The regions included in their study were *six3* CE5 and *six3* CE6, both are located within 4kb of the transcriptional start site of *six3* (Beccari *et al.*, 2015). The CE3 element is located ~25kb upstream of *six3*. Its activity in the original screen suggested that it was the dominant enhancer for *six3* based on the intensity of expression compared to other elements tested ((Hellsten *et al.*, 2010) and unpublished comments). We generated, using multiplex CRISPR, mutant lines for *six3* CE3 and CE5. We could not confirm germline transmission for *six3* CE5, so we did not pursue analysis of it further. We generated deletions of *six3* CE3 and identified a 1.1kb deletion which covered the core conserved region of *six3* CE3. Homozygous mutant lines for *six3* CE3 deletion did not show any apparent phenotype (data not shown) suggesting

the presence of redundant regulatory element which is able to recoup loss of the *six3* CE3 enhancer element. Further studies would be required to determine functionality and the multiplex CRISPR mutants of other identified (perhaps CE5, also having high levels of activity un driving eye expression in transgenic animals) to determine if a combinatory effect has a significant impact on the expression of *six3*.

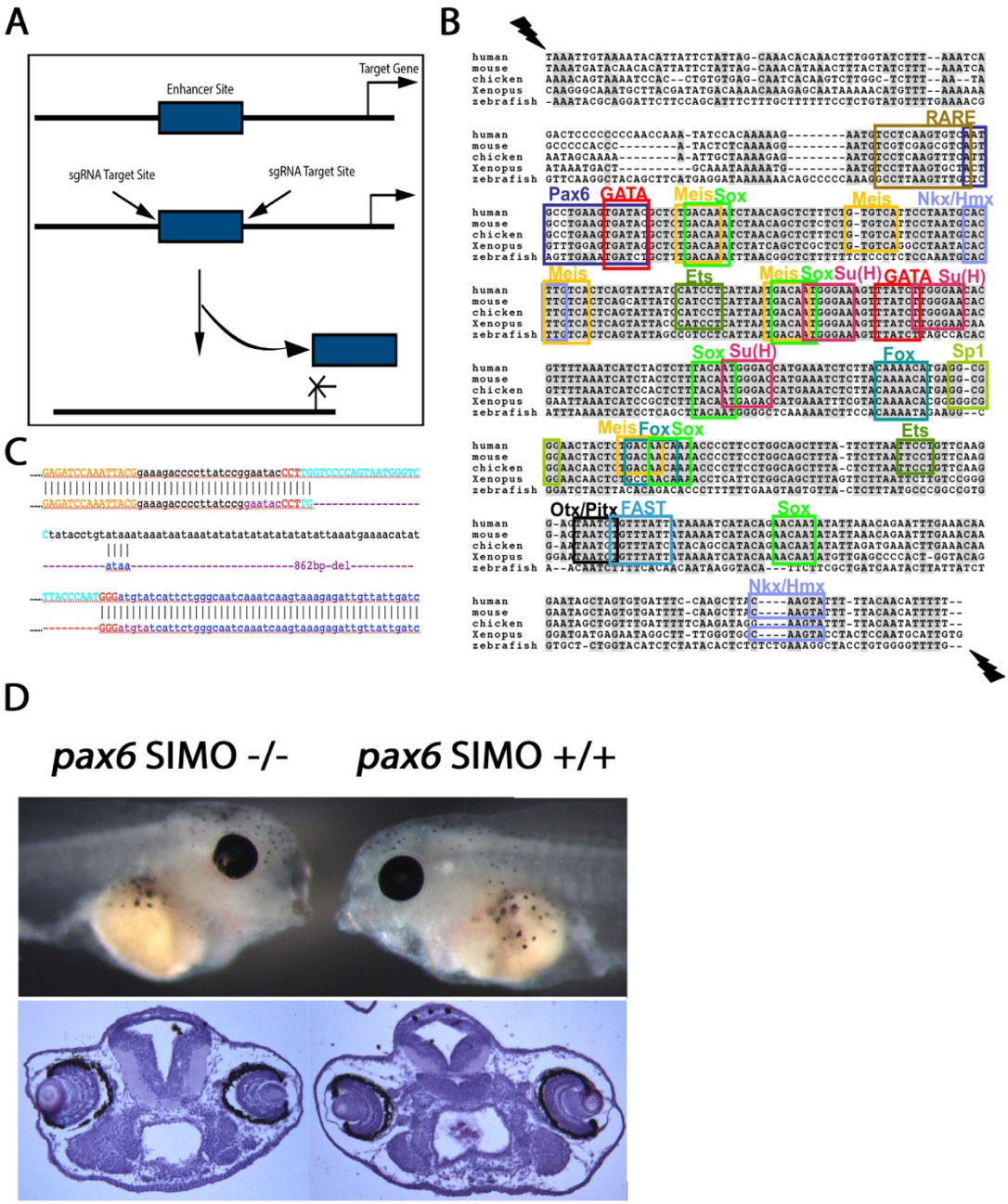

Figure. A-1 Description of *pax6* SIMO enhancer deletion in *Xenopus*. A. A generalized schematic of using multiplexed CRISPR deletions to remove a 1.1kb fragment from the genome. B. Phylogenetic footprint analysis of putative transcription factor binding sites. The lightning arrows indicate the boundaries of the core site and the deletion sites located upstream and downstream of the arrows. C. Sequence showing that the deletion line

established resulted in 1.1kb deletion and 4-base insertion. D. *pax6* SIMO null embryos showed no apparent phenotype (bottom panels – sections of embryos depicted in upper panels)

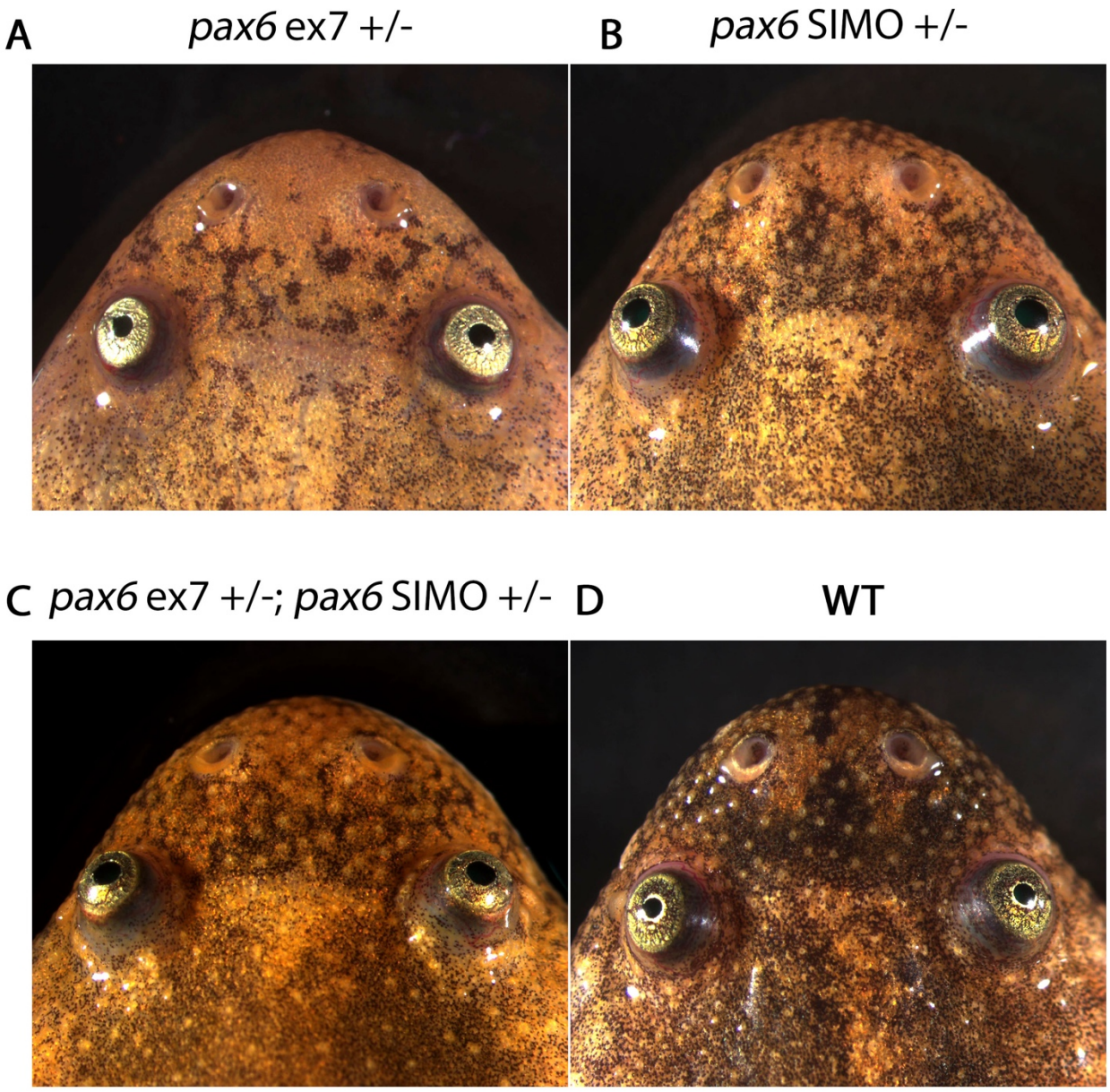

Figure. A-2 Head shots of juvenile of SIMO compound heterozygous eyes not displaying apparent phenotype or the possibility of a mild aniridia in B or C. A. Juvenile *pax6* ex7, ex9 +/- (Fig. 2B) *pax6* SIMO +/- Fig. 2C) *pax6* ex7, ex9 +/-; *pax6* SIMO +/- (Fig. 2D) WT frog for comparison.

Materials and methods

Xenopus tropicalis were housed and raised based on the protocol approved by the University of Virginia Animal Care and Use committee. Frogs used to generate CRISPR mutant lines were injected with priming levels of hormone (10U) 48hrs prior to boosting with inducing levels of hormone. Embryos were fertilized by in-vitro fertilization and injected with a cocktail containing the sgRNA, cas9 RNA and fluorescent dextran dye. The sgRNA was generated using the protocol described previously (Nakayama *et al.*, 2014). Example of cocktail used for injection is described below:

sgRNAs preparations

pax6 SIMO 5', 3.9 µg/µl

pax6 SIMO 3', 2.5 µg/µl

Diluted to 100 ng/µl

2. Cas9s

WT mRNA, 1.475 µg/µl

3. injections

pax6 SIMO 5' (100 ng/µl), 0.9 µl

pax6 SIMO 3' (100 ng/µl), 0.9 µl

Cas9 wt, 1.05 µl (ca. 1,500 ng)

RDDX70K, 0.15 µl (15 µg)

Nuclease free water, 0 µl

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3 µl: 30 ng gRNA ea + 500 ng Cas9 mRNA + 5 µg dye/µl

The embryos were injected in 6% Ficoll and injections were completed 40mins post fertilizations. Embryos were sorted 40 mins after last embryo injection and stored in 0.1X MBS in 22°C incubator. The next day, injected embryos were sorted to collect those showing fluorescence (i.e. definitively injected) and raised to maturity. Some siblings were selected for genotyping by lysing embryos overnight in complete lysis buffer:

50mM Tris (pH 8.8)

1mM EDTA

0.5% Tween 20

200µg/ml Proteinase K (PCR grade)

Genotypes were performed by PCR using the following primers:

*pax6*SIMOver5 – TGCCCGTCTCCATTTTAATC

*pax6*SIMOver3 – ACACCCCATCATCCTGTCAT

Mutations were identified by either variant band sizes by gel electrophoresis or by sequencing.

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Appendix II *in situ* hybridization and mRNA and CRISPR injection protocols in *Xenopus tropicalis*

As mentioned earlier in the previous chapters, the protocols were modified from (Harland, 1991) and modified by the Grainger lab to eliminate acetic anhydride treatment and the removal of the post fix step which interfere with genotyping assays. This is a typical protocol with modifications made depending the probe used. Further modification was also made to get better stain with older stage 41/42 embryos by nutating the embryos in 4°C as suggested by (Hollemann et al, 1998). This slows down the reaction rate but reduces the appearance of background stain considerably. Typical incubations times are on average 3 days (varies based on probe). It would be advisable to put an equivalent stage albino embryo along with the pigmented embryo in order to determine the end point for staining. The use of fresh 37% formaldehyde in both the initial fix and during the *in situ* process is highly recommended in particular for probes that stain lightly and for young embryo PLE and older embryo retina staining. A typical protocol used for *in situ* in the Grainger lab is shown below:

Day 1:

Rehydration: Place embryos in

75% EtOH / 25% (DEPC) H_20,

50%EtOH / 50% (DEPC) H_20,

25% EtOH / 75% PTw, (pTw = 1X PBS plus 0.1% Tween20 in either DEPC or sterile water)

3 X 100% PTw all for 5 minutes each

- **Proteinase-K step** Stock solution = 10mg/ml or 10ug/ul. Dilute the stock to (5ug/5ml or 1ug/ml). The amount of Proteinase K is varied depending on the batch of the stock and age of embryos used. Typically for stage 15 through to stage 28

embryos - 5min treatment at room temperatures (these were not typically controlled but ranged from 22°C - 25°C)

- Move embryos to 0.1M TEA and do an extra 1min wash
- **TEA**: Wash 2 x 5 min. in 0.1 M TEA pH 7-8.
- **Acetic Anhydride**: Add 12 µl of acetic anhydride/vial. Nutate for 5 min. Add 12 µl more of acetic anhydride and nutate for 5 min. This step is skipped if the embryos are to be genotyped.
- **Wash**: 2 x 5 min with PTw
- **Re-fix**: Nutate for 20 min in MEMFA.
- **Wash:** 5 x 5 min with PTw.
- **Wash:** briefly with 1ml PTw/250ul hybridization buffer
- **100% hybridization buffer:** Replace with fresh 500 µl Hybridization buffer. Place on shaker at Hybridization temperature (**60°C**) for 10 minutes
- **Pre-Hybridization**: Replace with fresh 500 µl of Hybridization Buffer. Pre-hybridize for 4-6 hours rotating at hybridization temperature.
- **Hybridize:** Replace with solution of 500 µl of probe solution (approximate amount of probe stock in hybridization buffer). Hybridize overnight at hybridization temperature

Day 2:

- Remove probe solution and save. It can be used 2-3 times. Return probe to -20°C
- Wash 1 x 10' with fresh hybridization buffer at hybridization temperature (I save and reuse the prehybridization buffer for this step)
- Wash 3 x 20 min with 2X SSC at 60°C. Use 1mL volume and nutate vertically

- Treat 30 min at 37°C with 300-500 µl RNAase (use 2 µL of stock per 1 mL of 2x SSC).
- Wash 10 min with 2X SSC at room temperature
- Wash 2X 30 min with 0.2X SSC at hybridization temperature.
- Wash 2 X 10 min with MAB at room temperature.
- Replace with 500µl of 2% BMB in MAB. Nutate for 10 min room temperature.

  To make 2% BMB in MAB: Add 10 ml of 10% stock BMB to MAB, bring to 50 ml.
- Replace with 500µl of 2% BMB + 20% HT lamb serum in MAB. Nutate for 1 hr room temperature.

  To make 2% BMB + 20% HT lamb serum in MAB:

  1 ml lamb serum + 1 ml 10% BMB + 3 ml MAB
- Overnight in 4°C in vertical nutation with 2% BMB + 20% HT lamb serum + anti-dig AP in MAB

  Use 500µl of Anti-Dig-AP (Roche 093 274 910) 1:2000 in 2% BMB + 20% HT lamb serum.

Day 3:

- Wash 5 x 1 hour (or 8 x 20 min) with MAB (full volume) at room temperature
- Incubate at 4°C overnight in MAB. (or start color reaction same day if 8 X 20 min wash was done)

Day 4: (Filter sterilize AP buffer, spin down BMPurple2 to remove precipitate)

Used BCIP/NBT only for embryos which were sectioned

- **Pre-color**: Wash 2 x 5 min with Alkaline phosphatase buffer with nutation. For 50.0 ml: 5.0 ml 1M Tris pH 9.5, 2.5 ml 1M $MgCl_2$, 1.0 ml 5M NaCl, 50 µl Tween 20, BTV with water. AP buffer must be fresh. No levamisol was used in this protocol.
- **Color Reaction**: Replace with 500 µl BCIP/NBT. Nutate at room temperature until color level is reached. (Perform color reactions at 4C for st.42 embryos to reduce background)

  For 1ml AP buffer:

  BICP 50 mg/ ml 3.5 µl

  NBT 100 mg/ml 3.375 µl
- For embryos which were to be sectioned - Color reactions were stopped, color reaction removed, and the embryos were washed with AP buffer. The tails were chopped using a razor blade and lysed overnight in complete lysis buffer (see Appendix I). The heads were fixed overnight in Bouin's fixative, washed in saturated $LiCO_3$ in 70% ethanol followed up with several 70% ethanol washes. Once genotyped, the mutant heads were combined together, and the embryos were bleached as shown below (Hydrate step)
- For embryos not being sectioned - Color reactions were stopped by removing the color reaction solution and adding 70% ethanol to the vials. The embryos were nutated for at least 3 hours or overnight under aluminum foil. This step was followed up with the bleaching protocol starting at the Hydrate step below.

- **Hydrate**: Nutate embryos in 50% EtOH, 25% EtOH 75% PTw, 100% PTw all 5 min each and a final rinse in 0.5X SSC (For 50ml 0.5X SSC—1.25 ml 20X SSC + 48.75 ml H_2O)
- **Make bleaching solution**:

  4.5ml H_2O, 0.16ml 30% H_2O_2, 0.25ml formamide, 0.125ml 20X SSC/ tube
- **Bleach**: Place vials on aluminum foil nutator under fluorescent light until bleached (whitened).
- **Wash bleach**: 3 x 15 (30) min with PTw. Embryos are now ready for scoring. If scoring within a few days, store at 4 ºC. If longer term storage is required, gradually dehydrate with 100% EtOH and store at -20 ºC

mRNA and CRISPR injections

Rescue mRNA was injected either at 4 or 8 cell stages in the dorsal blastomeres of *six3* mutant embryos. CRISPR injections were injected at the 1 cell stage and amounts used are listed in the respective chapters. Example provided here is for *mab21l1* mRNA and *six6* CRISPR injections. The protocol for CRISPR was developed in the lab and is described here (Nakayama *et al.*, 2013, 2014)

1. mRNAs

mab21l1 mRNA – 1496.5ng/ul

2. Mixture

FLDX – 0.30µl

Water –1.37µl

TOTAL – 2.0µl

Inject 2nl in each blastomere@ 4/8-cell stage. Final amount per embryo is 30ng dye and 1ng RNA

CRISPR injections

1. sgRNA's:

Xt six6 tar1 - 824 ng/µl

Xt six6 tar3 - 982 ng/µl

2. Cas9 Proteins:

WT, lot PC10162 (Protein obtained from PNA Bio)

3. Mixture: immediately after fertilization, sgRNAs and Cas9 protein are mixed, incubated at 37˚C (ca, 5 min) before adding dye, followed by immediate injection.

Xt six6 tar1, (final ca. 400 ng) – 0.48 µl

Cas9 WT pro (final 1000 ng) – 1 µl

Water – 0.22 µl

FLDX 40K (100 µg/µl) – 0.3 µl before adding, 5 min @ 37˚C

In 2 µl: 400 ng sgRNA + 1000 ng Cas9 pro + 30 µg /2 µl

Inject 4 nl: 800 pg sgRNA + 2000 pg Cas Pro + 60 ng dye @ 1-cell.

The embryos were injected in 6% ficoll (in 0.1X MBS) and transferred to larger petri dishes containing 0.1X MBS and stored overnight in 22˚C. Embryos were sorted the next day to pick injected embryos under fluorescence microscope and raised in 25˚C incubator.

References

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