DLX HOMEOBOX TRANSCRIPTIONAL REGULATION OF CRX AND OTX2 GENE EXPRESSION DURING VERTEBRATE RETINAL DEVELOPMENT

by

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Table of Contents

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The lessons learned through the course of this degree were not in science alone, and those are the lessons that are the most worthwhile. Here is where I raise my glass to those who have taught me the most.

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VIP, Winnipeg, 2010

ABSTRACT

DLX HOMEOBOX TRANSCRIPTIONAL REGULATION OF CRX AND OTX2 GENE EXPRESSION DURING VERTEBRATE RETINAL DEVELOPMENT

Objectives: We are interested in identifying and characterizing DLX transcriptional targets during retinal development. The *Crx* (Cone-Rod homeobox) gene is required for the differentiation and maintenance of cone and rod photoreceptors. *Otx2* (Orthodenticle homeobox 2) is a key regulator of photoreceptor cell fate. The *Dlx1/Dlx2* double knockout (mutant) mouse retina has a significant reduction of retinal ganglion cells with aberrant *Crx* expression in the neuroblastic layer and increased retinal *Otx2* expression. We hypothesized that the *Dlx* homeobox genes directly repress *Crx* and *Otx2* expression during retinal development.

Methods: CRX and OTX2 expression in mutants and wild-type littermates was detected at RNA and protein levels. Chromatin immunoprecipitation (ChIP) of embryonic retina was utilized to identify DLX protein-genomic DNA complexes *in situ*. Quantification of expression was assessed by qRT-PCR and cell counting. *In vitro* assays such as electrophoretic mobility shift assays (EMSA) and luciferase reporter assays were used to detect the direct binding and activity, respectively, of DLX2 on the *Crx* and *Otx2* promoters *in vitro*.

Results: Qualitative and quantitative assessment of the temporal and spatial expression of CRX demonstrates increased transcript and protein expression in the *Dlx1/Dlx2* double knockout retina at E18.5, suggesting that these DLX transcription factors may repress CRX expression, thereby restricting CRX expression to the outer nuclear layer. OTX2 expression is increased in the *Dlx1/Dlx2* knockout retina at E16.5 suggesting that DLX2 negatively regulates OTX2 expression. ChIP assays demonstrated that DLX proteins are bound to specific regions of the *Crx*

and *Otx2* promoters *in situ*, supporting a direct role for *Dlx* genes in repressing *Crx* and *Otx2* expression during retinal development.

Conclusion: The *Dlx1/Dlx2* knockout has aberrant and ectopic expression of CRX in the retina along with increased OTX2 expression. Our data supports our hypothesis that both CRX and OTX2 are transcriptional targets directly repressed by the DLX1 and DLX2.

LIST OF ABBREVIATIONS

cDNA-complimentary deoxyribonucleic acid

CKO- conditional knockout

DNA –deoxyribonucleic acid

DKO – Double knockout

Dlx – distalless homeobox gene, mouse

DLX - distalless homeobox protein, mouse and human

GCL – Ganglion cell layer

cGMP- cyclic guanosine monophosphate

CKO- Conditional knockout

Crx – cone rod homeobox gene, mouse

CRX - cone rod homeobox protein, mouse and human

CRX- cone rod homeobox gene, human

GDP- guanosine diphosphate

GFP- green fluorosecent protein

GTP- guanosine triphosphate

GOF- Gain-of-function

INL – Inner nuclear layer

LOF- Loss-of-function

NBL-Neuroblastic layer

ONL- Outer nuclear layer

Otx2 – orthodenticle homeobox 2 gene, mouse

OTX2- orthodenticle homeobox 2 protein, mouse and human

OTX2- orthodenticle homeobox 2 gene, human

PFA - paraformaldehyde

RT – room temperature

RGC- retinal ganglion cell

RNA-ribonucleic acid

RPC- retinal progenitor cell

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

WT- Wild-type

LIST OF TABLES

LIST OF FIGURES

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1. INTRODUCTION

It is said that beauty is in the eye of the beholder. However poetic an idea, it can only come to fruition if one has properly developed retinal photoreceptors. The photoreceptors, which include the rods and the cones, are involved in conveying the images we see to the brain for interpretation. The processes involved in development of the photoreceptors are tightly regulated and involve many players. Of these players,the homeobox transcription factors *Dlx2*, *Otx2* and *Crx* have been implicated. A general background of the mammalian retina and how these homeobox genes are regulated by one another will be discussed in this chapter.

1.1 RETINA: STRUCTURE AND DEVELOPMENT

1.1.1 Anatomical structure of retina

The retina is the inner-most layer of the eye. The retina itself is comprised of several layers as well as several cell types. Of these cell types, six are of the neuronal cell class and one is of the glial class. Belonging to the former class are the retinal ganglion cells (RGCs), amacrine, horizontal, and bipolar cells, and the photoreceptors, which include the cones and rods; the Müller glial cells are the sole member of the latter class. In the mature retina, these cells are arranged spatially in three nuclear layers and two plexiform layers (Dowling and Boycott, 1966) as shown in Figure 1.

The innermost layer is termed the ganglion cell layer (GCL). It is here that the displaced amacrine cells and RGCs, whose axons form the optic nerve, are found. The next nuclear layer, the inner nuclear layer (INL), contains the bipolar, amacrine and horizontal cells. Separating the GCL and INL is the inner plexiform layer (IPL). It is in this layer where synaptic connections between the axons of the bipolar cells and the dendrites of the amacrine and RGCs are made.

Following the INL is the outer plexiform layer (OPL) which separates the INL from the third nuclear layer, the outer nuclear layer (ONL). The ONL contains the nuclei of the cones and rods. Their cell bodies extend outward towards the retinal pigmented epithelium (RPE). The OPL houses the synaptic connections made between the axons of the photoreceptors and the dendrites of the bipolar and horizontal cells thus completing a synaptic relay between all three nuclear layers and the optic nerve. The Müller glial cells act as a structural support and their nuclei are located in the INL while the cell body extends between the ONL and the GCL (Dowling and Boycott, 1965; Dowling and Boycott, 1966; Dowling and Boycott, 1969).

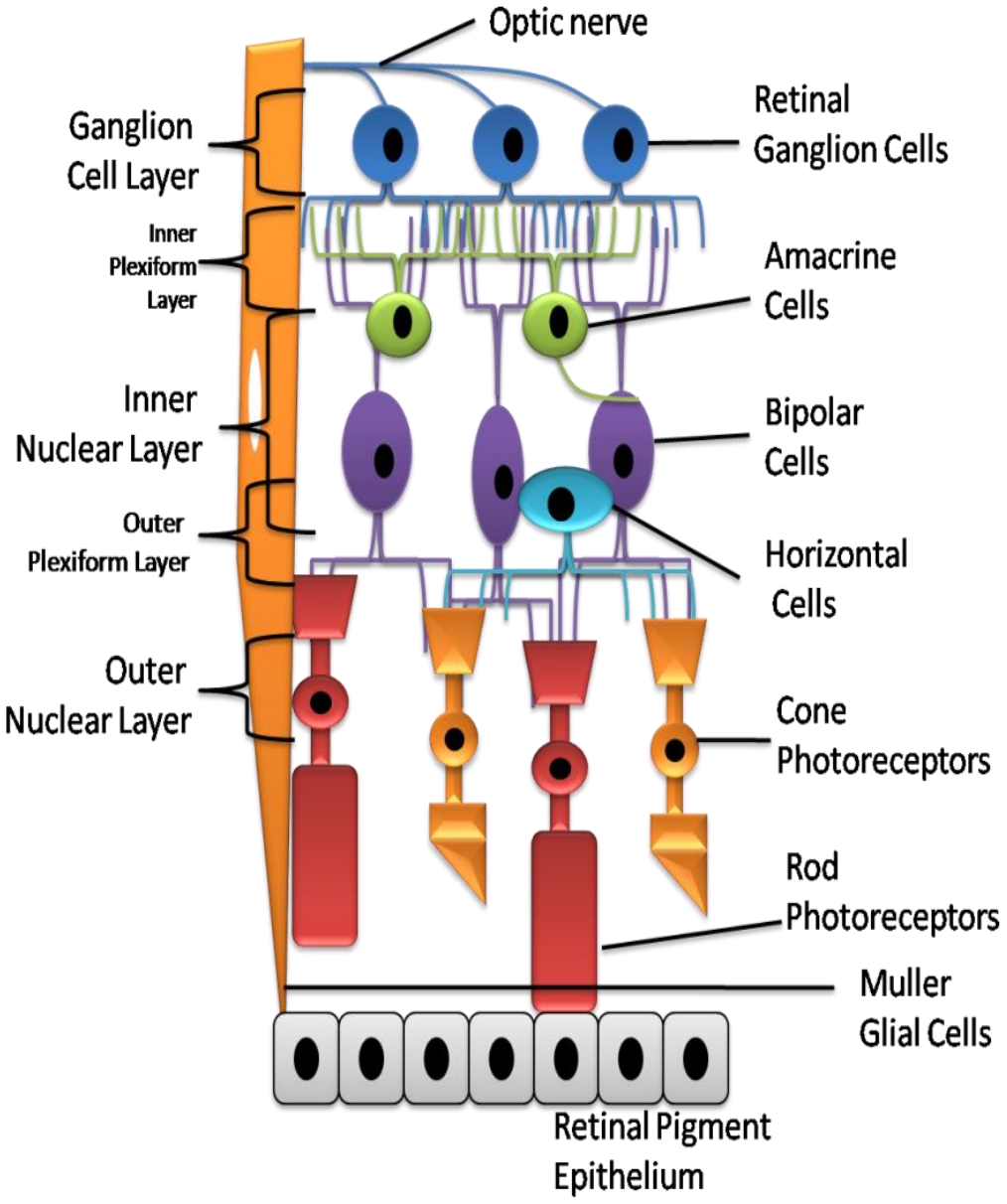


Figure 1: Laminar Structure of the Adult Retina.

Retinal ganglion cells (along with displaced amacrine cells) form the ganglion cell layer. The axons of the retinal ganglion cells form the optic nerve. The amacrine, bipolar and horizontal cells form the inner nuclear layer. The synaptic connections made between the amacrine and bipolar cells to the retinal ganglion cells are found in the inner plexiform layer. The connections made between the bipolar and horizontal cells to the photoreceptors are found in the outer plexiform layer. The nuclei and inner segments of the rod and cone photoreceptors are found in the outer nuclear layer. The outer segments extend towards the outer limits to the retinal pigment epithelium.

1.1.2 Retinal Function

The retina is the photosensitive layer of the eye which is responsible for relaying the images we see to the brain. Light enters the eye via the lens and transverses through the vitreous body in the centre of the eye where it first encounters the RGCs of the retina. Interestingly, processing of the image, via phototransduction, first takes place in the photoreceptors, the outermost cells.

Phototransduction is the biochemical process of converting light into a relay of electrical signals to convey the image we see into a message interpreted by the brain. When a photon of light first hits an opsin, a G-protein coupled receptor, the 11-*cis* retinal chromophore in the opsin undergoes a conformational change. This conformational change of the opsin activates transducin, whose alpha subunit exchanges its bound guanosine-diphosphate (GDP) for a guanosine-triphosphate (GTP) and dissociates from the beta and gamma subunits. The lone alpha subunit-GTP complex activates phosphodiesterase which is an enzyme that turns cyclic guanosine- monophosphate (cGMP) into 5'GMP. By doing so, activated phosphodiesterase lowers the concentration of cGMP, which is required for sodium channels. The decrease in concentration of cGMP results in the closure of these channels and results in the cell hyperpolarizing, due to the potassium influx, which in turn closes voltage-gated calcium channels. Calcium is needed to fuse cell membranes of the vesicles containing neurotransmitters and release their contents to relay the signal cascade. By this principle, the low calcium levels in the hyperpolarized cell decreases the levels of glutamate, an excitatory neurotransmitter, released by the cell. Due to the low amounts of released glutamate, the adjacent ON bipolar cells become depolarized along with OFF bipolar cells becoming hyperpolarized. This signal is then relayed

to the RGCs (and subsequently the axons of the RGCs which form the optic nerve) which uses the combination of ON and OFF signals, to send a message to the brain for interpretation (Neves and Lagnado, 1999).

1.1.3 Murine Retinal Development

Murine eye development begins at embryonic day (E) 8 which is marked by the formation of the optic placode on the inner surfaces of the cephalic neural folds. When the folds fuse at E8.5, the developing forebrain invaginates forming optic vesicles which grow laterally until they contact the surface ectoderm at E9. This contact initiates the surface ectoderm to thicken, giving rise to the lens placode, which will differentiate into the lens structure of the eye starting at day E10. In addition to this, the optic vesicles thicken and invaginate further creating dual-layered optic cups. The inner layer gives rise to the neural retina with the outer layer evolving into the non-light sensitive RPE.

In contrast to the adult retina, the embryonic retina is spatially divided in a different manner throughout retinogenesis. Initially it is a lone layer of cells but starting at E15.5, with the emergence of the GCL, there are two distinct cellular layers, the inner GCL as previously described, and the larger, denser outer neuroblastic layer (NBL). As retinogenesis progresses with differentiation into the various cell types, the NBL will divide into the outer and inner nuclear layers found in the mature retina (Diao et al., 2004; Graw and Loster, 2003).

1.1.4. Retinogenesis

The cell types of the neural retina have a specific birth order that is strictly regulated spatially and temporally in a manner that is conserved across all species. Species differences arise in regards to when during retinogenesis the birth of a specific cell type occurs; that is, if retinogenesis is complete by birth. In the human, the retina is fully developed when the child is born, as opposed to the mouse where retinogenesis is finalized postnatally (Dyer, 2003).

In the retina, the cell cycle is spatially regulated so that different phases of the cell cycle take place in different regions. Progenitor cells, originating from a common pool of neuroblastic cells, undergo mitosis at the apical part of the retina. The resulting cells migrate basally into the neural retina where they will either undergo DNA synthesis and return to the outer surface for addition mitoses, or will leave the cell cycle and undergo differentiation into one of the seven retinal cell types (Dyer et al., 2003b; Young, 1985). As retinogenesis progresses, the progenitor pool decreases with each round of mitosis. This leads to the temporal dependency of the cell type into which the post-mitotic cells will differentiate.

The first cells to differentiate are the RGCs, followed by the horizontal cells, cone photoreceptors, amacrine cells, rod photoreceptors, bipolar cells, and finally, the Müller glia (Dyer, 2003). In the mouse, this process spans from E10 to P14 with considerable overlap amongst the different cell types. All of the differentiated cell types originate from a retinal progenitor pool of cells. Over time, these retinal progenitor cells (RPCs) become restricted to what cell types they can differentiate into; in other words, RPCs are temporally competent.

1.1.5 Competency

What determines the fate of the RPCs, as to whether it is due to extrinsic or intrinsic cues, has come under much investigation. The simple answer is that both types of cues are required for proper differentiation into a specific retinal cell fate.

The balance between the decision to remain in the cell cycle or to differentiate lies with extrinsic and intrinsic influences. During the course of retinogenesis, different factors are present to regulate gene expression for proper differentiation. For example, earlier in retinogenesis, extrinsic cues such as Notch/Delta or sonic hedgehog (SHH) growth factors (Artavanis-Tsakonas et al., 1999; Jensen and Wallace, 1997), would favour cell-cycle progression relative to those for cell-cycle exit. In contrast, during later stages, cell-cycle exit and differentiation programs are favoured (Dyer and Cepko, 2001). It has been shown that intrinsic cues, such as regulation of differentiation genes, are more important to control proliferation in differentiating cells than in cycling stem cells (Belliveau and Cepko, 1999). In essence, temporal regulation changes the environment both intrinsically and extrinsically to promote one cell fate over the other or over mitosis.

The multi-potent RPC pool is believed to follow a competency model such that progenitors only respond to extrinsic cues depending on where they are temporally in retinogenesis. That is, cells in a given competency state are defined intrinsically and will differentiate into a specific subset of cell fates and not into fates that are temporally inappropriate, as illustrated in Figure 2. Extrinsic cues are required for proper generation into the appropriate cell types as the RPCs will respond to the extrinsic influences dependent on their current intrinsic program. For example,

experiments using mid-stage progenitors that are inclined to become cones or amacrine cells will still produce said cell-types regardless if they are placed in an environment where they are not scheduled to differentiate (Belliveau and Cepko, 1999; Belliveau et al., 2000).

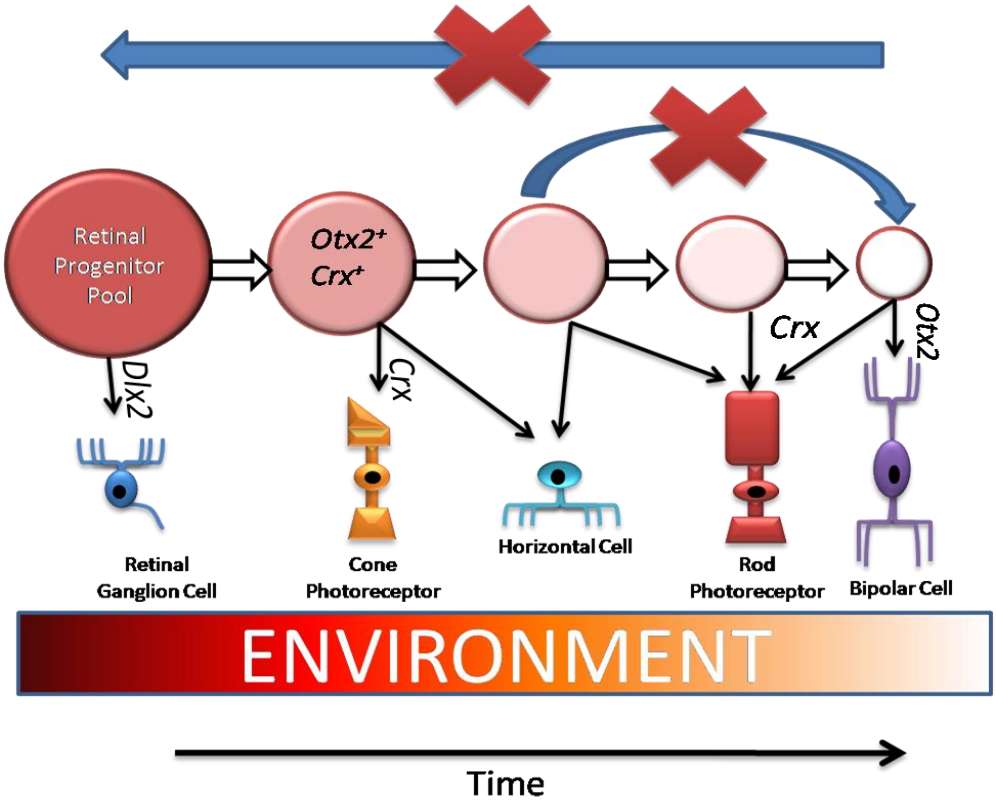


Figure 2: Retinal Competency Model

(Adapted from Cepko) The diagram illustrates that over time the environment changes so that the retinal progenitor cell (RPC) pool (coloured circles) becomes restricted in the both size of the pool and in the potential cell fates of the progenitor cells. As well, the progenitor pool cannot differentiate into cell types that are temporally inappropriate such as a bipolar cell during the time of horizontal cell differentiation which occurs at a later time-point nor can the RPC pool differentiate into an earlier cell fate. *Dlx2* is required to terminally differentiate retinal ganglion cells. *Otx2* and *Crx* are expressed in progenitors which are fated to become cones and rods, and as well *Crx* is required for their terminal differentiation. *Otx2* is also required for the terminal differentation of bipolar cells.

The pathways and interactions of the intrinsic players are complex and poorly understood. *Pax6* (paired homeobox gene 6) homozygous knockout mice present with lack of eyes, demonstrating a 'master regulator' role in establishing eye structure and subsequent retinal differentiation. A conditional knockout of *Pax6* after RPC identity has been established does not affect retinogenesis progression, yet the resulting retina is comprised of mostly amacrine cells at the expense of the other cell types (Marquardt et al., 2001). This lends credence to the theory that *Pax6* is required for maintaining RPC identity and negatively regulates amacrine cell differentiation programs in other cell types.

Similar to the *Pax6* mutants, the *Rx* (retinal homeobox gene) mutants fail to develop eyes. *Rx* has been implicated in a transcriptional network involved in photoreceptor differentiation (Kimura et al., 2000), but due to the severe phenotype of the knockout, little assessment can be made of *Rx*'s true functional role in the progression of retinogenesis until a conditional knockout can be generated successfully.

In addition to establishing and maintaining the RPC pool, the signal to exit the cell cycle is also intrinsically regulated as seen with *Prox1*, the vertebrate ortholog of Drosophila *prospero*. When absent, *Prox1* has a preferential effect on early born cell types (lack of horizontal cells with increased rods and Müller glia) (Cid et al., 2009). When it is misexpressed, an increased number of horizontal and amacrine cells are noted (Dyer et al., 2003b). This favouritism on early-born (horizontal and amacrine) vs. late-born cells (rods and Müller glia) is hypothesized as follows: once a cell begins to express *Prox1*, it leaves the progenitor pool and starts differentiating into a

horizontal cell fate (Cook, 2003; Wigle et al., 1999). If *Prox1* is not expressed, cells will cycle until a late-born signal is induced forcing a higher number of progenitors into becoming into a rod or Müller glia cell fate (Dyer et al., 2003a; Pinto, 2010). PAX6, RX and PROX1 are classic examples of how both extrinsic and intrinsic programs are temporally dependent and tightly regulated for proper retinal differentiation to occur.

1.1.6. Photoreceptor Development

The photoreceptors, comprised of the rods and cones, are required to initiate a phototransduction cascade that allows for proper vision. The rods are used for low-light vision and conversely, the cones are used for bright-light and colour vision. In the embryonic retina, there are two distinct cellular layers; the GCL and the NBL. As retinogenesis progresses, the NBL divides into two nuclear layers, the INL and ONL. In the mature retina, the nuclei of the photoreceptors are located in the ONL and the cell bodies extend towards the back of the retina to the RPE.

Development of the cone and rod photoreceptors occurs at different times during retinogenesis. Cone differentiation begins at E12 and ends at birth while the rods begin differentiation just prior to birth; however, rods are only fully differentiated by P7. Although some of the processes involved in the differentiation of these cell types have been elucidated, photoreceptor differentiation pathways are still under investigation to explain all the mechanisms and players involved.

The first step in photoreceptor differentiation is an extrinsic cue, the activation of the Notch1 transmembrane receptor. Once activated, it is able to bind to its ligand Delta1 (Jadhav et al.,

2006a). Notch1 is expressed in proliferating and non-differentiating cells throughout development with the glial cells, the last to differentiate, retaining Notch1 expression (Jadhav et al., 2009). Through both spatial and temporal conditional knockout studies, the Notch1 receptor acts to repress photoreceptor differentiation. By ablating Notch1 in early retinogenesis, a supernumeracy of cones was observed. Similarly, the absence of Notch1 in late retinogenesis produced only rod photoreceptors (Jadhav et al., 2006b). By over-expressing the Notch1 receptor, cells take on a glial fate; as well, by having a constitutively active Notch1, cells will favour gliagenesis rather than neurogenesis. The *Hes* family of basic helix-loop-helix (bHLH) genes are well explored downstream targets of the Notch pathway and null alleles of *Hes1* and *Hes5* have notable retinal phenotypes. *Hes1* mutants have a considerable decrease in eye size with increased RGCs, amacrine and horizontal cell numbers and decreased Müller glial cells. In addition *Hes1* null mutants have early horizontal and photoreceptor expression (Takatsuka et al., 2004; Tomita et al., 1996). Similar to *Hes1* mutants, *Hes5* knockouts have reduced Müller glial cell numbers but the eyes are of normal size (Hojo et al., 2000; Tomita et al., 1996). These results support the theory that the Notch-*Hes* relationship, along with additional downstream effectors of the Notch pathway, is important for maintaining the RPC population and in deciding between a neuronal or a glial cell fate.

The presumptive photoreceptor cells that are born late in embryogenesis are kept in an undifferentiated state by STAT3 activation (Ozawa et al., 2008; Ozawa et al., 2007; Ozawa et al., 2004). By birth, STAT3 becomes inactivated due to the down-regulation of its ligand CNTF (cillary neurotrophic factor) and increased levels of SOCS3 (suppressor of cytokine signalling 3)

(Ozawa et al., 2007). This inactivation is followed by the expression of rhodopsin and the subsequent differentiation into rod photoreceptor cells.

The opsin genes, rhodsopsin and cone-opsins, are required for final differentiation into either a rod or cone photoreceptor, respectively. However, in order for these genes to be expressed, critical transcription factors are required, CRX (cone-rod homeobox gene) and OTX2 (orthodenticle homeobox 2 gene) (Furukawa et al., 1997; Nishida et al., 2003). Therefore, the mechanisms and pathways of how these two transcription factors are regulated and their interaction with one another is essential to understanding proper retina and photoreceptor development.

1.2 DLX GENES IN DEVELOPMENT

Dlx (distalless homeobox gene) genes are the vertebrate orthologs of *Drosophila distalless* (Dll) and encode homeobox transcription factors. The third α-helix of the homeodomain recognizes and binds to ATTA/TAAT DNA sequences where, in complex with other transcription factors they act as a 'molecular switch' to either activate or repress target gene transcription (Levine and Hoey, 1988).

1.2.1 Dlx gene family

The *Dlx* gene family is comprised of six family members in the human and mouse; *Dlx1*, *Dlx2*, *Dlx3*, *Dlx5*, *Dlx6* and *Dlx7* (*DLX4* in Human). These six members can be divided into two clades based on the similarity of protein motifs encoded by the genes. *Dlx1*, *Dlx6*, and *Dlx7* belong to one clade with the *Dlx2*, *Dlx3* and *Dlx5* belonging to a second clade. In addition to the shared

homeodomain and carboxyl structures, the second clade have a common short conserved amino acid sequence at their amino-terminii (Akimenko et al., 1994; Roberson et al., 2001; Stock et al., 1996; Zerucha and Ekker, 2000).

It is believed that in the evolution of vertebrates, duplication events occurred resulting in 3 pairs of *Dlx* genes. The family members, in both human and mouse, arrange in 5'to 3', 3' to 5' bigenic clusters with each clade aligning vertically and linked to a *Hox* gene cluster (Stock et al., 1996). *Dlx1* and *Dlx2* are associated with *Hoxd*, *Dlx6* and *Dlx3* to *Hoxa* and *Dlx5* and *Dlx7*(4) to *Hoxb* (McGuinness et al., 1996; Nakamura et al., 1996; Stock et al., 1996).

The organization of the transcripts of each of the *Dlx* genes is also conserved as they all have 2 introns and 3 exons with exons 2 and 3 encoding the homeodomain. Studies have shown that multiple transcripts can arise due to splice variants or alternate transcriptional start sites. Localization of DLX proteins has also come under scrutiny as DLX5 has been found in the cytoplasm when not encoded with a nuclear localization signal (Eisenstat et al., 1999).

1.2.2 Expression and function of Dlx Genes

The *Dlx* genes are expressed in a variety of tissues and organ systems. In the mouse, about mid-gestation, all the *Dlx* genes are expressed, but primarily in the surface ectoderm and nervous system. DLX1, DLX2, DLX5 and DLX6 are all expressed, with overlap, in the developing forebrain (diencephalon and telencephalon). The *Dlx* genes are expressed in a temporal order, first with *Dlx2* followed by *Dlx1*, *Dlx5* and finally *Dlx6*. The genes are also regulated spatially as DLX1, DLX2 and DLX5 are all expressed in the cells of the subventricular zone (SVZ) with

DLX5 and DLX6 expression in post-mitotic cells (Anderson et al., 1997a; Anderson et al., 1997b; Eisenstat et al., 1999; Panganiban and Rubenstein, 2002).

In the branchial arches, an anlage for craniofacial skin and connective tissue, the same four *Dlx* family members implicated in the forebrain are also expressed along the proximodistal axis (Anderson et al., 1997b; Qiu et al., 1995). The temporal sequence follows as in forebrain. *Dlx* genes are also expressed in sensory organs such as the otic placode, olfactory bulb and the developing retina (Panganiban and Rubenstein, 2002). The overlapping and temporal similarities between tissues strengthen the hypothesis for redundancy amongst DLX family members.

The multiple roles of *Dlx* genes are elucidated by loss-of-function mouse models and gain-of-function studies. Unfortunately, these mouse models, especially those where both *Dlx* genes within a bigenic cluster (i.e. *Dlx1* and *Dlx2*) are knocked out, result in death at or shortly after birth (Anderson et al., 1997b). The cause of death is still unknown. By characterizing the various knockouts, one can determine the regulation by *Dlx* genes of downstream effectors, i.e. whether they are directly or indirectly activated or repressed by DLX transcription factors.

1.2.3 Dlx1/2 genes in retina and forebrain

Retina

DLX1 and DLX2 have been reported to be expressed in the developing murine retina with onset of expression at E11.5 (de Melo et al., 2008). Both transcription factors are localized to the nucleus and expressed throughout the embryonic neural retina, with greater numbers of cells

expressing these genes in the outer retina compared to the inner retina. Also, expression is limited to the central retina with distinct boundaries at the peripheral regions which become decreasingly distinct with age. By birth, DLX1 and DLX2 begin to differ in their retinal expression patterns; DLX1 becomes localized solely to the GCL, where DLX2 is expressed in the GCL and the innermost portion of the NBL. By adulthood, DLX1 expression is no longer detected whereas DLX2 is expressed in nuclei of the GCL and the now differentiated INL. The proportion of retinal cells that are DLX2 positive also changes over time with the highest expression values at E13 and in adulthood (de Melo et al., 2003).

Co-expression with a variety of cell-specific markers identifies in which subset of INL and GCL cells DLX2 is expressed. As mentioned, the INL contains the horizontal, amacrine and bipolar cells and the GCL contains the RGCs and displaced amacrine cells. DLX2 co-localizes with syntaxin, an amacrine cell marker, at P0 with increasing numbers of co-expressing cells by adulthood (de Melo et al., 2003). As well, DLX2 co-expresses with markers for horizontal cells both embryonically and in adulthood (de Melo et al., 2003). DLX2 also co-localizes with GAD65-, GAD67-, and GABA-expressing neurons in both the P0 and adult stage further implicating *Dlx* genes and the developing CNS (Le et al., 2007).

The majority of cells expressing DLX2 are found in the GCL. Using markers specific for RGCs, such as Brn3b, DLX2 is not co-expressed with Brn3b at E13. However, after E16, DLX2 and Brn3b co-localize without Brn3b single positive cells, supporting the hypothesis that DLX2 is required to differentiate and maintain `late-born` RGCs (de Melo et al., 2005).

The retinal phenotype of the *Dlx1/2* double knockout retinal phenotype was investigated. DLX2 is expressed in the horizontal, amacrine and RGCs. Of these cell types, *Dlx1* and *Dlx2* loss-of-function only affects the RGCs. Unaffected INL cell type differentiation may be explained by the redundancy of DLX5 that is expressed in the neural retina (G Du and D Eisenstat, unpublished observations). RGC numbers decrease by a third coinciding with a 33% and 66% increase in apoptosis at E13.5 and E16.5, respectively, in the DKO. As the axons of the RGCs form the optic nerve, comparison of optic nerve thickness between WT and DKO was analyzed and a 23% decrease was observed (de Melo et al., 2005). However, *Dlx1/2* DKO mice die at birth, so characterization is limited to those cell types which are born and differentiated by birth.

As *Dlx* genes are transcription factors, retinal genes are potential targets for *Dlx* gene regulation. Using assays such as chromatin immunoprecipitation (ChIP), one can determine which potential ATTA/TAAT sites DLX proteins occupy on promoter regions of target genes *in vivo*. DLX2 has been shown to occupy sites on the R1-pro promoter region of the *TrkB* gene. TrkB is a neurotrophin receptor that preferentially binds Brain Derived Neurotrophic Factor (BDNF) and is involved in RGC cell death during neurogenesis (de Melo et al., 2008; Ma et al., 1998). In addition, DLX2 binds to and activates the transcription of RGC differentiation genes *Brn3a* and *Brn3b* (Q. Zhang and D. Eisenstat, submitted)

The loss of *Dlx1/2* in the retina affects late-born cell types. Photoreceptors are classified as being ‘late-born’ and so, it was hypothesized that the photoreceptor lineage is the default pathway if the ganglion cell lineage was somehow interrupted. This is consistent with the competency model, so that the RPC pool at a given time in retinogenesis cannot differentiate into an ‘earlier’

cell-type. *Crx* is a homeobox transcription factor required to differentiate retinal progenitors into rods or cones (collectively termed 'photoreceptors'). In the *Dlx1/2* DKO, an increased and ectopic expression of the *Crx* transcript was observed at E18.5 shown in Figure 3 (de Melo et al., 2005). This suggests that either DLX1 and/or DLX2 play a role either directly or indirectly in the repression of *Crx* expression. Characterization of the *Dlx1* and *Dlx2* single knockout retinas are currently in progress.

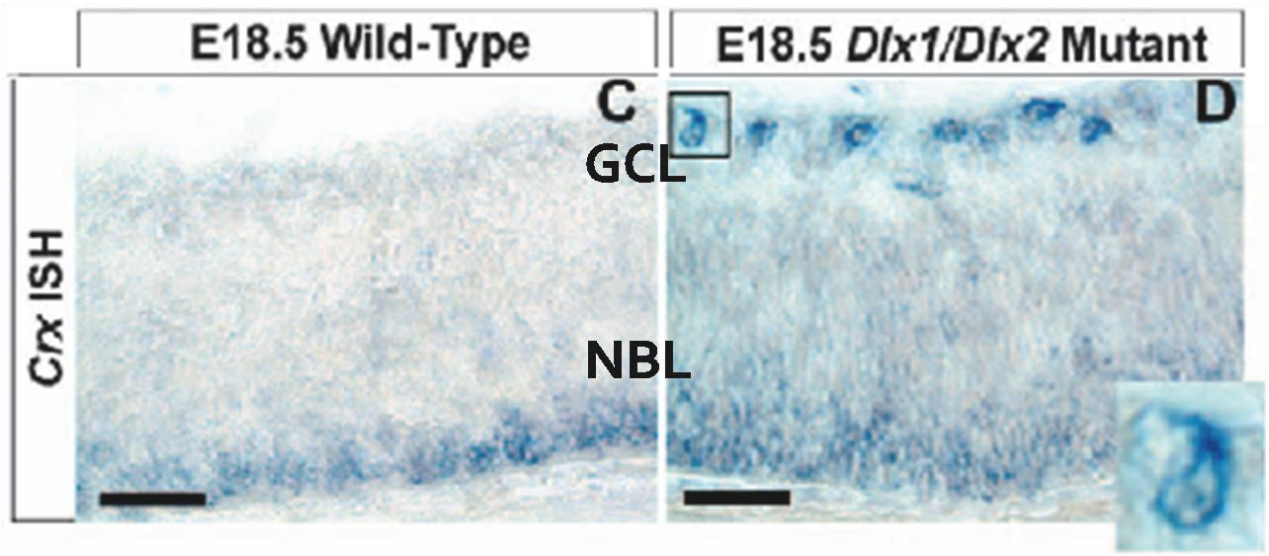


Figure 3: Comparison of *Crx* expression on E18.5 WT and *Dlx1/2* DKO Retina

In situ hybridization was performed on E18.5 wild-type and *Dlx1/2* double knockout tissue to visualize *Crx* expression using digoxigenin-UTP labelled *Crx* riboprobes (*Crx* cDNA courtesy of Dr. C Cepko). Wild-type tissue shows that normative *Crx* expression is limited to the outermost region of the neuroblastic layer (NBL). In the *Dlx1/2* DKO (panel D), *Crx* expression is upregulated in the NBL and ectopically expressed in the ganglion cell layer (GCL). Inset is a 3-fold magnification (de Melo et al, 2005).

Forebrain

In addition to DLX5 and DLX6, DLX1 and DLX2 are also expressed in the developing forebrain. *Dlx1* and *Dlx2* are expressed in the ventricular zone (VZ) and SVZ of the forebrain and are similar to each other in expression pattern, although more DLX2 expressing cells are

present in the VZ (Eisenstat et al., 1999). *Dlx5* and *Dlx6* are expressed in the SVZ and mantle zones. However, they are more localized to differentiated cells (Anderson et al., 1997b; Simeone et al., 1994). Loss-of-function mutants have been generated for *Dlx1* and *Dlx2*. With the exception of the *Dlx2* mutant having loss of tyrosine hydroxylase-expressing olfactory bulb interneurons, neither of these mouse models presented with major defects in the forebrain. This suggests redundancy between *Dlx* family members, albeit not complete redundancy (Anderson et al., 1997b; Qiu et al., 1995). To resolve this issue, a *Dlx1/Dlx2* double knockout mouse was generated and characterized (Anderson et al., 1997b). "Early-born" cells, those born before E12.5, are unaffected; however, those that arise after E12.5, specifically GABAergic interneurons, are not completely differentiated and remain in the SVZ due to tangential migration defects (Anderson et al., 1997a; Anderson et al., 1997b). Downstream targets are also affected as *Dlx5*, *Oct6* and *Brn4* are absent in the SVZ and *Lhx2* is no longer restricted to the VZ (Anderson et al., 1997b). DLX1/2 have been shown to directly bind to the *Dlx*5/6 intergenic enhancer and activate their expression (Zhou et al., 2004) . Conversely, expression of Neuropilin2 (*Nrp2*), a receptor involved in inhibition of neuronal migration, is up-regulated and aberrant in the *Dlx1/2* DKO. *In vivo* and *in vitro* assays determined that DLX1/2 protiens bind directly and repress *Nrp2* expression, respectively. This is the first report of *Dlx* genes acting as transcriptional repressors of target genes *in vivo* (Le et al., 2007).

1.3 OTX GENES (Pinto, 2010)

1.3.1 Family

The mammalian orthologs of the *Drosophila* orthodenticle (Otd) family, *Otx* (orthodenticle-like homeobox gene) genes encode paired-class homeodomain proteins. The family is comprised of three family members: *Otx1, Otx2* and *Crx* (cone-rod homeobox gene) located on mouse

chromosomes 11, 14 and 7, respectively. *Otx1* and *Otx2* have a high degree of homology; with a length of 355 and 289 aa, respectively, the first 98 residues inclusive of the amino-terminus are very similar (Simeone et al., 1993). In addition to the 37 aa upstream of the homeodomain, their homeodomains also share a high degree of homology. CRX also has great similarity to its family members (Figure 4). The 289 aa long CRX gene product has 45% homology to the 38 aa amino-termini of the other family members as well as 88% and 86% homology to the homeodomains of OTX1 and OTX2, respectively (Furukawa et al., 1997). In addition, the recognition helix of the homeodomain contains a lysine in the 9^{th} residue which is also found in other *Otx* family members. The residues on the carboxyl-end of the homeodomain share a 19% and 27% similarity to OTX1 and OTX2, respectively. A WSP motif, named in recognition of the high number of tryptophan (W), serine (S) and proline (P) residues, is a 10-residue domain at the carboxy-terminus with unknown function. 90% of this domain is shared between CRX and its other family members (Rivolta et al., 2001). Finally, at the carboxyl end of the protein, a DPLDYKDQSAWK sequence is found. With a slight discrepancy and tandem repetition, this sequence is also found at the carboxy-termini of OTX1 and OTX2 and has been named as the "Otx tail". This distinct 'tail' motif along with the homologous homeodomains, support the inclusion of these members in one family. (Furukawa et al., 1997)

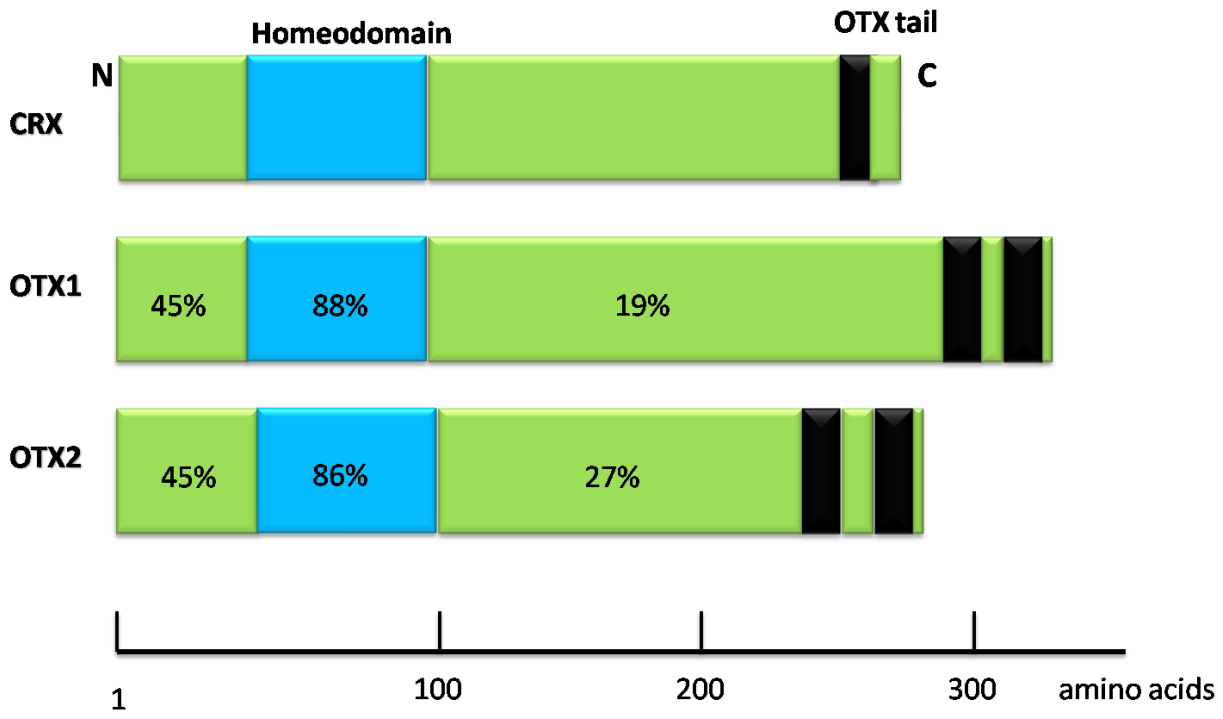


Figure 4: Homology between OTX Family Members

(Adapted from Furukawa 1997). Comparisons between CRX, OTX1 and OTX2 transcription factors. Shaded areas indicate the homeodomain along with 'Otx tail' regions (black boxes). CRX shares a 45% homology with the N-termini of both OTX1 and OTX2 in addition to 86-88% with regards to homeodomain similarity.

1.3.2 Expression

During embryonic development, OTX2 is the first family member to be expressed at E6.5. It is found in the epiblast and visceral endoderm before gastrulation and in the rostral neural plate and axial mesoendoderm post-gastrulation (Boyl et al., 2001). As development progresses, OTX2 expression becomes localized to the anterior neurectoderm which is fated to differentiate into the forebrain and midbrain. *Otx1* is transcribed after *Otx2* at E8.5 in the presumptive forebrain and midbrain neuroepithelium (Simeone et al., 1993) and shares expression domains with OTX2. OTX1 alone is expressed down the dorsal telencephalon starting at E11.5, and so, these neuronal cells are fated to become deep cortical layers (Acampora et al., 2000).

In addition, OTX1 and OTX2 are also expressed in primordial olfactory, acoustic and ocular sensory organs (Acampora et al., 2000). Specifically in the eye, OTX1 is expressed in the cillary process, primordial tear duct and iris (Simeone et al., 1993) and by E11.5, OTX2 expression is found in the retina and RPE (Fossat et al., 2007).

Crx is first expressed at E12.5 and is maintained throughout adulthood (Furukawa et al., 1997). As implied by the name, CRX is expressed in progenitors fated to become rod or cone photoreceptors. In addition to retinal expression, CRX is also expressed in the pineal gland (Chen et al., 1997). The pineal gland is the organ responsible for the sleep-wake cycle and also comprises of cells that contain photosensitive opsins.

1.3.3 Function

As all *Otx* genes encode a homeodomain, their primary function is to act as transcriptional regulators of downstream targets. The function of *Otx* genes during development has long been under investigation. Due to the phenotype of null mutants, conclusions have been mainly drawn from gene expression patterns. *Otx1* null mice developed epilepsy (Acampora et al., 1996). Morphological and histological analysis revealed abnormalities in brain development such as a decrease in the dorsal telencephalic cortex with an increase in the colliculi of the mesencephalon (Acampora et al., 1996). *Otx2* knockout mice are embryonic lethal and lack rostral neuroectoderm structures which are destined to be forebrain, midbrain and rostral hindbrain, including absence of eye development. As *Otx2* is required for proper anterior-posterior patterning, these mice also have abnormalities in their body plan such as a lack of anteriorization of the anterior visceral endoderm (Acampora et al., 1998; Acampora et al., 1995; Ang et al., 1996; Kimura et al., 2001). Specifically, *Otx* genes act at the midbrain-hindbrain boundary to properly position the isthmic organizer and *Fgf8* and *Shh* and are thus crucial for proper brain regionalization (Acampora et al., 2005). The function of *Otx* genes in the retina will be discussed in more detail in the following section.

1.3.4 Otx genes in retina: Otx2 *and* Crx

In mammalian retinal development, the family members *Otx2* and *Crx* are critical for proper photoreceptor formation.

Otx2 is first expressed in the mouse at E6.5 throughout adulthood (Fossat et al., 2005), but is first detected at E11 in the embryonic murine retina. In the adult mouse retina, OTX2 is expressed in the nuclei of the bipolar cells and in the retinal pigment epithelium but has a peri-nuclear localization in the photoreceptors (Fossat et al., 2007). As *Otx2* is critical for anterior patterning in early embryogenesis, a traditional knock-out mouse is problematic to study a retinal phenotype as these mice are embryonic lethal and lack rostral brain structures (Matsuo et al., 1995). A conditional knockout (CKO) mouse in which *Otx2* is down-regulated under the control of the *Crx* promoter, a gene expressed in the retina and pineal gland, has been constructed to ablate *Otx2* expression solely in the retina (Nishida et al., 2003). In the $Otx2^{-/-}$ CKO retinas, there was an increase in amacrine cells at the cost of the rods and cones. Also, the eyes from the $Otx2^{-/-}$ CKO were half the size of the control mice, a condition known as microphthalmia, which was determined to be due to an increase in apoptosis. There have been reports of this condition in humans being associated with a deletion on chromosome 14 near the *OTX2* gene (Hever et al., 2006). It has been theorized that besides being a transcriptional regulator, *Otx2* is also required for the survival of photoreceptor precursors and those precursors which evade apoptosis become committed to an amacrine cell fate. The genetic regulation by OTX2 on *Crx* expression was also evident in the $Otx2^{-/-}$ CKO mice as *Crx* transcript was down-regulated in the absence of *Otx2* expression, supporting an activator role on *Crx* expression. Retroviral over-expression of *Otx2* demonstrated an increase in rods and a decrease in amacrine and Müller glial cells. In addition, ectopic *Otx2* decreased the quantity of bipolar cells (Nishida et al., 2003). This indicates that not only is there a critical role for *Otx2* in the regulation of photoreceptor fate but also for bipolar cells. The relative spatial and temporal expression levels of OTX2, such as mis-expression versus a complete loss, is essential in determining the cell fate to which a retinal progenitor will

commit. That is, in normal development, OTX2 is required for the cell fate determination and terminal differentiation of photoreceptors; however, relative to *Crx*, OTX2 plays a minimal role in the latter function (Figure 5A). Conversely OTX2, in concert with CRX, is required for terminal differentiation of bipolar cells. Misexpression of *Otx2* causes postmitotic cells to choose a photoreceptor cell fate and so, results in a supernumeracy of photoreceptors at the cost of the bipolar cells and amacrine (Figure 5B). In the case of the complete absence of *Otx2* (Figure 5C), photoreceptor-fated cells are obligated to become amacrine cells. As OTX2 is an activator of *Crx*, CRX becomes downregulated thus resulting in a decrease of photoreceptors and increasing amacrine cell numbers. Bipolar cells also decrease in number when *Otx2* is absent as it is required for terminal differentiation (Koike et al., 2007).

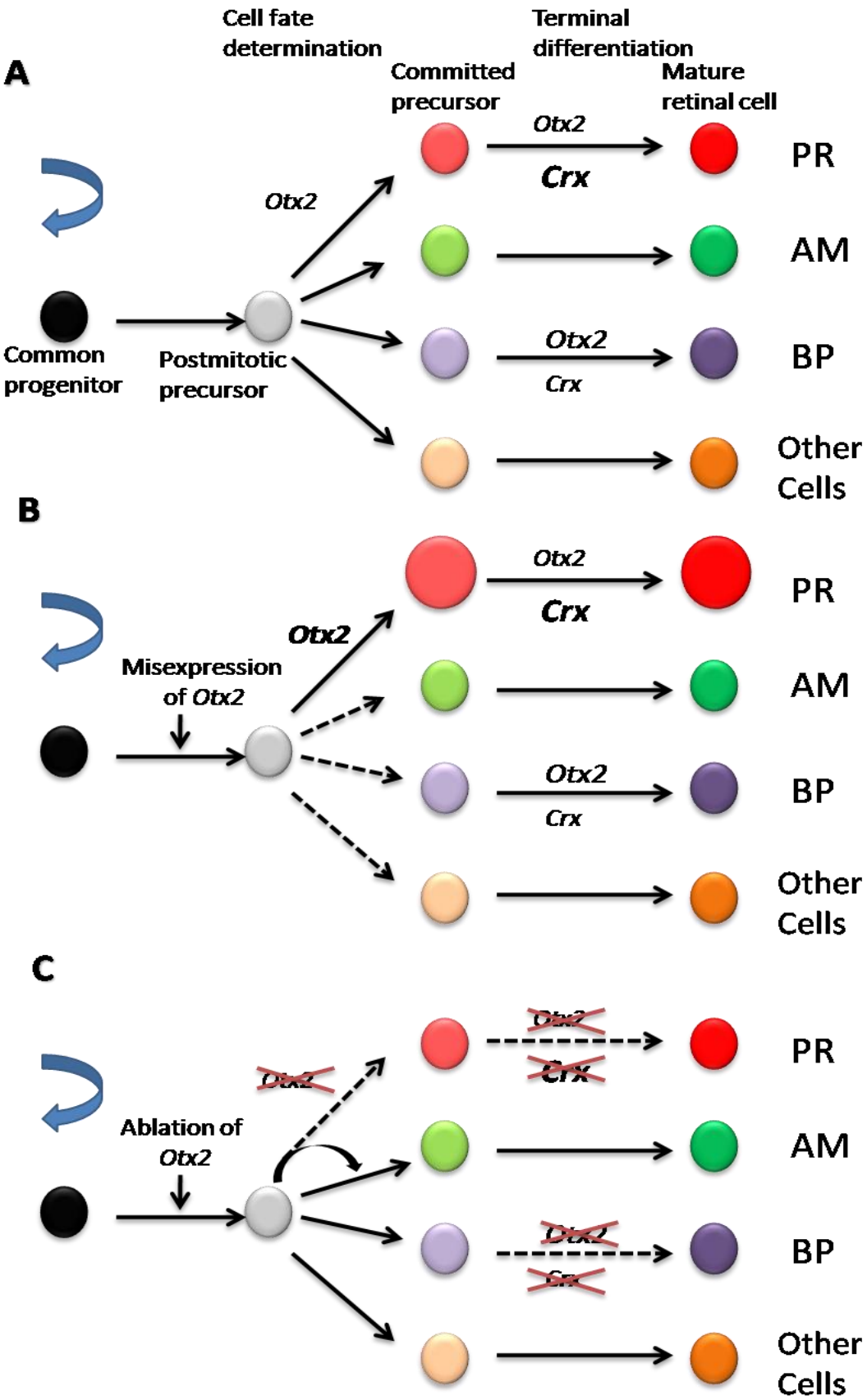

A
Cell fate determination
Terminal differentiation
Committed precursor
Mature retinal cell
Otx2
Crx
PR
Otx2
AM
Otx2
Crx
BP
Common progenitor
Postmitotic precursor
Other Cells
B
Otx2
Crx
PR
Otx2
Misexpression of Otx2
AM
Otx2
Crx
BP
Other Cells
C
Otx2
Crx
PR
Otx2
Ablation of Otx2
AM
Otx2
Crx
BP
Other Cells

Figure 5: OTX2 Function in photoreceptor and bipolar differentiation

(adapted from Koike, 2007) **Panel A** demonstrates that during normal development, OTX2 is required to commit and terminally differentiate cells to a photoreceptor fate in cooperation with CRX. However CRX has a larger role relative to OTX2 in photoreceptor differentiation. In the bipolar lineage, however, OTX2 (along with CRX) is required for terminal differentiation of cells rather than determination. **Panel B** illustrates the consequence of OTX2 misexpression. Post-mitotic cells 'switch' to the photoreceptor pathway increasing their numbers at the cost of the other cell fates OTX2 is involved with. When OTX2 is ablated as illustrated in **Panel C**, CRX is absent as a result and so cells fated to become photoreceptors switch to the amacrine cell fate. As OTX2 and CRX are required for terminal differentiation of bipolar cells, these cells eventually become downregulated. Both insults to these pathways results in an increased amount of amacrine cells. Photoreceptors (PR), Amacrine cells (AM), Bipolar Cells (BP)

Crx is first expressed at E12.5 in the mouse retina and is localized to the nuclei of the NBL found near the RPE (Furukawa et al., 2002). The cells expressing *Crx* are considered to be progenitors committed to a photoreceptor cell fate. By binding to a conserved sequence of C/TTAATC/T, known as the CRX binding element (CBE 1) CRX regulates downstream targets such as rhodopsin, arrestin and interphotoreceptor retinoid-binding protein (Chen et al., 1997; Furukawa et al., 1997). Depending on its protein binding partners, CRX can either act as a transactivator or as a repressor. For example, when bound with Nrl it acts synergistically to activate rhodopsin (Chen et al., 1997) . Conversely when CRX interacts with Nr2e3, it acts to repress cone opsin expression to promote rod differentiation (Oh et al., 2008). Interestingly, in the *Crx* homozygous knockout mouse, the retinal phenotype is comparative to that of wild-type retinas until 2 weeks postnatally where the outer segments fail to form followed by degeneration of the photoreceptors (Furukawa et al., 1999). This suggests that the role of CRX is critical for terminal differentiation and that there is another player required for the determination of a photoreceptor cell fate. Alternatively, by overexpressing CRX, via retroviruses, a marked increase of rod photoreceptors was observed along with a significant decrease in amacrine and Müller glial cells. However, unlike *Otx2* overexpression, no difference was observed in bipolar cells (Furukawa et al., 1997).

As OTX2 mutations have been linked to human ocular malformations, so has CRX been implicated (Rivolta et al., 2001). *CRX* dominant mutations, whether frameshift or missense, have been linked to degenerative disorders such as cone-rod dystrophy and retinitis pigmentosa. Leber's congenital amaurosis, a cause of congenital blindness, has also been linked to CRX

mutations (den Hollander et al., 2008; Rivolta et al., 2001). However, genotype-phenotype predictions are still under study.

Investigation into the complex network that regulates these genes is ongoing. As stated, OTX2 has been elucidated to activate *Crx* whereas another homeobox protein, PAX6, has been shown to repress *Crx* expression (Oron-Karni et al., 2008). There has been evidence to suggest that the regulation of *Otx2* expression in the retina is under direct control of the Wnt/β-catenin pathway (Fujimura et al., 2009). Interestingly, naturally occurring antisense transcripts for both *Otx2* and *Crx*, as well as other retinal genes, also function to properly control the expression of these genes (Alfano et al., 2005).

1.4 Hypothesis and Research Aims

Hypothesis

We hypothesized that the DLX1/2 homeobox genes restrict the expression of *Otx2* and *Crx* both temporally and spatially during retinal development.

Specific Aim 1: Determine the expression of OTX2 and CRX in normal development as well as in *Dlx1/Dlx2* mutant embryonic retina.

The expression patterns of OTX2 and CRX will be demonstrated by immunohistochemistry and immunofluorescence during embryonic retinogenesis. All analysis will be done on mouse retina tissue sections using antibodies specific for OTX2 and CRX.

Specific Aim 2: Characterization of molecular interactions of DLX2 on the *Otx2* and *Crx* gene promoters.

Chromatin immunoprecipitation (ChIP) studies will be performed to determine whether DLX2 binds to the promoter regions of *Otx2* and *Crx*. To confirm this binding, electrophoretic mobility shift assays (EMSA) using recombinant DLX2 protein will be utilized.

Specific Aim 3: Demonstrate the functional consequences of DLX2, interacting with the *Otx2* and *Crx* promoter *in vitro* and *in vivo*

The functional consequences of DLX2 protein binding to the *Otx2* and *Crx* promoter will be determined by luciferase reporter gene assays using human embryonic kidney cells (HEK 293). RNA isolated from wild-type and mutant eyes will be quantified by qRT-PCR to assess the loss of *Dlx2* function on the expression of *Otx2* and *Crx* transcripts *in vivo*.

2. MATERIALS AND METHODS

2.1. Animal and tissue preparation

All tissues were obtained from the mouse animal model (*Mus musculus*) with wild type and mutant tissues in the CD-1 (ICR) BR Swiss background (Charles River Laboratories, Worcester, MA, USA). Dr. John Rubenstein (University of California at San Francisco, USA) graciously provided *Dlx1/Dlx2* double heterozygous mice to establish the DKO colony at the University of Manitoba. All animal protocols were conducted in accordance with guidelines set by the Canadian Council on Animal Care and were approved by the University of Manitoba Animal Care Committee.

To collect embryos at various points during development, timed-pregnant mice were used and embryonic age was determined by presence of a vaginal plug after breeding (deemed as embryonic day 0.5). Pregnant mothers were euthanized by cervical dislocation before removing embryos which were euthanized via decapitation. Tissues were kept in cold PBS and eyes were dissected using a stereomicroscope. Tails of each embryo were kept for genotyping, which was accomplished via PCR with specific primer pairs for the *Dlx1/2* DKO mice (Anderson et al., 1997b). The tails were incubated overnight at 65^{o}C in 80 µl of tail lysis buffer (100 mM NaCl, 50 mM Tris-HCl, 50 mM EDTA, 0.5% SDS, pH 8.0) in addition to 0.1 µg/µl Proteinase K. The next day, in order to deactivate the Proteinase K, samples were heated in boiling water for 10 minutes and then centrifuged at maximum speed (13,000 rpm) for 1 minute. Supernatant was collected and 0.5µl of this solution was used as template for each PCR reaction. PCR reactions were carried out using HotStar DNA polymerase (Qiagen).

2.2. Tissue embedding and sectioning

Eyes were removed (enucleated) from embryos of developmental stages E15 and older. Whole-embryonic heads were used for eyes needed at younger embryonic ages as the eyes were too small to be enucleated. Once dissected, tissues were washed in 1xPBS and then fixed in 4% paraformaldehyde (PFA) on a rotating shaker at 4^{o}C for 1 hour (E18.5), 45 mins (E15.5-16.5), overnight (heads) or for 3 hours (adult eyes). After fixation, tissues were prepared for cryopreservation via a sucrose gradient (10%, 15%, and 20%) at 4^{o}C until the saturated tissue sank to the bottom of the tube. Before embedding, the tissue was incubated at 4^{o}C in a 1:1 solution of 20% sucrose and Optimal Cutting Temperature compound (OCT) for 30 mins. Using dry ice and cold 2-methyl-butane, plastic embedding moulds were cooled and filled with OCT, with the eyes oriented with the optic nerves facing each other and the lens facing outwards. This was to mimic the orientation of the eyes as they are in the mouse, and to optimize the maximum amount of sections within each retina. These blocks were then stored at -80°C. The ThermoShandon Cryostat was used to cut frozen blocks of tissue into thin sections (12µm). Cut sections were placed on Fisherbrand® Superfrost Plus slides; every tenth slide was kept aside for reference staining by cresyl violet with the remaining slides stored at -80°C.

For immunostaining of gain-of-function (GOF) retinas, full-length *Dlx2* cDNA was cloned into a pCIG-GFP plasmid. At E14.5, plasmids were injected into each embryo's eye through the uterine wall and electroporated. The embryos were replaced into the mother and allowed to gestate until sacrifice date (E18.5). Eyes were enucleated and fixed as described above (Zhang Q et al, submitted).

2.3 Histological staining and immunofluorescence (IF)

Cresyl Violet staining (for reference slides)

To stain the slides with cresyl violet for reference purposes, slides were immersed in the stain for 1-2 minutes followed by a rinse with double distilled water. Dehydration of the tissue was achieved by putting the slides through an ethanol gradient (50-100%) at 2 minutes in each concentration. After a final immersion in Xylene, slides were mounted using Permount (Fisher Scientific) and cover slips.

Immunostaining

Slides were removed from -80°C and are left to air-dry for 5-10 mins. Using a waterproof PAP pen, the tissue was encircled to contain the solutions to be used. The tissue first went through a blocking process to prevent non-specific binding. Slides were incubated for 2 hours at room temperature in blocking solution (0.1% BSA, 0.2% Triton X-100, 0.02% Sodium Azide, 5% Horse Serum in 1xPBS pH7.4). After blocking, the primary antibody (Table 1) diluted in blocking solution was applied and incubated at 4°C overnight. As a negative control, one slide remained with the blocking solution and without primary antibody. The following day all slides, at room temperature, including the negative control (which are processed with the experimental slides from this point forward), were washed 3 times with 1xPBS for 5 minutes. After washing, the secondary antibody (Table 2) was diluted in blocking buffer, applied and incubated for 2 hours at room temperature in the dark followed by washes as previously described. To mount the slides with cover slips, VectaShield with DAPI was used. Commercial, clear nail-polish can be

used to adhere edges of the cover slips to the slide. The covered slides were stored at 4°C until ready for imaging.

Double immunofluorescence

The procedure for a dual immunofluorescence (DIF) is similar to a single immunofluorescence experiments. The tissue was dried, marked and blocked for 2 hours at room temperature with the first primary antibody incubating at 4°C overnight. Washing and application of the secondary antibody followed on Day 2 as previously described. Instead of mounting, the second primary was applied and incubated overnight at 4°C. Day 3 followed with washing and application of the second secondary antibody which has a different fluorochrome to avoid overlapping wavelengths which result in false positives. After the second secondary antibody, one last round of washing was performed and then the slides were mounted as described previously.

If the primary antibodies originated from the same host, the method described previously (DIF over 3 days) was used with a slight amendment. Instead of using a fluorochrome-conjugated secondary, a biotinylated secondary antibody was used to detect the first primary antibody. After a 2-hour incubation at RT, slides were washed and are then incubated with a tertiary flurochrome for another 2 hours at RT. After washing, the second primary antibody was applied and the procedure was followed as before. Slides were viewed and imaged using an Olympus BX51 Microscope, Olympus DP70 Digital Camera System and ImagePro Plus software (Media Cybernetics).

Gain-of-function assays (GOF)

To achieve over-expression of DLX2, full-length *Dlx2* cDNA was cloned into a pCIG-GFP plasmid. This plasmid contains a chicken β-actin promoter with the CMV enhancer to drive *Dlx2* expression. At E14.5, plasmids were injected into the eyes of each embryo of C57/BL6 mice (with pigmented RPE) through the uterine wall and electroporated as in previous studies (Garcia-Frigola et al., 2007). Embryos were replaced into the mother and allowed to gestate until the sacrifice date (E18.5). A plasmid with GFP-only was used as a control.

Table 1: Primary antibodies used for immunofluorescence

Primary Antibody	Dilution	Source
Rabbit anti-DLX2	1:400	Purified in-house by Dr. Eisenstat and Dr. J. Rubenstein
Rabbit anti-CRX	1:1000	Gift from Dr. Cheryl Craft
Rabbit anti-PAX6	1:1000	Covance
Goat anti-OTX2	1:200	Santa Cruz
Goat anti-Brn3b	1:200	Santa Cruz
Mouse anti-Brn3a	1:200	Santa Cruz
Mouse anti-Syntaxin	1:10000	Sigma

Table 2: Secondary antibodies used for immunofluorescence

Secondary Antibody	Dilution	Source
Biotinylated goat anti-rabbit	1:200	Vector Labs
Biotinylated rabbit anti-goat	1:200	Vector Labs
FITC-conjugated donkey anti-rabbit	1:200	Jackson Immunoresearch
FITC-conjugated donkey anti-goat	1:200	Jackson Immunoresearch
Alexa-594 conjugated donkey anti-rabbit	1:200	Invitrogen
Alexa-594 conjugated donkey anti-goat	1:200	Invitrogen

2.4. Cell Counting

Cell counting was performed on E18.5 WT and *Dlx1/2* DKO tissue to quantify CRX protein expression that was visualized by IF. Tissues were sectioned and immunostained as previously described. As the optic nerve is the only point of reference in the globe-like eye, we used the section containing the optic nerve as "point 0". From there we counted, immunostained, and imaged every 5^{th} slide in either direction from point 0. For each slide, cells were then counted with the observer blinded as to the genotype and numbers were tabulated using ImageProPlus software.

2.5. Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) is a useful tool to determine the interaction between a protein of interest and a desired fragment of DNA *in situ*, as in this case, to isolate which putative homeobox binding sites on the *Crx* and *Otx2* promoters were occupied by the DLX2 protein in retinal tissues. As there is no DLX2 expression in the hindbrain, this anatomic region was used as a negative tissue control.

E18.5 retinas and hindbrains were dissected from CD-1 mice and washed twice with cold 1xPBS using a swinging bucket centrifuge at 2000 rpm at 4ºC for 5 mins. Tissues were then gently dissociated into single cells by pipetting and were pelleted at 2000 rpm at 4ºC for 5 mins. Supernatant was removed and the pellet was fixed in 1% PFA + 1x Protease Inhibitor Cocktail (PIC) and incubated for 30 mins at RT with rotation. Fixed cells were spun at 2000 rpm at 4ºC for 5 mins, the supernatant was removed and the pellet was stored at -80ºC.

The following day, pellets were thawed on ice and 60µl of Pierce UltraLink Protein A/G beads (Cat#53132) were 'primed'. Beads were primed by adding Dilute buffer (0.01% SDS, 1.1% Triton X-1001.2mM EDTA16.7mM Tris-HCl (pH 8.1)167mM NaCl) + 1xPIC to the total amount of beads for a final volume of 1ml. The solution was spun at RT 2000 rpm for 2 mins and supernatant removed and wash was repeated. To make 50% beads, X volume of Dilute Buffer + PIC (X = original volume of added beads) was added. Thawed pellets were washed cells 2X with cold 1x PBS. After the last wash, 400µl of freshly made Lysis Buffer (1% SDS, 10mM Tris-HCl (pH 8.1),10mM EDTA) + PIC was added and chromatin was sonicated on ice 10-20X at 15 sec intervals (40% pulse strength and 3 output) with 30 sec rest periods using a Brandon cell disruptor. 1-3 µl of sonicated sample was run on a 1% agarose gel to ensure that chromatin was sheared with 300-700bp DNA fragments. Once the desired size sheared fragments were obtained, the 400µl of sonicated chromatin were split into 200µl in each tube.

To each tube, 60µl of 'primed' beads was added to pre-clear the solution, to remove any binding of 'background' IgGs. After a 1 hr incubation at 4ºC with rotation, tubes were centrifuged for 2000 rpm at 4ºC for 5 mins. Supernatant was transferred into a new tube and BSA and tRNA were added to a final concentration of 500 µg/ml. 1-2µg of DLX2 Ab was added to the tubes designated to have Ab; the "no Ab" tubes remained as is. Tubes were incubated at 4ºC O/N with rotation. Beads were primed again, as described previously; however, this time BSA and tRNA were added to a final concentration of 500µg/ml to make 50% beads. This step is to prime beads to the same conditions as the sonicated chromatin with Ab. These tubes were also incubated at

4ºC O/N with rotation. The next day, 60µl of primed beads were added to each tube (including the no Ab tubes) and were incubated at 4ºC O/N with rotation.

The following day, the beads were pelleted at 2500 rpm at 4ºC at 5 mins. Supernatant was transferred to a fresh tube (this supernatant contained all unbound chromatin and was used as a "Total Input" sample). Beads were washed with rotation at 4ºC with 1ml of low salt wash buffer (0.1% SDS, 1%Triton X-100, 2mM EDTA, 20mM Tris-HCl (pH8.1) and 150mM NaCl) for 5 mins, high salt wash buffer (0.1% SDS, 1%Triton X-100, 2mM EDTA, 20mM Tris-HCl (pH8.1) and 500mM NaCl) for 30 mins, LiCl Buffer (0.25M LiCl, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl (pH8.1) and 1% NP-40) for 30 mins followed by two washes with commercial TE buffer (pH 8.0) for 5 mins.

After the last wash 250µl of freshly prepared Elution Buffer (1% SDS, 0.1M $NaHCO_3$, pre-heated to 65ºC) was added. Tubes were incubated for 15 mins at RT with agitation and then centrifuged at 13 000 rpm for 5 mins at 4ºC. The supernatant was transferred to a fresh tube and elution steps were repeated with the two supernatants combined to give a final volume of approximately 500 µl. To digest any RNA molecules, 1µl of RNaseA and 25µl of 5M NaCl was added to combined supernatants. Tubes were incubated at 68ºC O/N.

The next day, 10µl of 0.5M EDTA, 20µl 1M Tris-HCl (pH 6.5) and 2µl Proteinase K (Invitrogen) were added and incubate at 65ºC for 2hrs. The reaction was "cleaned up" using Qiagen PCR Purification Kit and products were used as a template for PCR.

The 3kB Crx promoter (Figure 6A) contains 17 ATTA/TAAT homeodomain motifs. To encompass all these potential sites, the promoter was divided up into 7 regions and oligonucleotide primers, summarized in Table 3, were designed to flank these regions.

For the 3kb *Otx2* promoter, 26 potential homeodomain binding sites were found (Figure 6B). Primers, summarized in Table 4, were designed to flank all putative sites creating 10 regions in total.

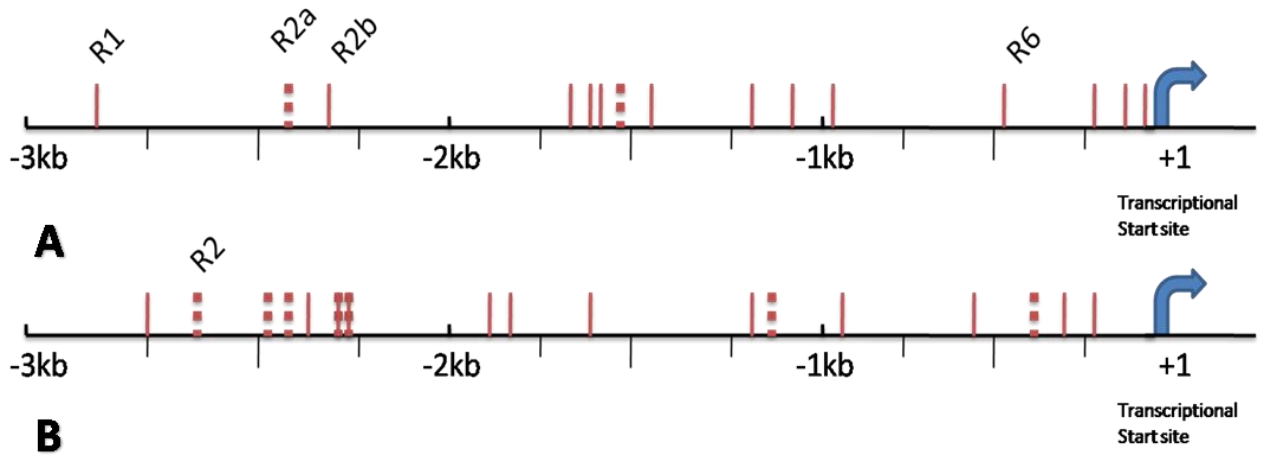


Figure 6: Schematic diagram of *Crx* and *Otx2* promoters with potential homeodomain binding sites.

(A) *Crx* promoter map with 17 potential DLX binding sites. Dashed lines indicate 'doublet' ATTA/TAAT sites. Labelled marks, R1, R2a, R2b and R6 are the sites pulled down by the ChIP assay. **(B) *Otx2* promoter map** with 26 potential DLX binding sites. Dashed lines indicate 'doublet' ATTA/TAAT sites, dashed and solid line indicate 'triplet' sites. The labelled mark, R2, indicates the site pulled down by the ChIP assay.

Table 3: Primers used for ChIP for the *Crx* promoter (M.Pind and D. Eisenstat, unpublished)

Region (location in promoter)	Primer sequence detail
I (160bp-321bp)	F 5'ggggctatctgtggaggtctgt3'
	R 5'tgatgaaggctgagagaatgagtg3'
II (451-653bp)	F5'tctgcaggttgtctttcgt3'
	R5' acctaacttggctctccctc3'
III (1237-1438bp)	F 5'cgtcagacaactcctcc3'
	R 5'gttctgcttctctaaacacc3'
IV (1428-1859bp)	F 5'tgcacggcccggtgtttaga3'
	R 5'tcctgcaatccttgagctgaaatga3'
V (1859-2104bp)	F 5'accagggctccattctc3'
	R 5'ggttctggctcttttcctc3'
VI (2419-2588bp)	F 5'gaggaagtgagggaagaaggga3'
	R 5'tgtggcccaggctagtattga3'
VII (2777-2914bp)	F 5'ggcaggagttgggcttt3'
	R 5'ggagtgatgtcatctattgtgg3'

Table 4: Primers used for ChIP for the *Otx2* promoter

Region (location in promoter)	Primer sequence detail
I (221-329bp)	F 5'ggacttttccggccactga3'
	R 5'cggtgttaggtctggaagg3'
II (311-479bp)	F 5'ccttccagacctaacaccg3'
	R 5'gcgggcatttggaaaacag3'
III (461-740bp)	F 5'ctgttttccaaatgcccgc3'
	R 5'atctaccagttgctgtgtcc3'
IV (1114-1330bp)	F 5'gcaagcggtgaaagttaggt3'
	R 5'gaggtccttcttggagagtc3'
V (1311-1482bp)	F 5'gactctccaagaaggacctc3'
	R 5'cccttggacagttctgacc3'
VI (1728-1979bp)	F 5'ctggaagaaatcacagctgtt3'
	R 5'ggcacttaaagcgctctctc3'
VII (2001-2150bp)	F 5'cagccttacacacattgcct3'
	R 5'taaccgcacttctctctgc3'
VIII (2402-2542bp)	F 5'gccctgtgctagtcttgaag3'
	R 5'ggaggagtttgattacatgtg3'
IX (2522-2680bp)	F 5'cacatgtaatcaaactcctcc3'
	R 5'cttgctgaacaacaaacttgtg3'
X (2659-2910bp)	F 5'cacaagtttgttgttcagcaag3'
	R 5'gattggggccatttgcagag3'

2.6. Electrophoretic Mobility Shift Assays (EMSA)

Oligonucleotides (oligos) of 20-25 bp were designed to encompass the sites of the gene promoter where DLX2 was shown to occupy by ChIP (Table 5). Both the forward and reverse (compliment) oligos were ordered for each desired binding region (Sigma) and were annealed with annealing buffer as described by Sigma-Aldrich Protocol for Annealing Oligos. Oligos were designated as *CRX1*, *CRX2a*, *CRX2b* and *CRX6*. Annealed oligos were radiolabelled using $P^{32}\alpha$ (Perkin Elmer) by T4 Polynucleotide Kinase (Invitrogen) and purified using GE HealthCare Illustra Micro-spin G-25 spin columns. Radioactivity was measured using a Beckman Coulter scintillation counter. Samples were prepared as previously described (Zhou et al., 2004) and incubated for 30 mins without the radiolabelled oligos, then for another 20 mins after addition of the oligos. Samples were run on a 4% non-denaturing acrylamide gel for 45 mins at 280V and vacuumed-dried for 1.5 hours using a gel drier (BioRad). The gel was exposed to autoradiography film (Kodak) overnight at -80^{o}C.

Table 5: EMSA Oligos designed for *Crx* ChIP-positive regions

Oligo Name	Sequence Detail (**potential binding site**)
CRX1	F 5'gcagga**atta**tagcaaggacatc3'
	R 5'gatgtccttgcta**taat**tcctgc3'
CRX2a	F 5'catgaattca**taatta**tggatg3'
	R 5'catcca**taatta**tgaattcatg3'
CRX2b	F 5'gacacatttca**atta**tttgggtagag3'
	R 5'ctctacccaaa**taat**tgaaatgtgtc3'
CRX6	F 5'cacatttga**atta**gttcactcag3'
	R 5'ctgagtgaaca**taat**tcaaatgtg3'

2.7. Luciferase Reporter Assays

Cloning

To design plasmids for transfection, inserting the desired regions of the promoter into the pGL3 basic vector was required. This was achieved by designing primers to the full length promoter and also for the regions pulled down as "positives" from the ChIP assay summarized in Table 6. The forward primers had a *Mlu1* site incorporated at the 5' end where the reverse primers had an *Xho1* site at their 5'end. Using these primers and genomic DNA as a template, regions of interest were amplified by PCR (Qiagen HotStar). The pGL3 basic vector and resulting PCR products were digested using these enzymes (New England BioLabs) for 3 hours to overnight at 37^{o}C and were purified using a PCR Clean-up Kit (Qiagen). The inserts with complimentary 'sticky ends' to the digested plasmid were ligated using T4 DNA Ligase (New England Biolabs) and transformed into DH5α *E.coli* cells (Invitrogen). Transformed bacteria were plated onto ampicillin/carbenicillin-containing agar plates and incubated at 37^{o}C overnight. Colonies will only grow if the plasmid has re-circularized; in this case, the digested vector will only reform if the insert is ligated (as it contains the compatible ends to complete the circle). Colonies were picked, amplified overnight in LB broth with antibiotic, and purified with a Mini-Prep Kit (Sigma). Resulting plasmids were sequenced and aligned with original sequence to ensure correct ligation of inserts.

Table 6: Primer design for inserts for luciferase plasmids

Region	Primer sequence detail
CRX Full Length Promoter	F 5'cagacgcgtacaacacaaacaaaaagg3'
	R 5'gtcctcgagtaggtcccctcacacgg3'
CRX Region 1	F 5'cagacgcgtggggctatctgtggaggtctgt3'
	R 5'gtcctcgagtgatgaaggctgagagaatgagtg3'
CRX Region 2	F 5'cagacgcgtggggctatctgtggaggtctgt3'
	R 5'gtcctcgagtgatgaaggctgagagaatgagtg3'
CRX Region 6	F 5'cagacgcgtgaggaagtgagggaagaaggga3'
	R5'gtcctcgagtgtgcccaggctagtattga3'

Luciferase reporter assays

The HEK293 cells were grown in 75cm^2 cell culture flasks (Nalgene) to 80% confluence. Cells were then trypsinized (Gibco Invitrogen) and stained with Trypan Blue for counting using a hemocytometer. 1X10^5 cells were plated into each well of two 12-well culture plates (Nalgene) with 1 ml of DMEM+10%FBS media (Invitrogen). Cells were cultured overnight at 37°C at 5% CO_2 (Fisher Scientific IsoTemp Incubator) to reach 70% confluence. Cells were then washed with warmed 1xPBS and then incubated with 1ml of warmed OPTIMEM media (Gibco-Invitrogen) at 37°C until the transfection mixture was ready. Transfection mixture per well was prepared as follows: 0.5µg of designed reporter pGL3 plasmids (empty pGL3 vector was used as a control), 0.5µg of pcDNA$_3$-DLX2 (empty pcDNA$_3$ as control) and 0.1µg of β-galactosidase to measure transfection efficiency in 125µl of OPTIMEM. 2.5µl of Lipofectamine 2000 reagent (Invitrogen) was incubated with 125µl OPTIMEM for 5 mins at RT in a separate tube. The two mixtures were combined and allowed to incubate at RT for 20 mins before adding 250µl of the mixture to each well. After a 48 hr incubation, the transfected cells were lysed using 100µl of 1x Lysis Reporter Buffer (Promega).

Luciferase activity measurements were performed using an Lmax Luminometer (Molecular Devices) by combining cell lysates (10µl) with Luciferase assay substrate buffer (Promega). β-galactosidase activity was measured to determine transfection efficiency at 420 nm using the SpectraMax 190 instrument (Molecular Devices) and after addition of ONPG buffer (4mg/ml).

2.8. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA Isolation

Eyes were enucleated from E18.5 WT and *Dlx1/2* DKO embryos and were kept in 500µl of RNAlater (Qiagen). RNAlater maintains RNA integrity in the tissue. Samples were kept at -20°C until RNA extraction. To extract RNA, tissues were then frozen in liquid nitrogen and ground to a powder. RNA was isolated using the QiaShredders and RNEasy Mini Kit (Qiagen) and concentrations were measured.

cDNA Synthesis

To 1µg of RNA, 1µl of Oligo $d(T)_{50,}$1µl 10mM dNTP and ddH_2O final volume 13µl were added and heated to 65ºC for 5 mins followed by a 1 min incubation on ice. Samples were briefly centrifuged followed by the addition of 4µl 5x First Strand Buffer, 1µl 0.1M DTT, 1µl RNase Out, and 1µl SuperScript III. Samples were mixed by pipetting and then incubated at 50ºC for 50 mins and then at 70ºC for 15 mins. Resulting cDNA was stored at -20ºC. All reagents were acquired from Invitrogen.

qRT-PCR

Primers were designed to flank exons 2 and 3 of *Otx2* and exons 1 and 2 of the *Crx* genes (Table 7). Samples set up as per protocols described using the Qiagen QuantiTech SYBR Green RT-PCR Kit and run on an Applied Biosystems 7500 Real Time PCR System. Cycling conditions were an initial denaturing step of 95°C for 15 mins followed by 40 cycles of 95°C for 30 secs, 60°C for 30 secs and 72°C for 33 secs and finally a default dissociation curve. Data was

collected at the 72°C step. Analysis was performed using the Applied Biosystems Sequence Detection Software Version 1.4.

Table 7: Primers for qRT-PCR

Primers (Location in coding sequence)	Sequence detail
Otx2 (480-708bp)	F 5’ tcgacgttctggaagctct 3’
	R 5’cactctctgaactcacttcc 3’
Crx (118-255bp)	F 5’gatggcatatatgaacccgc3’
	R 5’gtgaatgtggtccgctccc3’

3. RESULTS

3.1 Expression Patterns of CRX and OTX2 in the Developing Retina

DLX2 is expressed in the developing murine retina starting at E11.5. Through characterization it has been demonstrated that DLX2 is localized to GCL of the neural retina and so it is expressed in RGCs as well as the displaced amacrine cells found in the GCL (de Melo et al., 2005). As previously described, the *Dlx1/2* DKO has a 33% reduced RGC count in addition to a 23% decreased optic nerve thickness. Upon comparison of *Crx* expression, in increased expression in transcript was found in the DKO retina when compared to wild-type littermate controls.

To observe protein expression profiles, comparison of WT and *Dlx1/2* DKO tissue during embryogenesis was performed. This was accomplished by means of IF at embryonic stages 13.5, 16.5, and 18.5. As mentioned, the embryonic retina is anatomically arranged differently than the adult retina. The developing retina consists of two layers, the outer is a large dense nuclear layer (or the neuroblastic layer (NBL)) that is circumscribed by the retinal pigment epithelium (RPE), while the inner layer is a thinner nuclear layer called the ganglion cell layer (GCL) as highlighted in the DAPI images of Figures 7G, H and 8G, H. Over the course of embryogenesis, there is a margin where OTX2 is expressed in the WT; the outer NBL and RPE. There are some OTX2-positive cells in the inner NBL that are more dispersed and situated towards the inner retina (GCL) (Figure 7 C, E). CRX has a similar expression profile except that there are fewer ‘dispersed’ CRX-positive cells in the inner NBL (Figures 8 C, E) relative to OTX2.

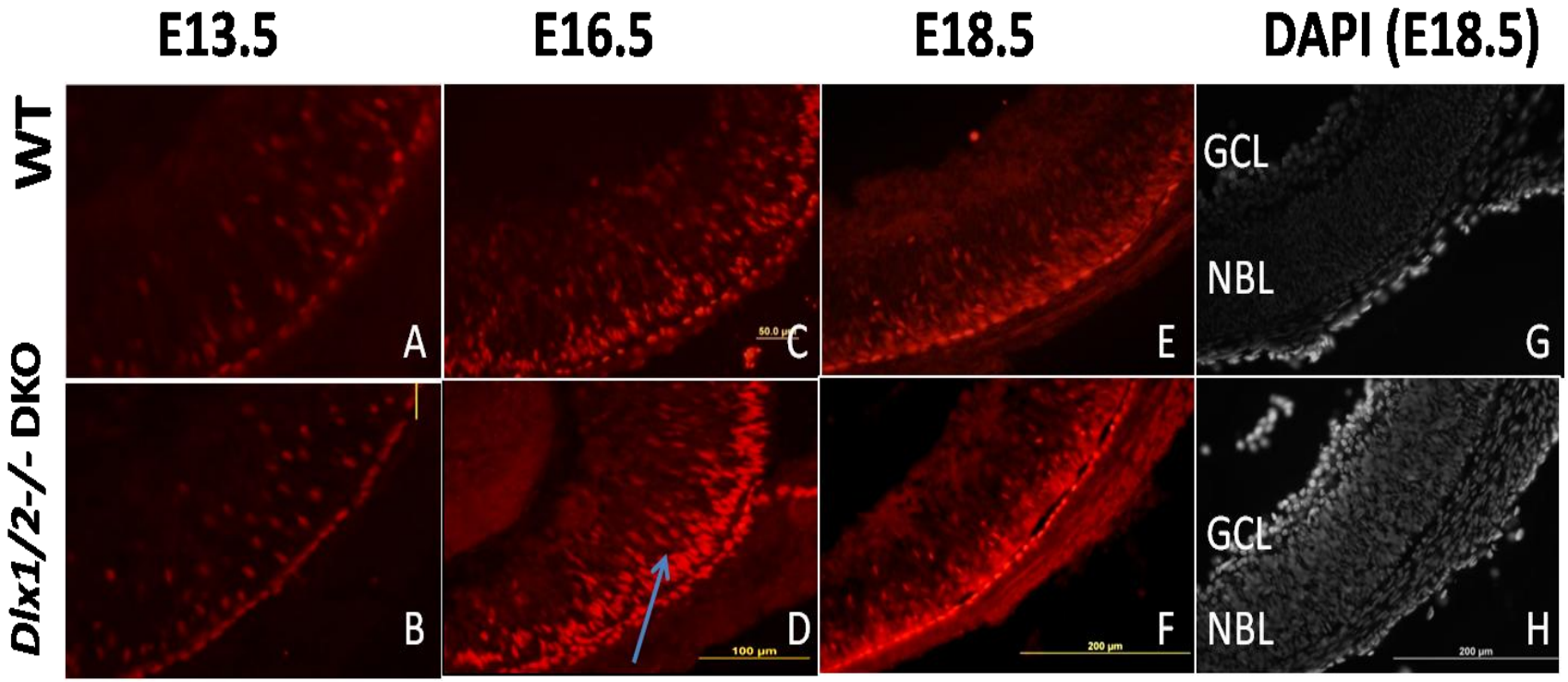


Figure 7: Comparing OTX2 expression during embryogenesis between wild-type and *Dlx*1/2 DKO retinas

OTX2 expression during embryogenesis between wild-type (A, C, E) and *Dlx1/2* double knockout (DKO) (B, D, F) retinal tissue. At E16.5, OTX2 expression is increased in the DKO (D) compared to WT (C) (arrow). Panels G and H are DAPI images of the corresponding E18.5 staining. Wild-type (WT), Double knockout (DKO), Ganglion cell layer (GCL), Neuroblastic layer (NBL)

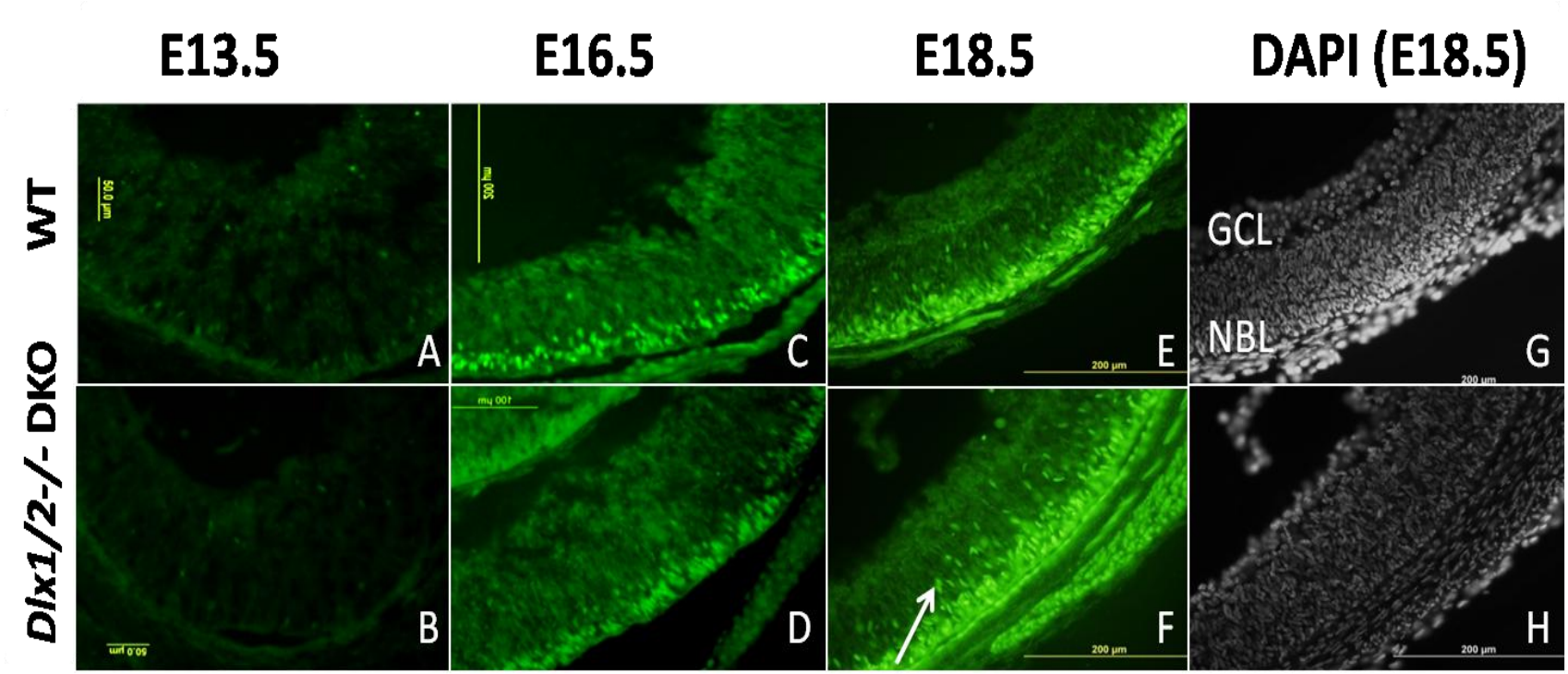


Figure 8: Comparing CRX expression during embryogenesis between wild-type and *Dlx1/2* DKO retinas

CRX expression during embryogenesis between wild-type (A, C, E) and *Dlx1/2* double knockout (DKO) (B, D, F) retinal tissues. At E18.5 CRX expression is increased in the DKO (F) compared to WT (E) (arrow). Panels G and H are DAPI images of the corresponding E18.5 staining. Wild-type (WT), Double knockout (DKO), Ganglion cell layer (GCL), Neuroblastic layer (NBL)

Comparison between the WT and mutant tissue reveals a slight difference in protein expression during embryonic retinogenesis. There appears to be an increase in OTX2 expression at E16.5 in the *Dlx*1/2 DKO (indicated by arrow in Figure 7D). However, at E13.5 and E18.5, no upregulation is apparent (Figures 7B and F). Noting the differences in CRX expression, a similar increase is found as the dispersed CRX-positive cells that are found in the NBL appear to be in higher number in the DKO at E18.5 (arrow in Figure 8F). To quantify the protein expression, cell counting was performed. Preliminary data shows that cells which immunostained for CRX are increased in the *Dlx*1/2 DKO compared to WT littermates by an approximately 1.5 fold increase (Figure 9A). Similar counting was performed on OTX2-positive cells (Figure 9B). However, at E18.5, there is no apparent difference.

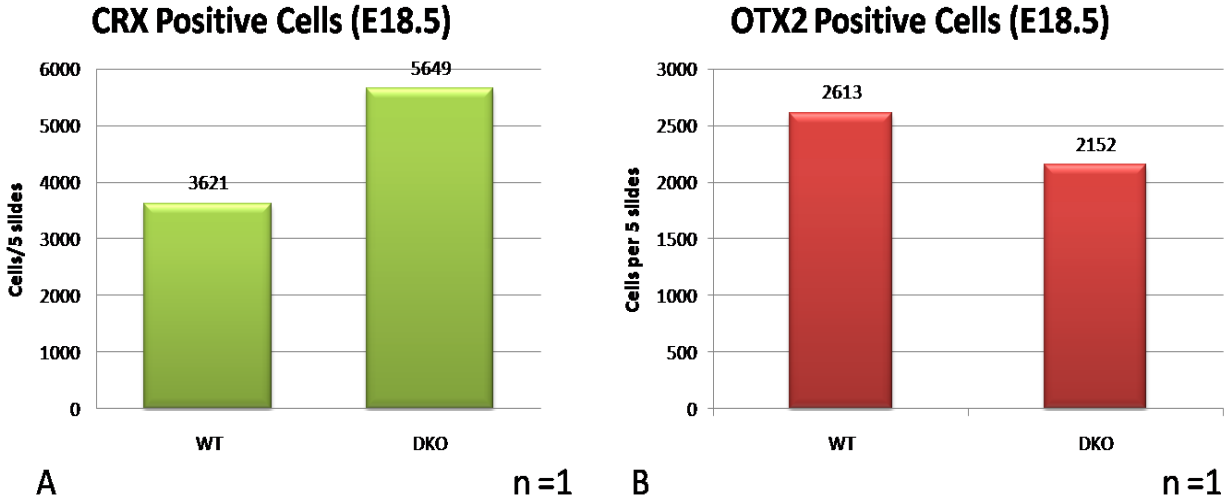


Figure 9: Quantification of CRX-positive and OTX2-positive cells in the E18.5 WT and *Dlx1/2* DKO retinas

Cell counting of CRX-positive **(A)** and OTX2-positive cells **(B)** cells in the E18.5 wild-type (WT) and *Dlx1/2* double knockout (DKO) retinas. Cell counting shows preliminary results of an approximately 1.5x increase in CRX-positive cells in the DKO compared to the WT. Preliminary counting of OTX2-positive cells does not show a discernable difference between WT and DKO counts at E18.5 (n=1 eye).

3.1.1. Dual Immunoflurorescence (DIF)-OTX2 and CRX

Since OTX2 is a direct activator of *Crx* expression, and both genes are required for photoreceptor cell fate (Koike et al., 2007; Nishida et al., 2003), we wanted to see if there was a differential expression with the co-localization of OTX2 and CRX in the WT versus the *Dlx1/2* DKO. In order to determine whether a retinal cell is co-expressing both markers under investigation, a yellow signal will appear when the individual red and green images are merged. Of the time-points studied, in both wild-type and mutant tissues, all CRX-positive cells also co-expressed OTX2 (Figures 10 A, D, G, J, M, P). There are cells, however, which express OTX2 alone (Figure 10M, arrow). Interestingly at E18.5, cells that are only expressing CRX are observed (Figure 10P, double arrows). In particular, in the DKO, there appears to be more of these CRX single "+" cells than in the WT. The proportion of cells that are co-expressing OTX2 and CRX in both tissues at E16.5 and E18.5 is yet to be quantified.

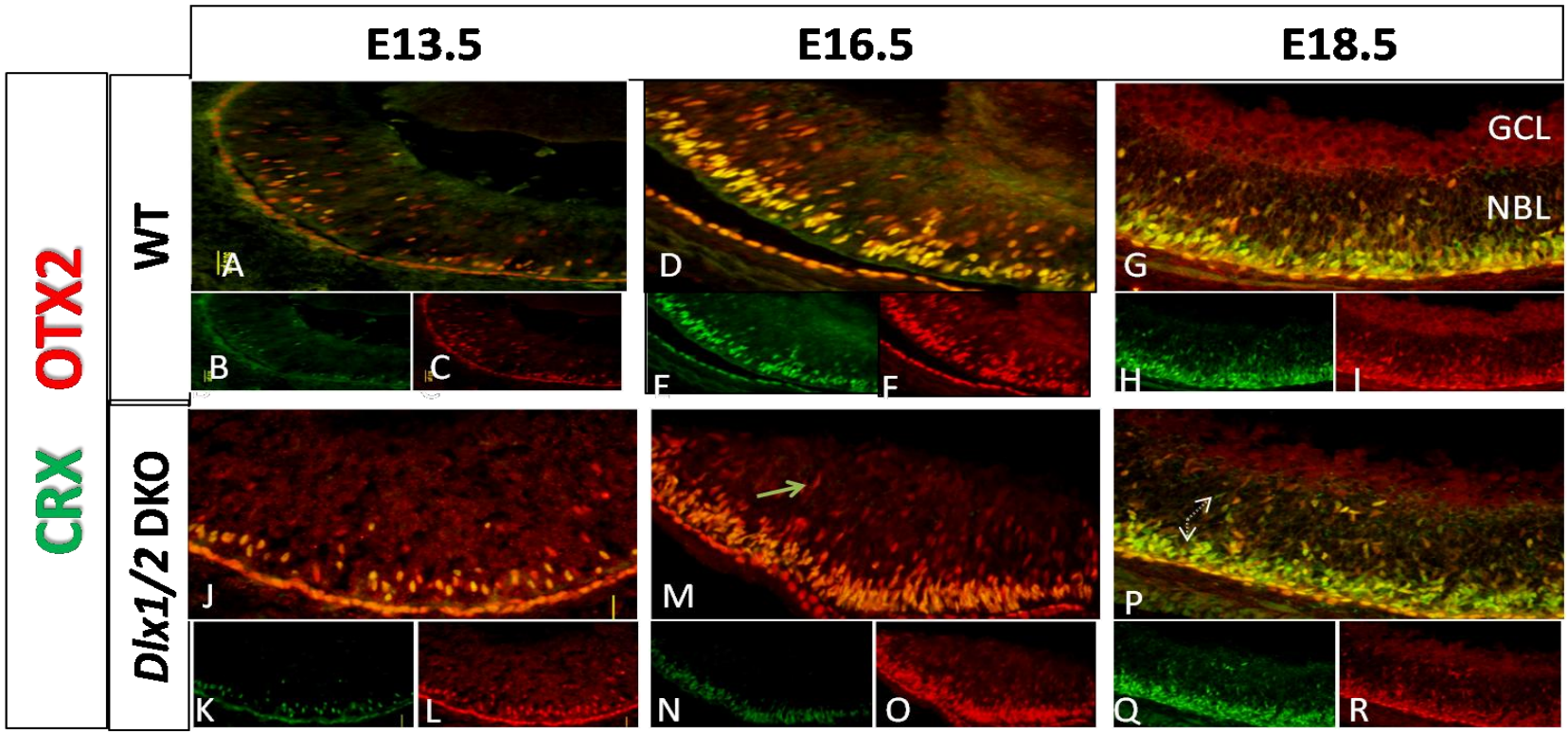


Figure 10: Comparing CRX and OTX2 co-expression during embryogenesis between WT and *Dlx1/2* DKO tissue

CRX expression shown in green (B, E, H, K, N, Q) OTX2 shown in red (C, F, I, L, O, R), merged images (A, D, G, J, M, P) indicate co-expression via yellow signals. At E16.5, there is an increased amount of OTX2 expression in the DKO; however, the aberrant OTX2-positive cells in the DKO (M) do not co-express with CRX (indicated by arrow) as they do the in WT (D). At E18.5 the additional CRX-expressing cells in the DKO (P) do not co-express with OTX2 (double arrows) to the extent they do in WT (G). Wild-type (WT), Double knockout (DKO), Ganglion cell layer (GCL), Neuroblastic layer (NBL)

3.1.2. DIF – OTX2 and DLX2

OTX2 is a transcriptional activator of cells destined to become photoreceptors and DLX2 is a transcriptional activator of RGC genes (Zhang et al., submitted). We hypothesized that through concurrent DLX2 repression of photoreceptor gene expression, DLX2 promotes a RGC fate over that of a photoreceptor cell fate. So we wanted to determine whether there were any embryonic retinal cells that co-expressed both transcription factors. Assessments were performed on E13.5, E16.5 and E18.5 WT retina. At E13.5 we observed some cells co-expressing both markers (Figure 11A, arrows). However at later timepoints, the proportion of co-expressing cells were not as apparent (Figures 11D, G). The proportion of cells that co-expressed OTX2 and DLX2 at each time stage has yet to be quantified.

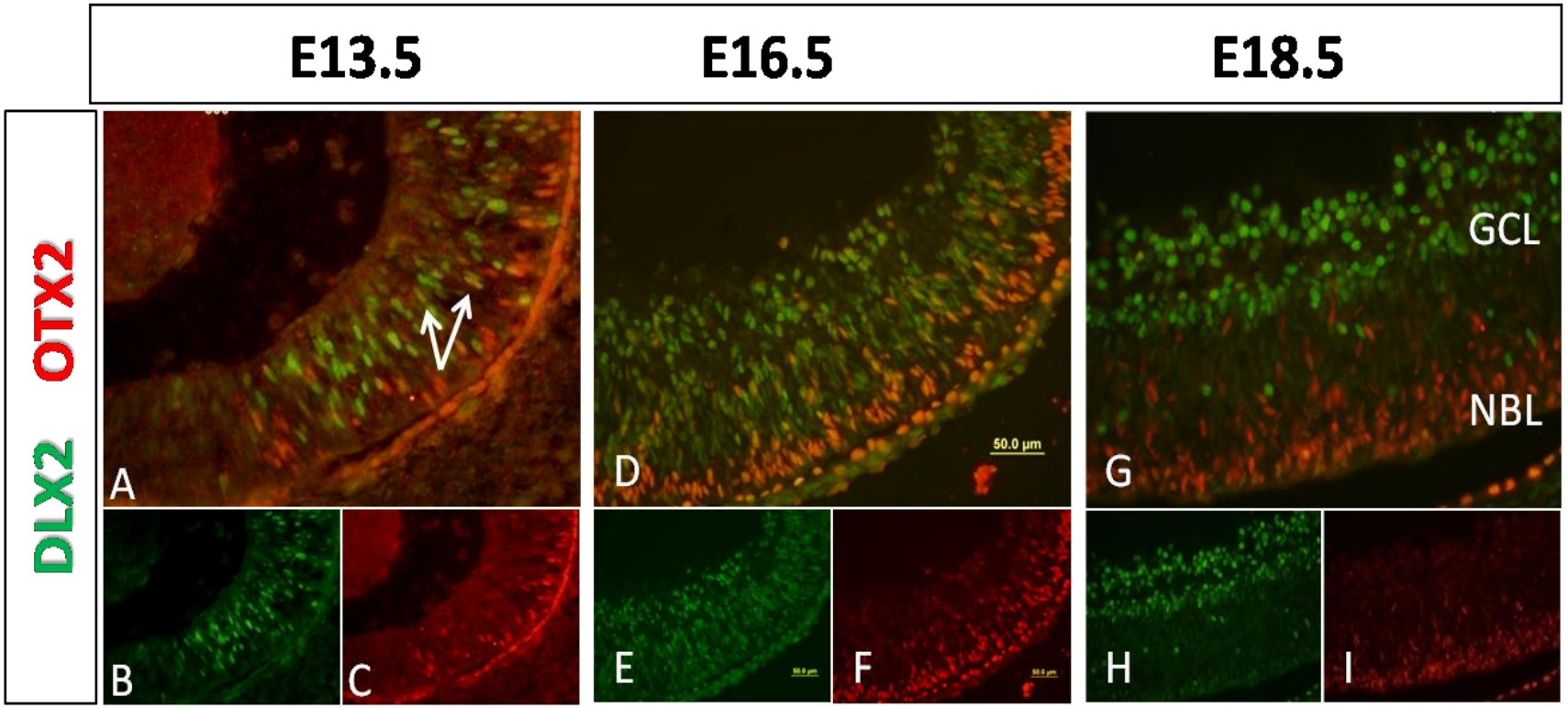


Figure 11: Comparing DLX2 and OTX2 co-expression during embryogenesis

DLX2 expression shown in green (B, E, H), OTX2 shown in red (C, F, I); merged images (A, D, G) indicate co-expression via yellow signals. At E13.5, there appears to be more cells that co-express DLX2 and OTX2 (arrows) compared to later time-points. Ganglion cell layer (GCL), Neuroblastic layer (NBL)

3.1.3. DIF – CRX and other markers

To determine whether the CRX-positive cells in the *Dlx1/2* DKO are expressing markers for other retinal cell lineages, co-expression studies through dual immunofluorescence (DIF) were conducted. As differential expression was observed at E18.5, all DIFs were performed on this time-stage. Using the previous described BRN3a and BRN3b antibodies as specific markers for RGCs, we were able to ascertain that the CRX-expressing cells in the *Dlx1/2* DKO were not expressing markers of a RGC fate (Figure 12 A, D, G, J). As well, DIFs with markers for amacrine (Syntaxin) and for RGCs, amacrine, and horizontal cells (PAX6) also demonstrated an absence of CRX co-expressing cells (Figure 13A, D, G, and J).

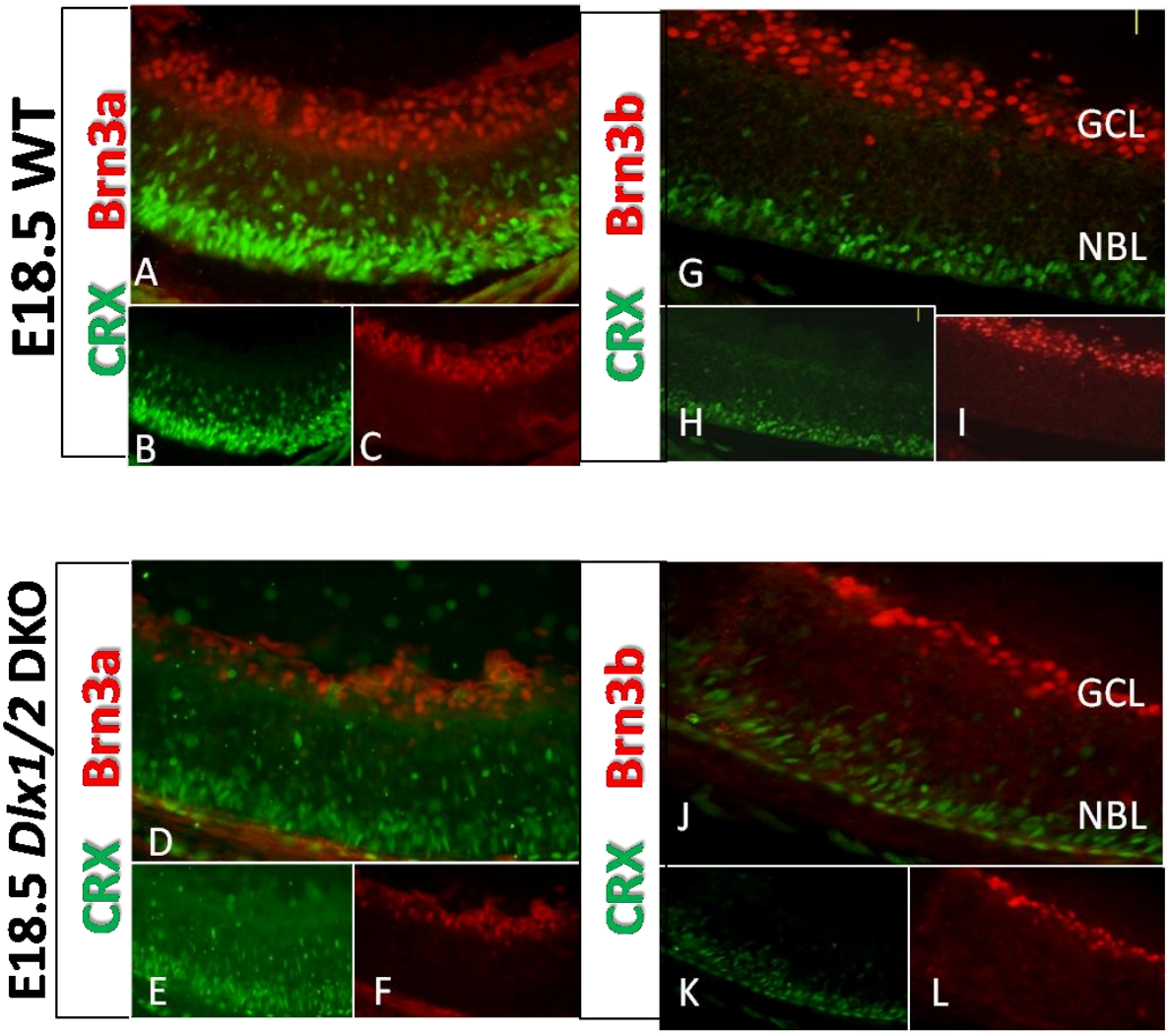


Figure 12: Comparing CRX and RGC specific markers BRN3a and BRN3b co-expression at E18.5 between WT and *Dlx1/2* DKO tissue

CRX and RGC markers BRN3a and BRN3b co-expression at E18.5 were compared between wild-type (WT) and *Dlx1/2* double knockout tissue (DKO). CRX is stained in green (B, E, H, K) with BRN3a or BRN3b in red (C, F, I, L). Absence of yellow signals in merged images (A, D, G, and J) indicates that CRX does not co-express with these markers in WT or DKO tissue. Ganglion cell layer (GCL), Neuroblastic layer (NBL)

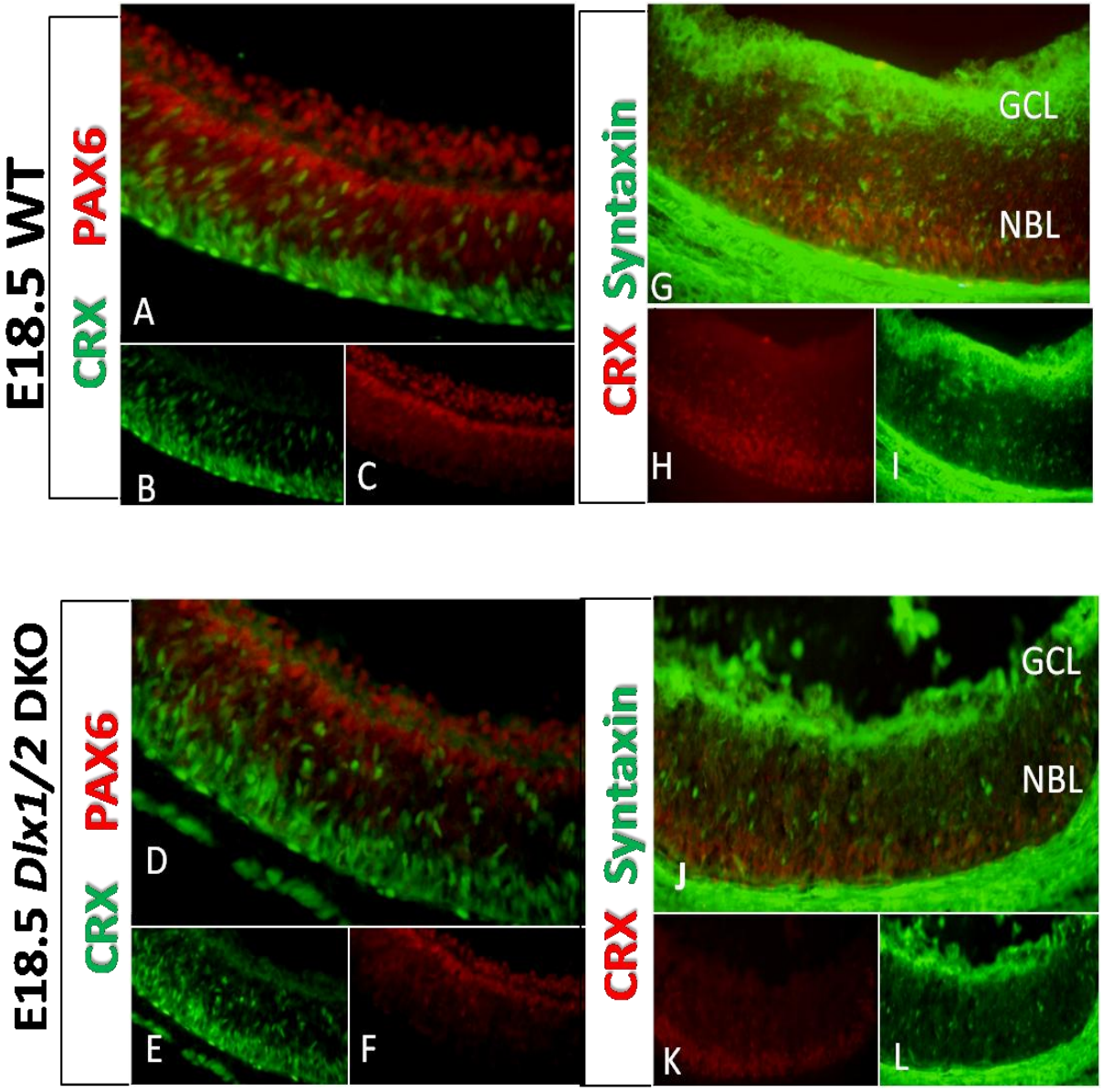


Figure 13: Comparing CRX and PAX6 or Syntaxin Expression in WT and *Dlx1/2* DKO tissue at E18.5

CRX and PAX6 or Syntaxin expression in wild-type and mutant tissue at E18.5. CRX is stained in green (B, E) and PAX6, a marker for RGCs, amacrine, and horizontal cells, is in red (C, F). Syntaxin is stained in green in (I, L) with CRX in red (H, K). Absence of yellow signals in merged images (A, D, G, J) indicates that CRX does not co-express with these markers in WT or DKO tissue. Wild-type (WT), Double knockout (DKO), Ganglion cell layer (GCL), Neuroblastic layer (NBL)

3.1.4. Gain-of-Function

The alternative to assessing gene function by studying loss-of-function models is to use gain-of-function (GOF) studies *in vitro* and *in vivo (in utero).* We used GOF assays to assess the effects on OTX2 and CRX expression when DLX2 was over-expressed.

IFs were performed on the GOF tissue to assess any differences in OTX2 and CRX expression. Upon visual assessment, an unexpected increase in OTX2 expression was observed in the GOF retina (Figure 14F) compared to retinas electroporated with an empty GFP plasmid (GFP-only) in Figure 14B. Assessing CRX expression in the GOF tissue (Figure 15F), demonstrated a similar (unexpected) increase in expression compared to the control experiment (Figure 15B).

Interestingly, there happens to be a differential expression of CRX depending upon the strain of mouse from which the retina was procured. In the 'control' retinas (which were obtained from C57/BL6 mice with pigmented RPE) for the GOF studies, baseline CRX expression was lower (Figure 16A) than compared to the WT retina (Figure 16B) collected from the *Dlx1/2* colony (albino CD1 strain with non-pigmented RPE). However, there appears to be similar levels of OTX2 immunostaining between the two mouse strains (Figure 16 E and F).

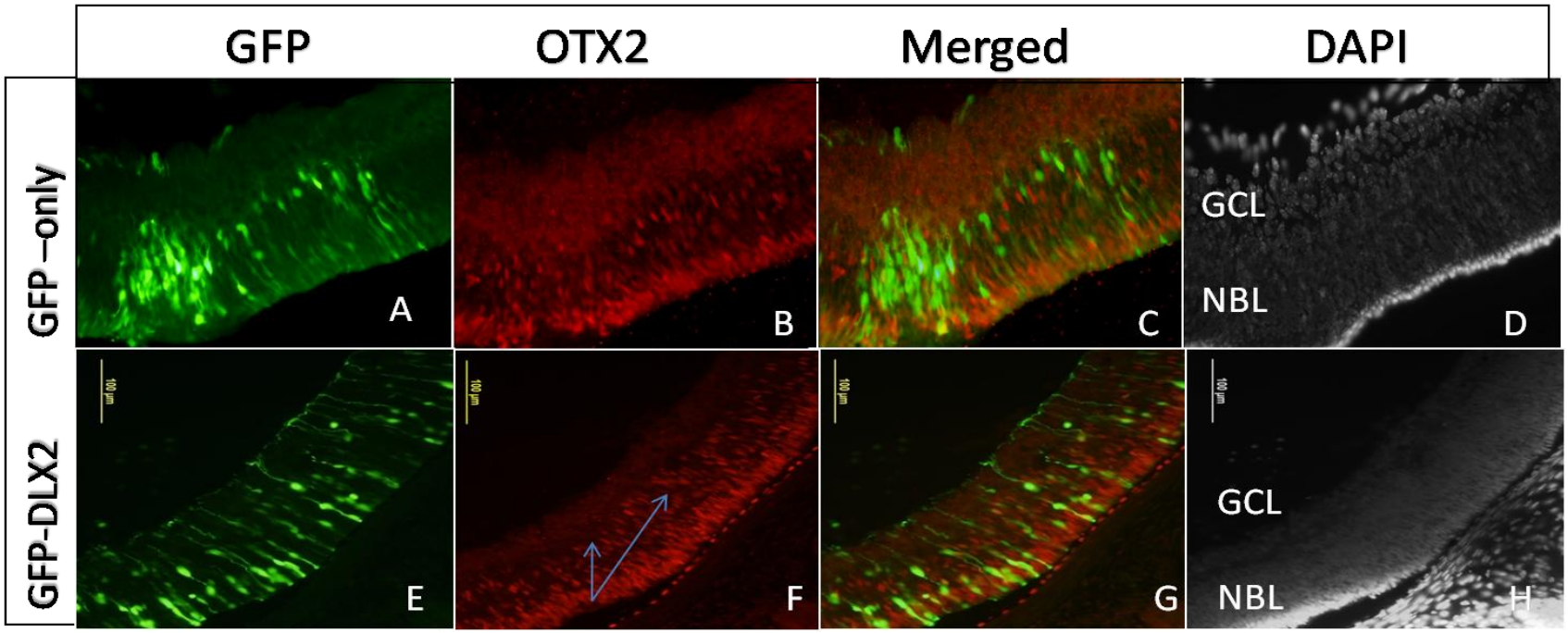


Figure 14: OTX2 expression in wild-type and *Dlx*2 *in utero* gain-of-function (GOF) in E18.5 retinas

OTX2 expression in wild-type and *Dlx2 in utero* gain-of-function in E18.5 retinas. Panels A–D are control tissues. Panels E to H are from the *Dlx2*-GOF retina. Panels A and E indicate the expression of the GFP plasmid with and without *Dlx2* in the expression vector, respectively. Comparison of OTX2 expression shows an apparent increased expression (arrows) in the NBL in the GOF retina (F). Panels D and H are DAPI images for each retina. Green fluorescent protein (GFP), Ganglion cell layer (GCL), Neuroblastic layer (NBL)

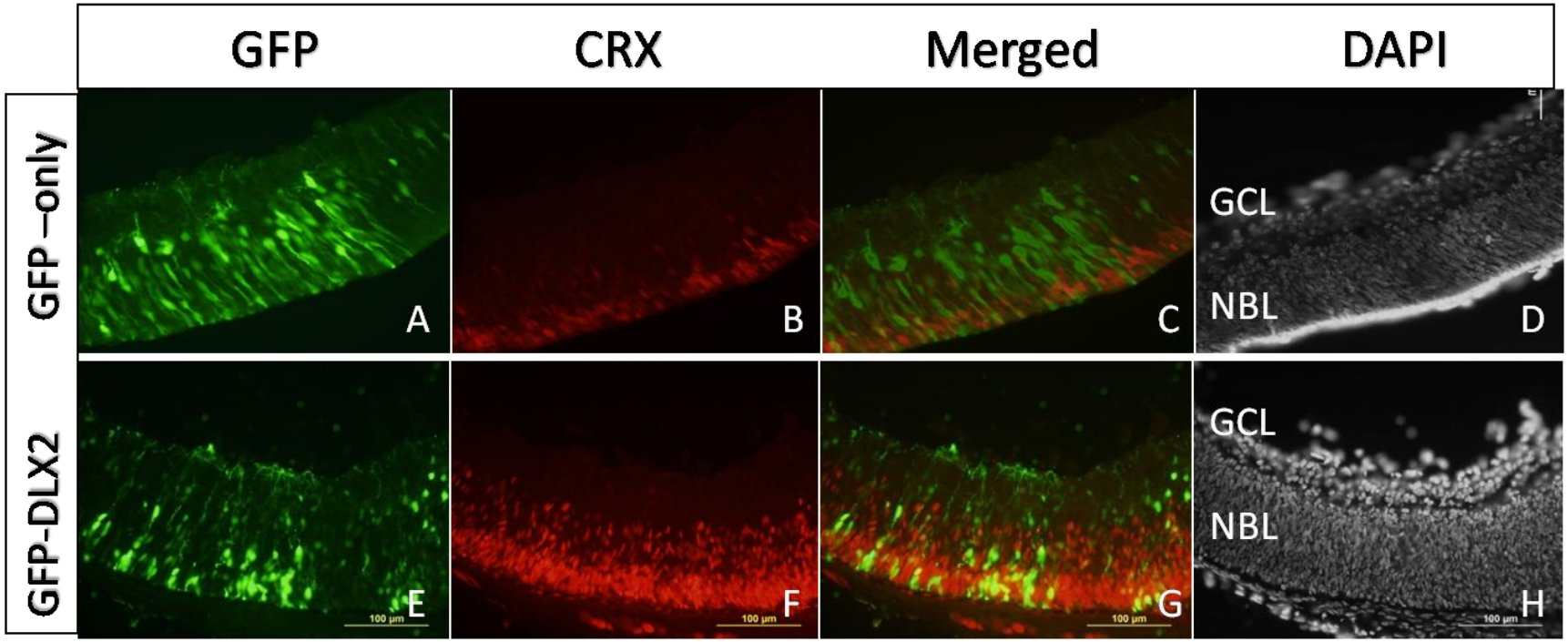


Figure 15: CRX expression in wild-type and *Dlx2 in utero* gain-of-function in E18.5 retinas

CRX expression in wild-type and *Dlx2* gain-of-function in E18.5 retinas. Panels A-D is control tissues. Panels E-H is from the *Dlx2*-GOF retina. Panels A and E indicate the expression of the GFP plasmid with and without *Dlx2* in the expression vector, respectively. Comparison of CRX expression shows increased expression in the GOF retina (B vs. F). Panels D and H are DAPI images for each retina. Green fluorescent protein (GFP), Ganglion cell layer (GCL), Neuroblastic layer (NBL).

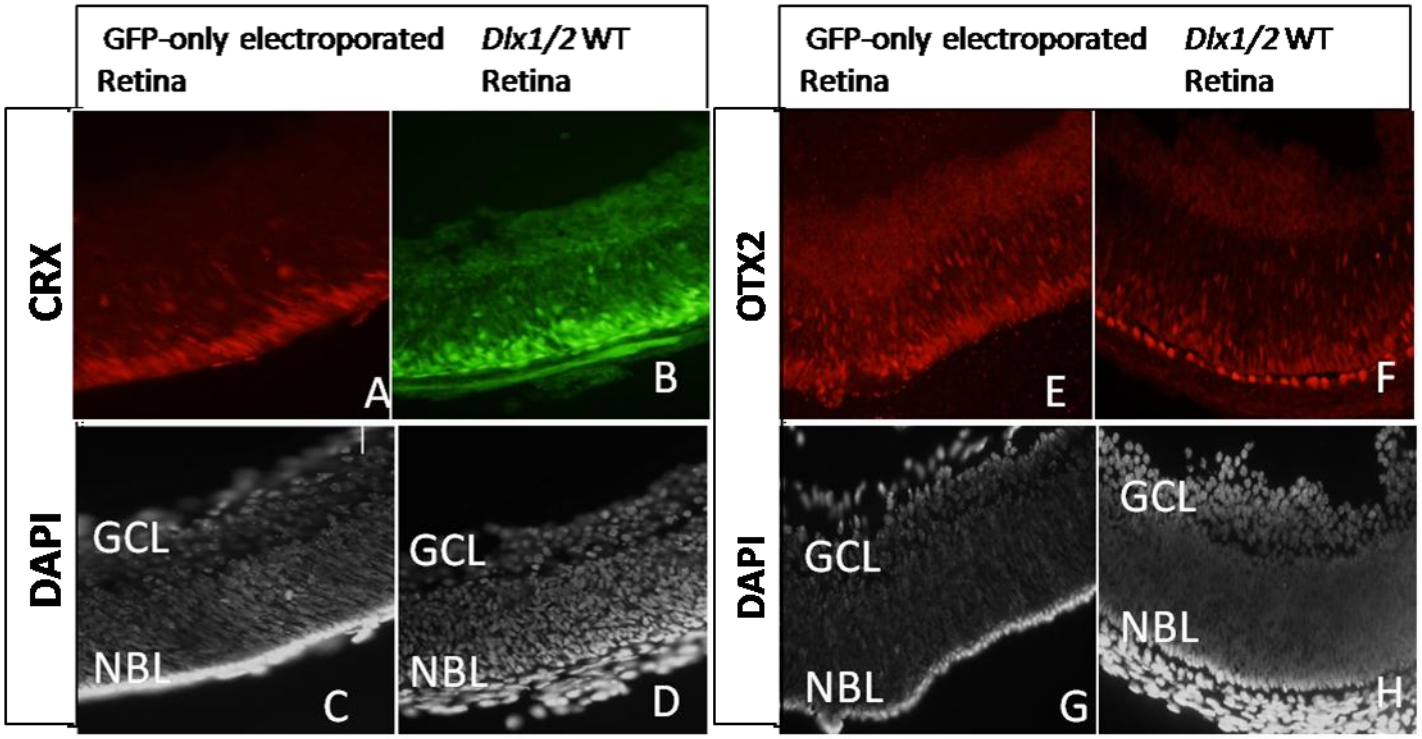


Figure 16: CRX and OTX2 expression in electroporated and *Dlx1/2* WT control retinas

Panel A is the expression pattern of CRX in the control tissue of the electroporated retinas (C57/BL6 mice with pigmented RPE). Panel B shows CRX expression in the WT tissues from the *Dlx1/2* DKO colony (albino CD1 mice with non-pigmented RPE). Panels E and F compare OTX2 expression between the two control retinas. Unlike CRX, OTX2 has similar expression patterns between strains. Panels C, D, G and H are the respective DAPI images for each retina. Ganglion cell layer (GCL), Neuroblastic layer (NBL).

3.2. DLX2 Binds to the *Otx2* and *Crx* Gene Promoters

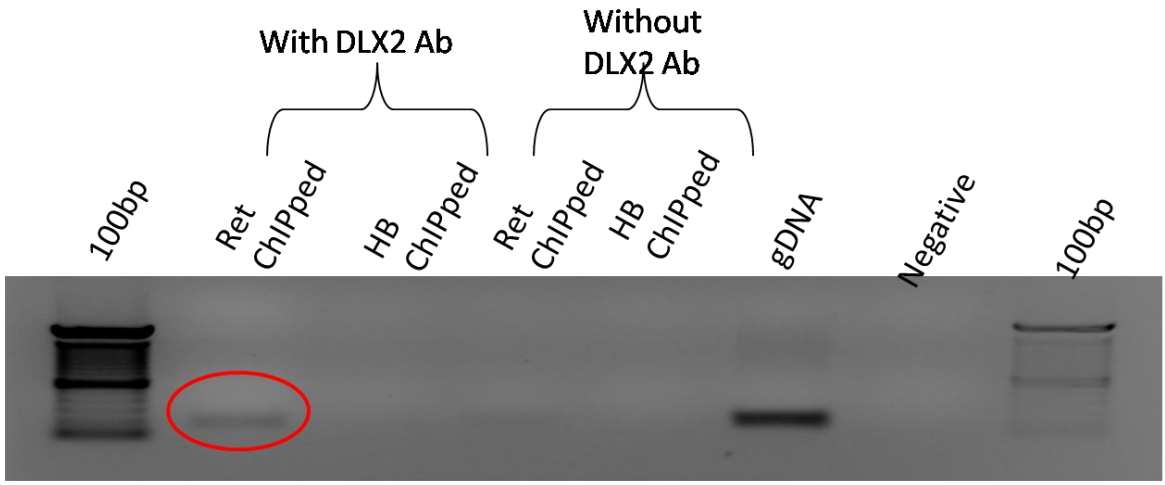
The regulation of *Otx2* and *Crx* is required for proper photoreceptor development. It has been shown that OTX2 is a direct activator of *Crx* expression ; as well, CRX acts in an auto-positive (feed-forward) feedback mechanism to regulate itself (Koike et al., 2007). Increased OTX2 and *Crx* expression in the *Dlx1/2* DKO (de Melo et al., 2005) suggested a role for the *Dlx* transcription factors on the regulation of these retinal genes. Analysis of the *Otx2* and *Crx* gene promoters revealed 26 and 17 putative DLX homeodomain binding sites, respectively (Figure 6).

3.2.1. DLX2 Binds to the Otx2 *and* Crx *Promoters in vivo*

To determine which, if any, of these putative sites are occupied by DLX2 *in situ*, chromatin immunoprecipitation (ChIP) assays were performed. By isolating E17.5 and E18.5 retinas from CD-1 time-pregnant mice, protein-DNA complexes were cross-linked with PFA and sonicated into fragments. Using a polyclonal antibody specific for DLX2, we were able to isolate the complexes that the DLX2 protein was associated with. As DLX2 is not expressed in the embryonic hindbrain, this tissue, along with a non-specific IgG, were used as negative controls. Reverse cross-linking and amplification by PCR determined which of the regions of the *Otx2* and *Crx* promoters DLX2 occupied.

Out of the possible 10 regions designed to encompass all 26 potential sites on the *Otx2* promoter, the first 3 regions have been tested so far. Region 2 was the only one which was pulled down by the ChIP assay, indicating DLX2 promoter occupation in E18.5 retinas (Figure 17).

For the *Crx* promoter, E17.5 and E18.5 retinas were ChIPped and 3 regions: Region 1, Region 2 and Region 6, were positive for DLX2 promoter occupation (encircled bands in Figure 18). Although this ChIP shows that DLX2 occupies Region 1 in E17.5, subsequent ChIPs have demonstrated DLX2 binding in E18.5 retinas as well (data not shown). It is interesting to note the differential binding of Region 2 at E17.5 (absent) and E18.5 (present).

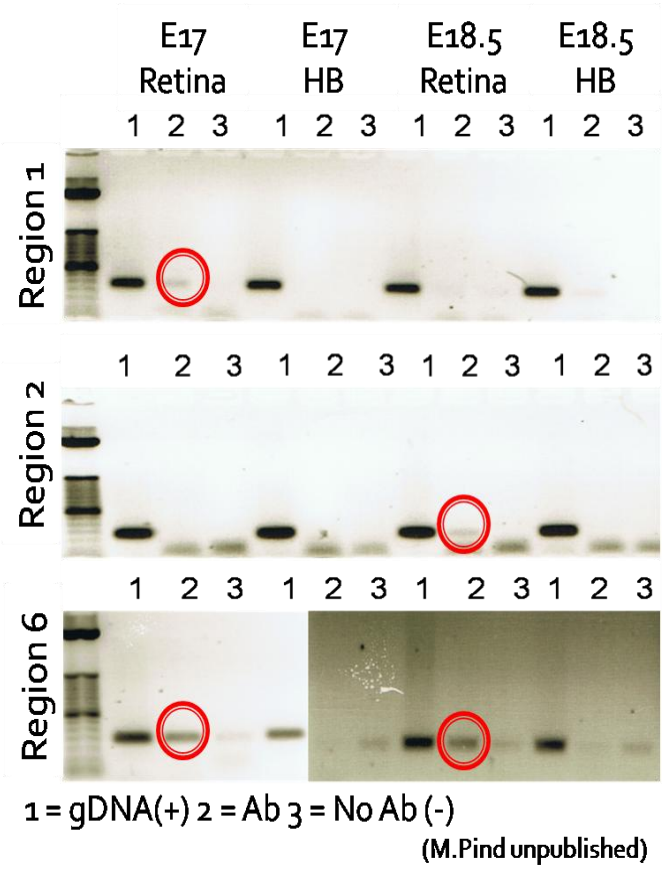


Otx2 Region 2 Sequence Detail:

311 CCTTCCAGAC CTAACACCGG GGCTTCTTCG AACTATAATT AATAGTCAAG
361 AGCAACCCTT TGGCACATTT CTTTCTCCCT CCCTCGCGGG TGGTGATGTT
411 GATTCCCCGA GGCCAGAAAC TTTCACTCGA GTTGGCGGGC CTGGCGCCGG
461 CTGTTTTCCA AATGCCCGCG

Figure 17: *Otx2* promoter ChIP assays with sequence detail

Using an antibody (Ab) specific for DLX2, occupation by DLX2 was observed at *Otx2* Region 2 *in vivo* in E18.5 retina (Ret) as indicated by the encircled band. DLX2 is not expressed in the hindbrain (HB) which is used as a negative tissue control. gDNA (genomic DNA) was used as a PCR positive control. Sequence detail reveals a 'doublet' site in this DLX2 occupied region (highlighted in red).



***Crx* ChIP Positives: Sequence Detail**

Region 1

(160)**GGGGCTATCTGTGGAGGTCTGT**CC
TTGGGGGGGCAAGGCAGGA**ATTA**TAG
CAAGGACATCTGCTCTCAGCAGCCACC
AGCTCCTCCTCCATGCTTCCCAGTGACA
CCATAGGAGCTCCCAGAACCACCAGGG
AGGTTTCCTCATCTATTCCAGTCAGTCT
CACTCATTCTCTCAGCCTTCATCA(321)

Region 2

(451)**TCTGCAGGTTGTCTTTCGT**CTCTTC
CATGTGAACATCCTGCTTTCCACAAACA
AACAAACAAACAAACAAACAAACAAAC
AATGTAGTTAAACCAATTCAGACATGTC
ATGAATTCA**TAATTA**TGGATGAAAAGA
CAGTCCCTATAGAGAGGGGACACATTT
CA**ATTA**TTTGGGTAGAGGCATCTAGAG
CTAGGGTGATCCT**GAGGGAGAGCCAAG**
TTAGGT(653)

Region 6

(2491)**GAGGAAGTGAGGGAAGAAGGG**
AGAAAGACAGAGAGGCACAGAGAAAG
AGAGAGAAACTGAGGCAGAAAAAGGG
AGAACCATCAAGAACTACACATTTGA**A**
TTAGTTCACTCAGTGGGGTCACAGATCT
CTCTTCCCAGTTATTCTAGCTGTGTCAG
AAGACTTAAAAGTT**CAATACTAGCCTG**
GGCCACA(2588)

Figure 18: *Crx* promoter ChIP assays with sequence detail

Using an antibody (Ab) specific for DLX2, occupation by DLX2 was observed at *Crx* promoter regions 1, 2 and 6 *in vivo* in E18.5 retina as indicated by the encircled bands. DLX2 is not expressed in the hindbrain (HB) which is used as a negative tissue control. gDNA (genomic DNA) was used as a PCR positive control. Sequence detail reveals a total of five potential binding sites (highlighted in red) by DLX2, including one 'doublet' site in Region 2.

3.2.2. DLX2 Binds to the Otx2 and Crx Promoters in vitro

To determine the specificity of DLX2 binding *in vitro* to the regions of the *Otx2* and *Crx* promoters identified by ChIP *in vivo*, recombinant DLX2 protein and radiolabelled oligonucleotides from the positive ChIP regions were used in electrophoretic mobility shift assays (EMSA). EMSAs exploit relative migration rates in a polyacrylamide gel to determine complex size. A complex of antibody-protein-DNA will migrate more slowly than just a protein-DNA complex compared to a 'free' DNA probe (Garner and Revzin, 1981). Upon analysis of the three positive regions from the *Crx* ChIP, there are 4 potential homeodomain binding sites under investigation; one in each of Regions 1 and 6, and two from Region 2 (the oligos were designated as Region 2a and 2b). Of these sites, only Region 2a 'shifted' in presence of the recombinant DLX2 protein. To ensure specificity of both protein binding and protein identity, cold competition and incubation with DLX2-specific antibody were performed. Cold competition entails incubation with both a radiolabelled probe and non-labelled probe, the latter being at a 100-fold increased concentration to 'out-compete' the labelled probe for protein binding. Including an anti-DLX2 antibody creates a 'supershift' as the complex consists of DNA, protein and antibody. Region C of the *Tenascin-3* promoter, a DLX2 transcriptional target, was used as a positive control (Y. Tan and D. Eisenstat, unpublished observations). These results show that recombinant DLX2 binds to Region 2a of the *Crx* promoter *in vitro* (Figures 19 and 20).

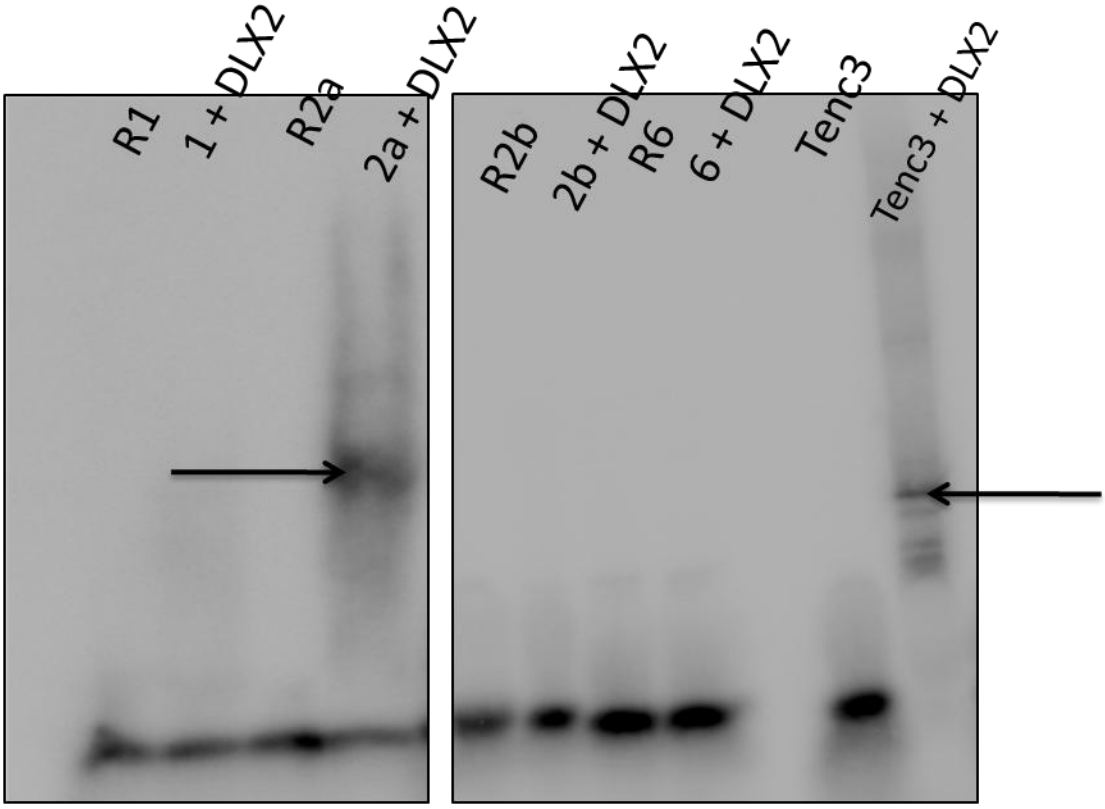


Figure 19: EMSA of recombinant DLX2 and *Crx* promoter oligos

Using recombinant DLX2 protein and radioactively labelled oligonucleotide probes designed for the positive *Crx* ChIP regions, only Region 2a was shown to bind directly to DLX2 as protein-DNA complexes will 'shift'(arrows) in a non-denaturing polyacrylamide gel relative to a free DNA probe. The Tenascin C3 promoter region was used as a positive control.

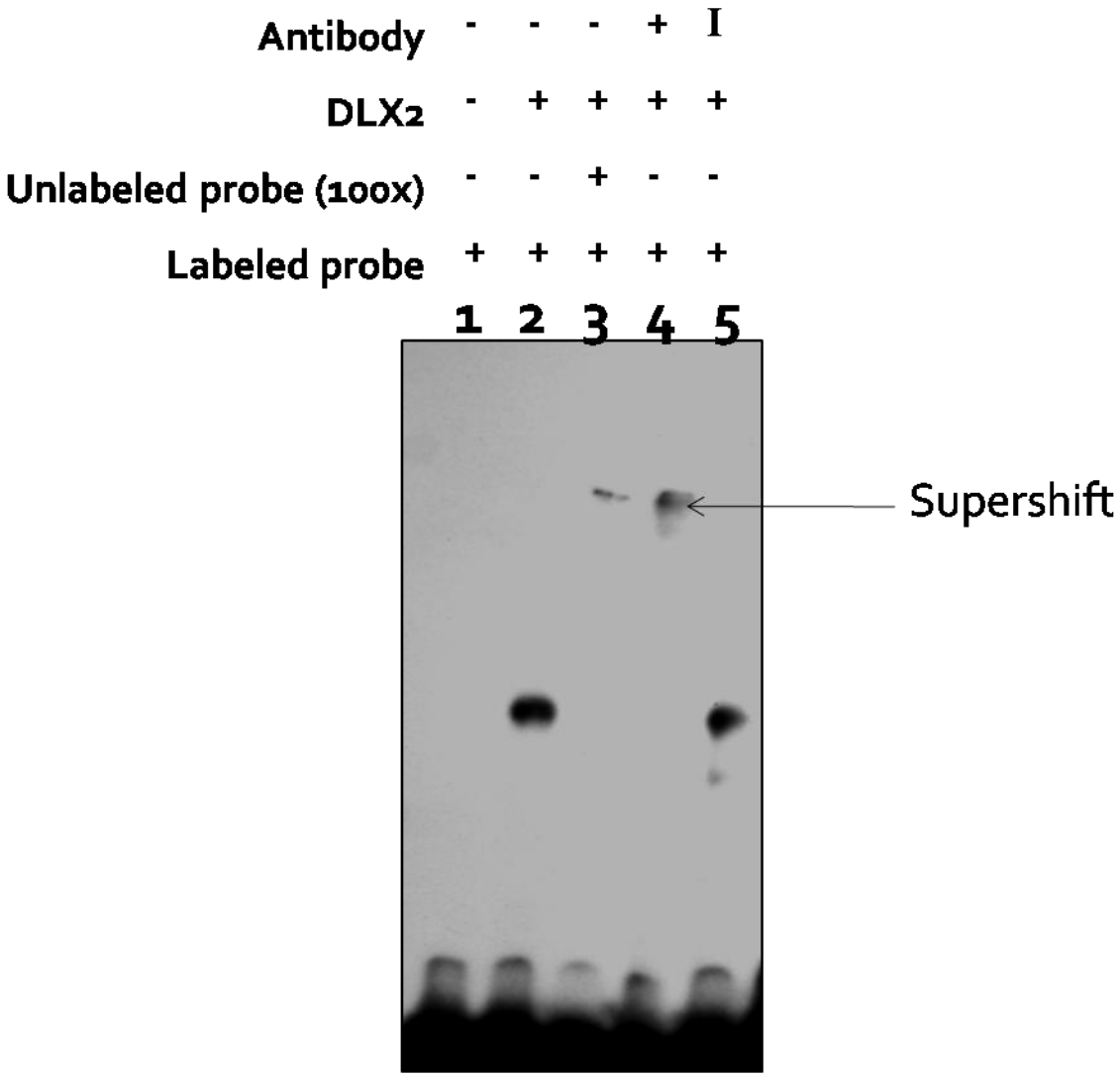


Figure 20: EMSA Supershift Assay with anti-DLX2 Antibody on Region 2a of the *Crx* promoter

By means of 100x cold competition (Lane 3) and using an irrelevant (I) antibody (Lane 5) the specificity of the DLX2 protein/DNA interaction can be confirmed by gel shift (Lane 2) and supershift (Lane 4). The band in Lane 3 represents "spill-over" from the sample loaded in Lane 4.

3.3. DLX2 regulates *Otx2* and *Crx* expression *in vivo* and *in vitro*

3.3.1. DLX1 and DLX2 repress Otx2 *and* Crx *expression in vivo*

To quantify the effects of *Dlx1/2* loss of function on *Otx2* and *Crx* transcription, quantitative real-time PCR (qRT-PCR) was performed on E18.5 WT and *Dlx1/2* DKO eyes. Primers were designed to flank exons 1 and 2 of the *Crx* transcript and exons 2 and 3 for *Otx2*. Expression was normalized to glyceraldehyde-3-phosphate dehydroxylase (*Gapdh*). Preliminary data shows that there was a 1.72 fold increase of *Crx* expression in the *Dlx1/2* DKO relative to the WT. *Otx2* expression was also increased in the *Dlx1/2* DKO by a factor of 1.56 (Figure 21).

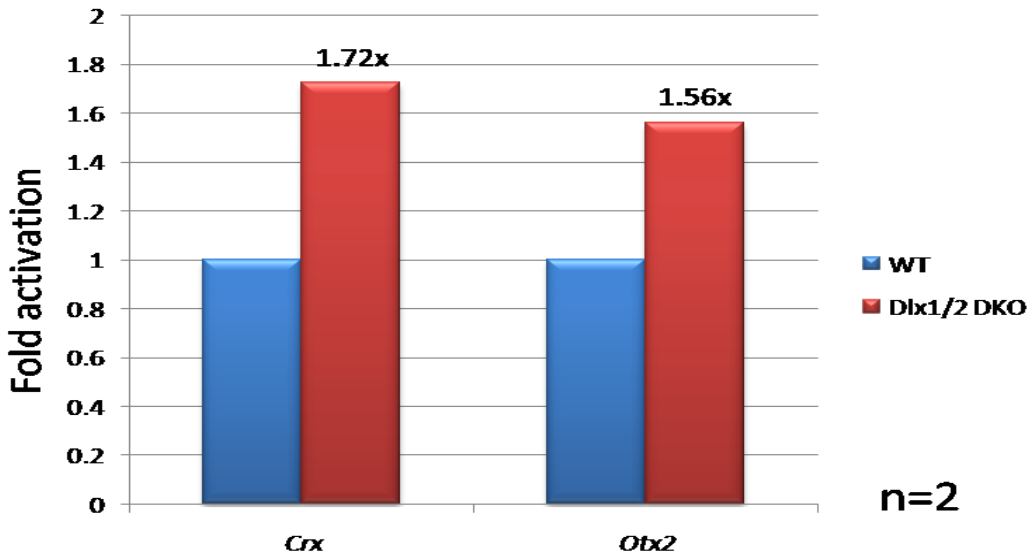


Figure 21: Quantification of *Crx* and *Otx2* transcripts in WT and *Dlx1/2* DKO retina tissue

By means of qRT-PCR, an increase in both *Crx* and *Otx2* transcripts is observed in the DKO (red bars) compared to the WT (blue) at E18.5. The *Crx* transcript is increased by approximately 1.72 times, whereas *Otx2* is increased 1.56 times. n = 2 eyes (1 litter).

3.3.2. Dlx2 regulates Crx *expression in vitro*

Luciferase reporter gene assays were used to determine the functional consequence of *Dlx2* co-expression with the *Otx2* and *Crx* promoters *in vitro*. Transfection plasmids were designed to have either the entire promoter or only the ChIP positive regions cloned upstream of the luciferase gene of the pGL3 vector. The luciferase reporter gene product, under control of the promoter in question, will be expressed and its protein product will bioluminesce and thus provide a quantitative measurement of *in vitro* expression. HEK 293 cells were transfected with either the 'empty' (no insert) pGL3 plasmids, or plasmids containing the various promoter inserts. pcDNA3 was used as the vector for the *Dlx2* cDNA plasmid (Le et al., 2007). An empty pcDNA3 plasmid was used as a control. All transfected cells were normalized for transfected β-galactosidase activity.

When comparing plasmids transfected in the HEK 293 cell line (Figure 22), the whole *Crx* promoter along with Regions 1 and 2 demonstrated an unexpected activation in the presence of *Dlx*2 (by a 9.3, 1.5 10.8 fold increase respectively). Region 6, however, which is located within 1kB of the transcriptional start site, has repressive effects on gene expression (5.5 fold decrease) relative to empty pGL3 in the presence of *Dlx2*. This suggests that *Dlx2* can be either activating or repressing gene expression on different regions on the same promoter. However, this is an *in vitro* assay and may not be an absolute representation of what is occurring *in vivo.*

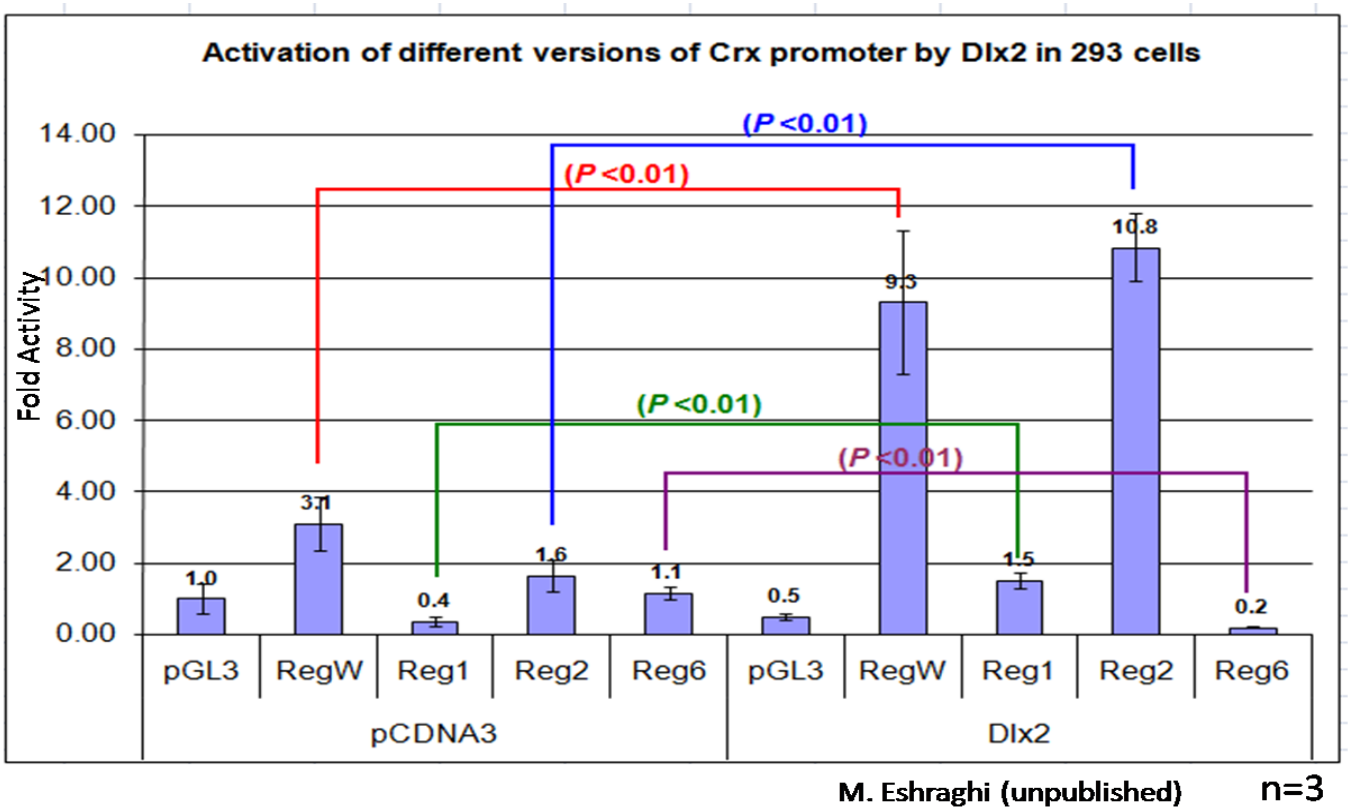


Figure 22: Luciferase reporter assays with *Crx* ChIP-positive regions in HEK 293 cells

Cells were transfected with pGL3 reporter vectors that contained either no insert (pGL3), the whole *Crx* promoter (RegW), Region 1 (Reg1), Region 2 (Reg2) or Region (Reg6) as well as with empty pcDNA3 or Dlx2-pcDNA3 expression vectors. In the presence of DLX2 co-expression, the whole promoter, Region 1 and Region 2 were all activated to varying degrees. However, *Crx* promoter Region 6 is repressed in the presence of *Dlx2*. All results were normalized to β-galactosidase readings (M. Eshraghi, V. I. Pinto and D. Eisenstat, unpublished observations).

4. DISCUSSION

We have found that *Otx2* and *Crx* are direct transcriptional targets of the DLX2 homeobox transcription factor during retinal development. DLX2 binds to promoters of *Otx2* and *Crx* and restricts their expression in the developing retina. Loss of *Dlx1/2* gene function results in an increase in the expression of *Otx2* and *Crx* transcripts, especially at E18.5. It is interesting to note that even with increased gene transcription, the OTX2 and/or CRX protein expression varies at each time-point when comparing WT and *Dlx1/2* DKO retinal tissues. This section will discuss the potential mechanisms through which DLX2 regulates *Otx2* and *Crx* expression in the developing photoreceptors in the mammalian retina.

4.1. DLX2 restricts *Otx2* and *Crx* gene transcript expression and, to a lesser extent, protein expression.

Otx2 and *Crx* are a part of a gene regulatory network involving the activation of *Crx* expression by OTX2 as well as an auto-positive feedback mechanism on CRX itself (Koike et al., 2007). Given that the *Crx* KO only displays significant photoreceptor degeneration postnatally, this suggests that the roles of the players involved in photoreceptor development are temporally regulated. DLX2 has been implicated as being a part of this complex network with ectopic aberrant expression of *Crx* in both the NBL and GCL of the *Dlx*1/2 DKO (de Melo et al., 2005).

By quantifying transcript expression, through qRT-PCR of E18.5 WT and mutant retinas, both *Otx2* and *Crx* expression are increased in the *Dlx1/2* DKO. However, increased transcription does not necessarily imply increased translation to the same degree. Through immunofluorescence and cell counting, protein expression is less discordant when comparing WT and *Dlx1/2* DKO retinas during embryogenesis. It is interesting to note that the observed

differences between protein expression patterns occur at different time-points for OTX2 (increased at E16.5) and CRX (increased at E18.5). This is consistent with the hypothesis that the respective roles of these transcription factors are temporally regulated; that despite the expression of DLX2, OTX2, and CRX during a large potion of retinal development, the effect of their presence, or absence, may not be of significance at any given time during retinogenesis. In fact, the increased levels of OTX2 and CRX protein expression observed at different time-points could also be explained if loss of *Dlx1/2* function is resulting in a general developmental delay. However, prior studies from the laboratory demonstrate specific defects in RGC differentiation and survival (de Melo et al., 2005).

Another explanation for this 'disconnect' between transcription and translation of *Otx2* and *Crx* are natural antisense transcripts (NATs) (Alfano et al., 2005). One mechanism of regulating translation is through RNA interference. This tool can be utilized in the lab to 'knock-down' protein levels of a given transcript by having a complimentary RNA which will recognize and bind to its counterpart and create a double-stranded fragment of RNA. Double stranded RNA (dsRNA) initiates a cellular 'immune' response and results in cleavage of the dsRNA, thereby inhibiting the initial transcript from undergoing translation (Fire et al., 1998). Natural antisense transcripts act in the same fashion; however, they are derived from *cis*-transcription from the opposite strand of DNA *in vivo* (Bartel, 2004). This creates the 'complementary' strand that will bind to either the primary transcript, or the processed mRNA initiating its degradation (Faghihi and Wahlestedt, 2009; Faghihi et al.). In the case of *Otx2* and *Crx*, their NATs, *Otx2Os* and *CrxOs* respectively, have almost complimentary expression patterns to their sense transcripts. *In vivo* studies of over-expression *CrxOs* postnatally resulted in a 26% decrease in *Crx* and,

interestingly, *Otx2* expression as well (Alfano et al., 2005). It could be hypothesized that the absence of *Dlx1/2* increases *Otx2* and *Crx* expression, and by doing so, correspondingly increases their NATs, which in turn, 're-regulate' transcript levels for translation.

The GOF results support the importance of temporal regulation in retinal development. We had expected to see a noticeable decrease in both OTX2 and CRX expression upon the over-expression of *Dlx2*; however, this was not the case. If our hypothesis concedes that DLX2 is acting as a transcriptional repressor, both OTX2 and CRX expression should have decreased in the presence of ectopic *Dlx2*. It is important to keep in mind the timeline for the onset of expression of these transcription factors as well as the competence model of retinal cell fate. In the retina, *Dlx2* and *Otx2* are first expressed at E11.5 followed by *Crx* at E12.5 (de Melo et al., 2008; Koike et al., 2007). The *in utero* electroporation of *Dlx2*-GFP occurred at E14.5, after the expression of the aforementioned genes has been initiated. Perhaps by this time in embryonic retinogenesis, the supernumerary DLX2 is occupying all the 'activating' sites of the CRX promoter that we have identified *in vitro* resulting in an overall increase in *Crx* expression. A similar explanation could be offered for the *Otx2* promoter, although we have more limited information about the interaction of DLX2 with this promoter both *in vitro* and *in vivo*. Also, in these GOF retinas, only *Dlx2* was overexpressed, not *Dlx1*. As our *in vivo* LOF model includes both *Dlx1* and *Dlx2*, who may have redundant functions, at least in forebrain development, these transcription factors may be working in concert with one another to properly regulate photoreceptor differentiation. By not over-expressing *Dlx1*, we are excluding the cooperative effect of *Dlx1* and *Dlx2*. Another important note is the expression differences between strains of mice. The C57/BL6 strain was used for electroporation as the RPE is pigmented providing a

landmark for *in utero* injections. Albino strains, such as CD1, are not ideal for such procedures since the eyes are not pigmented and precision in electroporation becomes problematic. Our *Dlx1/2* DKO colony has been bred into the CD1 strain of mice. There have been reports of differences and rescues in retinal phenotypes in transgenic mice depending on the strain, such as the *ocular retardation* (orJ) mouse (Bone-Larson et al., 2000) and so, comparing the effects of *Dlx1/2* LOF and GOF among different mouse strains may be of interest.

Co-expression studies demonstrated that cells that express CRX do not express markers for other cell types at E18.5. This indicates that the CRX-expressing cells are committed solely to a photoreceptor cell fate at this time-stage. However, because this is a relatively late stage in relation to the types of cell-type specific markers we investigated (RGC, amacrine and/or horizontal), these CRX positive cells may be co-expressed in cells that are destined for 'late-born' or postnatal cell fates such as bipolar cells. Further investigation using other 'late-born' cell type specific markers is warranted.

OTX2 is not co-expressed with DLX2 at E16.5 and onwards. As the initiation of retinal expression for both these genes is at E11.5, these transcription factors would only be expressed in the same cells before E16.5 (as demonstrated at E13.5) to establish programs to commit the RPCs to either a RGC or photoreceptor cell fate. Once these 'commitments' have been made and the neural retina begins to differentiate into distinct layers, DLX2 and OTX2 are localized to their respective inner and outer retinal regions and act to repress photoreceptor cell fate in the RGCs in the GCL or to activate them in the nascent ONL, respectively.

4.2. The DLX*2* transcription factor binds to the promoters of retinal genes *Otx2* and *Crx*

Analysis of the *Otx2* and *Crx* promoters has shown potential binding sites for homeodomain transcription factors such as DLX2. By using *in vivo* methods such as ChIP of developing retina, we have been able to isolate three regions, containing a total of 5 putative homeodomain binding sites, where DLX2 is present on the *Crx* promoter. Of the 3 regions tested to date for the *Otx2* promoter, one region has been isolated using ChIP, Region 2, which contains a 'doublet' binding site. By *in vitro* means (EMSA), recombinant DLX2 was specifically bound to Region 2a of the *Crx* promoter; further EMSAs using retinal nuclear extracts will confirm the direct binding of DLX2 to this region. These results are consistent with the hypothesis that *Otx2* and *Crx* are direct transcriptional targets of DLX2. It is interesting to note a difference in binding of DLX2 on *Crx* Region 2 between E17.5 and E18.5 retinas. As a ChIP assay only 'captures' a moment in development, it is quite plausible that the presence of DLX2 on the *Crx* promoter is temporally regulated.

The functional consequence of DLX2 occupation of the *Otx2* and *Crx* promoters was assessed *in vitro* by the luciferase reporter assay. This *in vitro* assay showed that *Dlx*2 co-expression activated *Crx* expression and supports our GOF experiments *in utero* but not our LOF data from the *Dlx1/2* DKO retina *in vivo*. However not all *Crx* promoter regions which were isolated as bound to DLX2 *in vivo* by the ChIP assay were activated *in vitro*. Co-expression of Region 6, which is most proximal to the transcription initiation site, led to a 5-fold repression of the reporter gene *in vitro*. Although Region 6 of the CRX promoter did not bind recombinant DLX2 in an *in vitro* assay (EMSA), we cannot completely rule out that DLX2 is not binding directly to

this region *in vivo*. Given that the oligonucleotides for the EMSA experiments were designed to solely encompass the binding site, whereas the inserts for the luciferase assays contained the sequence amplified from the ChIP "positives", the absent flanking sequences may be required for proper DLX2 binding and activity. Also, development is not considered a process that has a linear 'x-y' relationship, but moreso an 'x-y-z' relationship where z is space and time. The spatial-temporal influences are not accounted for in luciferase assays. Another example of the spatial importance is a promoter's capability to form secondary 'loops'. These loops allow for protein binding at one site but due to physical dynamics, can regulate sites located in other regions of the promoter (Ogata et al., 2003).

Through our results, we have demonstrate the temporal influence of DLX2 plays an important role in the regulation of *Otx2* and *Crx* genes. We propose a role for the *Dlx* homeobox genes in the regulation of these retinal photoreceptor genes; through negative regulation, *Dlx* genes repress the differentiation of progenitor cells to a photoreceptor cell fate and by doing so, promote a RGC fate (Figure 23).

5. CONCLUSIONS AND FUTURE DIRECTIONS

In closing, the *Dlx2* homeobox gene binds to and directly restricts the *Otx2* and *Crx* genes during retinal development. Over a variety of experiments, many interesting results have indicated that the role of DLX2 itself is temporally regulated, having specific effects at certain time-points during development. Results from the *Dlx2* GOF studies have further complicated our initial hypothesis that DLX2 restricts the expression of OTX2 and CRX from cells destined to become RGC. Also, there is some discordance between *Otx2* and *Crx* transcript levels and their protein expression in the *Dlx1/2* DKO.

Some of the methods used were *in vitro* assays designed to mimic what is potentially occurring *in vivo*. The *in vitro* methods such as luciferase reporter and gel shift assays using recombinant DLX2 protein are useful but are not necessarily reflective of what is occurring naturally in mouse retinal development. Using *Crx* reporter mice (Furukawa et al., 2002) crossed with the *Dlx1/2* DKO, will give an *in vivo* readout of *Crx* reporter gene expression. The *Crx* lacZ/*Dlx1/Dlx2* DKO reporter mice are currently being established in the lab. An *Otx2*-GFP reporter mouse (Fossat et al., 2007) will also be bred with the *Dlx1/2* DKO colony for characterization of reporter gene expression *in vivo*. Designing loss-of-function knock-in mouse models specific for putative sites regulated by DLX2 would provide us with *in vivo* determinations of DLX2 function on a candidate target gene's transcriptional regulation, specifically which sites would be necessary and/or sufficient. From the deleted fragments used to create these particular loss-of-function mice, we can determine the length, sequence and subsequently, other regulatory elements, required for proper interaction with DLX1/2. As a difference was noted in DLX2 binding between E17.5 and E18.5 in the *Crx* ChIP, it would be

worthwhile to pursue a time-course ChIP to determine when each region is occupied by DLX2 *in situ*. Use of retinal nuclear extracts for EMSAs would also provide additional insights into DLX2 binding at regions of the *Otx2* and *Crx* promoters identified by ChIP.

As noted, our mouse model has both *Dlx1* and *Dlx2* genes deleted. Despite having reported redundant functions in forebrain development, perhaps during retinal development they act individually and have prominent roles at different times during embryogenesis. Characterization of *Otx2* and *Crx* as DLX1 transcriptional targets, over-expression of *Dlx1* studies *in vitro* and *in utero*, along with analysis of the *Dlx1* and *Dlx2* single knockout retinas, will provide more insight into these relationships. The *Dlx1/2* DKO mice die at birth, which becomes problematic for studying retinal development as in the mouse, retinogenesis is completed postnally. By generating a conditional *Dlx1/2* DKO mouse in the retina, we can determine the effects of the absence of both of these transcription factors during retinal development in the postnatal and adult retina. An alternative is to use short interfering or small-hairpin RNAs to 'knock-down' *Dlx1/2* expression in retinal explants to mimic the effect of *Dlx1/2* loss postnatally.

As there is an apparent doscordance between transcript levels and protein levels of Otx2 and CRX in the *Dlx1/2* DKO, it would be interesting to investigate when during embryogenesis this 'disconnect' occurs by comparing transcript to protein levels at earlier time-points. Exploring the expression of *Otx2* and *Crx* NATs in the *Dlx1/2* DKO would also be of interest to determine whether these are being affected directly and that the alterations of *Otx2* and *Crx* expression are actually a downstream result of NAT mis-regulation.

To further the *Dlx2*-GOF studies *in utero*, electroporation and assessments at different time-points during development should be conducted. Ideally, if electroporating increased amounts of exogenous *Dlx2* could coincide with onset of endogenous *Dlx2* expression, the effects would be more relevant to assess. As the genetic strains of our *Dlx1/2* DKO colony and the GOF mice are not the same, it would be ideal to use the same strain for both transgenic lines. However, due to technical limitations with regards to the size of embryonic eyes at E11.5, and the difficulty of performing *in utero* injections in albino mice, these experiments will remain a future direction until technology enables these studies.

Overall, the transcriptional network involving *Dlx2, Otx2* and *Crx* is dynamic and relies heavily on temporal and/or spatial regulation. The investigation of the regulatory relationships between these three players has proved to be quite a complex, perplexing and yet interesting quest. The experiments and results discussed in this thesis provide an insight into the vast regulatory network that is vertebrate retinal development.

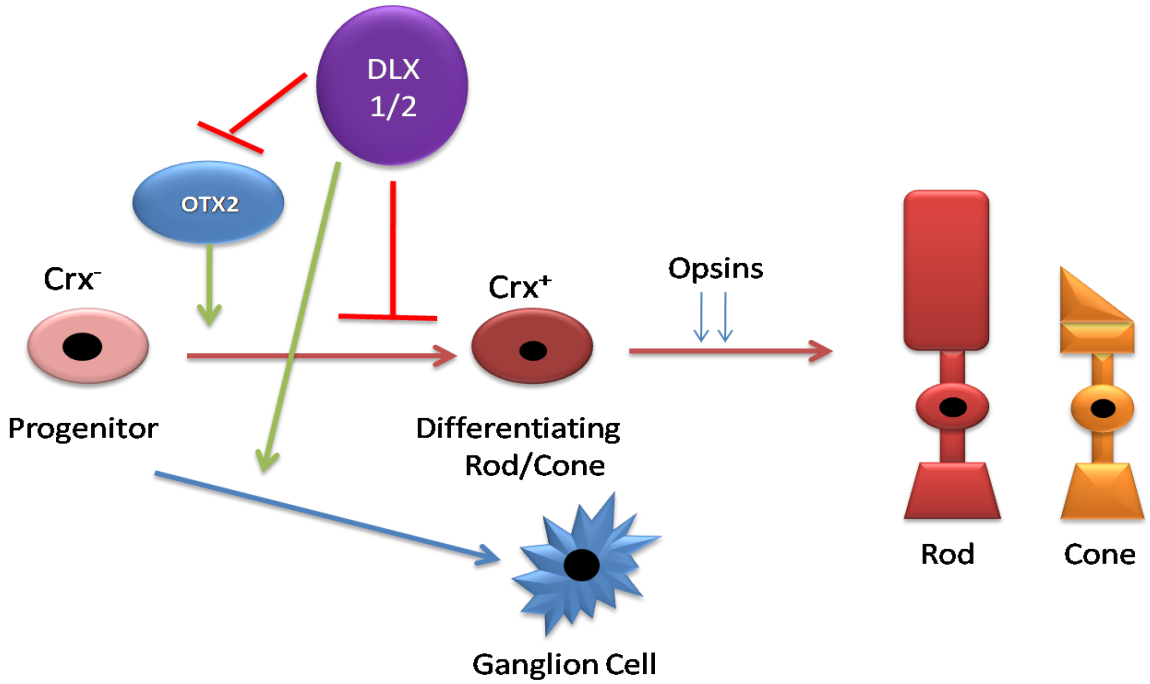


Figure 23: Proposed Model for the Regulation of *Otx2* and *Crx* by DLX1/2 transcription factors

The activation of *Otx2* in a progenitor cell activates the expression of *Crx* committing the cell to a photoreceptor cell fate. Downstream targets of *Crx*, such as opsins, are activated to differentiate into either a rod or cone photoreceptor. DLX1/2 acts to repress the expression of *Otx2* and *Crx* and by doing so, promotes retinal ganglion cell fate in progenitor cells. This proposed model does not include the hypothesized mechanism supported by the GOF studies.

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