TRANSCRIPTIONAL REGULATION OF THE RETINOBLASTOMA FAMILY MEMBER P107 BY DLX HOMEOBOX GENES DURING CENTRAL NERVOUS SYSTEM DEVELOPMENT

by

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Acknowledgements

"If I could offer you only one tip for the future, sunscreen would be it. The long term benefits of sunscreen have been proved by scientists whereas the rest of my advice has no basis more reliable than my own meandering experience…" – Mary Schimich

I recently heard a song on the radio that was released when I was 13 years old; "Wear Sunscreen" performed by Baz Luhrmann; a verbatim speech written by Mary Schimich (and don't worry, permission was obtained. No plagiarism) titled "Advice, like youth, probably just wasted on the young". I recall hearing this song years ago but of course, never really put much of a second thought to the lyrics. Now, as I write my thesis, this song provides inspiration for these acknowledgements.

First and foremost, I would like to thank my supervisor, **David Eisenstat**. I'm truly grateful you offered me a position in your lab. I will never forget our first meeting. During our meeting, it was very apparent that not only were you interested in me as a student; you were truly interested in me as a person. Your dedication to your students is incomparable. I can't thank you enough for all you have done for me and continue to do. I'm looking very much forward to continuing my studies under your guidance in Edmonton. I only hope that with time your jokes improve. Nobody has seen "The Fly". That is nobody born after 1980. May I suggest the following for your repertoire: Where do retina researchers get their music? Eye-tunes. Maybe mine aren't much better.

"Get to know your parents, you never know when they'll be gone for good" - Next, I would like to thank my parents, **James** and **Sharon** for all their love and support. A portion of the credit for my successes goes to you for making me the person I am, both by what you've taught me, and the genes you've passed on to me through the incredible process of meiosis. I truly hope I've made you both very proud.

To my "other" family members, **Barney** and **Sophie**: No matter how terrible the day, you two always make me laugh and smile.

"Dance…even if you have nowhere to do it but in your own living room". In our case, in the lab! - I've been very fortunate to work with an amazing group of people. **Sari**: Hey B! Life is short; buy the shoes. Ha ha! The lab was truly better with you in it! It is not the same without you. No matter where life takes us, I hope we will remain friends…as long as your keen sense of fashion never waivers! If I see you wearing runners and a dress, it's over. **Andrea**: I didn't think there was anyone who was a bigger "keener" than I… then I met you! Ha ha! But it's all love. We are a dying breed. Never change. I look forward to attending neuroscience meetings with you in the future. **Qi**: QI!!!! You're so very handsome. I always said I aspire to be the She-Qi. I've always looked up to you and always will. I can only hope I will be remotely as good as you one day. Also, watch your back. You never know when I may come up behind you and scare you ☺. **Mario**: Thanks for teaching me so many bad words and funny phrases in Spanish. Not only have you taught me that, but much of my laboratory techniques you've taught me as well. So if they're no good, it's your fault. Chupacabra! **Shunzhen** – I always thought David described you best: you are Yoda - powerful and very wise. When I joined the lab, I felt

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"Understand that friends come and go, but for the precious few you should hold on." - I've also been fortunate to make many wonderful friends outside of the lab since moving to Winnipeg. **Raj**, **Carrie, Jason and Jonie**: Words cannot express how much your friendship has meant to me. You were all there for me when I needed friends the most. I will never forget that. I love you all! I will always cherish our friendship. You four are like family to me. I will miss you guys so much when I head out West. Please come and visit!

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"Be nice to your siblings; they are the best link to your past and the people most likely to stick with you in the future" - Finally, I want to thank the most special person in my life: my brother **Matthew**, AKA "Mr. Cool". You are and always will be my best friend. It's funny how our relationship has evolved. We fought like crazy as kids (remember the sandwich incident?) then went on to live together as we both went to the University of Manitoba. I didn't think living with you would be very fun at first (sorry), but it turned out to absolutely be the best time of my life. I always have so much fun with you and moving away from you will truly be the most painful. I'm so incredibly proud of you and all you have accomplished. Mom and Dad have some pretty awesome kids! Love you, baby brother <3.

As I finish this chapter in my life in Winnipeg at the University of Manitoba to start anew at the University of Alberta, another line from "Wear Suncreen" – "sometimes you're ahead, sometimes you're behind…the race is long, and in the end, it's only with yourself."

GO JETS GO!!!

Jamie Lauren Zagozewski
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ABSTRACT

TRANSCRIPTIONAL REGULATION OF THE RETINOBLASTOMA FAMILY MEMBER P107 BY DLX HOMEOBOX GENES DURING CENTRAL NERVOUS SYSTEM DEVELOPMENT

Objective: The *Dlx* homeobox genes are important for the proper development of vertebrate forebrain, retina, craniofacial structures and limbs. The absence of *Dlx1/Dlx2* results in defects in neural differentiation and migration in the developing forebrain and retina. To further understand genetic regulation of neural differentiation and migration by *Dlx* genes, we have examined transcriptional regulation of the cell cycle regulator *p107* by DLX2. We hypothesize that DLX2 directly binds to the *p107* promoter, thereby activating its expression in the developing forebrain and retina.

Methods: Chromatin immunoprecipitation was carried out to confirm occupancy of DLX2 at the *p107* promoter *in situ*. Electrophoretic mobility shift assays were performed to determine specific binding to sites found in the *p107* promoter *in vitro*. *Dlx1/Dlx2* double knockout murine tissues were examined for *p107* expression by immunofluorescence and qRT- PCR. Reporter gene assays were conducted to examine the functional effect of DLX2 binding to the *p107* promoter *in vitro.* Site directed mutagenesis (SDM) of putative DLX2 binding sites in the *p107* promoter was done to identify critical sites for activation of *p107* expression and direct binding.

Results: DLX2 occupies the *p107* promoter *in situ* in both the developing forebrain and retina and binds directly to sequences for the *p107* promoter *in vitro*. In the absence of *Dlx*1*/Dlx2*, we observe reduced *p107* expression. DLX2 binding to the *p107* promoter results in significant activation of luciferase reporter gene expression *in vitro*. DNA

binding sites in *p107* promoter region 1 and *p107* promoter region 8 have been identified as critical sites for activation of and direct binding by DLX2 *in vitro*.

Conclusions: DLX2 binds to the *p107* promoter *in situ* and *in vitro*, resulting in the activation of *p107* expression in both the developing forebrain and retina. These findings contribute to our knowledge of genetic regulation of neurogenesis by *Dlx* homeobox genes and may contribute to identification of novel targets for disorders of neuronal differentiation and migration.

LIST OF ABBREVIATIONS

αDLX2 – DLX2 high affinity antibody

bp – base pair

BrdU - Bromodeoxyuridine

BSA – bovine serum albumin

CNS – central nervous system

cDNA- complementary deoxyribonucleic acid

CDK – cyclin-dependant kinase

CDKI – cyclin-dependant kinase inhibitor

ChIP – chromatin immunoprecipitation

CKO – conditional knockout

Ct – cycle threshold

DIF – double immunofluorescence

DKO – double knockout

Dll – distal-less homeobox gene (invertebrate)

Dlx – distal-less homeobox gene (vertebrate)

DLX – distal-less homeobox protein

DNA – deoxyribonucleic acid

DTT – Dithiothreitol

E – embryonic

E. coli – *Escherichia coli*

EGF – epidermal growth factor

EMSA – electrophoretic mobility shift assay

FB – forebrain

G_1 – first gap phase

G_2 – second gap phase

GABA – γ aminobutryic acid

GCL – ganglion cell layer

gDNA – genomic deoxyribonucleic acid

GE – ganglionic eminences

HDAC – histone deacetylase

IF – immunofluorescence

INL – inner nuclear layer

IPL – inner plexiform layer

IPTG – isopropyl-β-thiogalactopyranoside

ISH – *in situ* hybridization

kb – kilobase

KO – knockout

LGE – lateral ganglionic eminence

LB – lysogey broth

M phase – mitotic phase

MGE – medial ganglionic eminence

mRNA – messenger ribonucleic acid

MZ – mantle zone

NBL – neuroblastic layer

NCX –neocortex

O.C.T. – optimal cutting temperature

O/N – overnight

ONL – outer nuclear layer

OPL – outer plexiform layer

PIC – protease inhibitor cocktail

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PFA – paraformaldehyde

PPP – Primary proliferative population

P0 – postnatal day 0

p107 – *p107* gene

P107 – *p107* protein (gene product)

p130 – *p130* gene

P130 – *p130* protein (gene product)

pRb – retinoblastoma protein (gene product)

qRT-PCR – quantitative real-time polymerase chain reaction

Rb- retinoblastoma gene

rDLX2 – recombinant DLX2 protein

RGC – retinal ganglion cells

RNA – ribonucleic acid

RPC – retinal progenitor cells

RT – room temperature

S phase – synthesis phase

SEM – standard error of the mean

SDM – site directed mutagenesis

SPP – secondary proliferative population

SVZ – subventricular zone

TEMED - N,N,N',N'-Tetramethylethylenediamine

TGFβ - transforming growth factor β

TGFβRII – transforming growth factor β receptor II

tRNA – transfer ribonucleic acid

VZ – ventricular zone

WT – wild-type

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INTRODUCTION

Development of the forebrain

Early development of the forebrain

The vertebrate prosencephalon (forebrain) is arguably the most complex biological structure containing a remarkably heterogeneous collection of neurons. The forebrain arises from the anterior neuroectoderm, positioned above the notochord which forms during the process of gastrulation (Rubenstein, Shimamura et al. 1998; Wilson and Houart 2004). Inductive signals from the notochord drive neuroectodermal cells to differentiate into neural precursors initiating the process of neurulation. It is the resulting neural tube from which the brain and other CNS structures are derived. The forebrain is divided into the rostral telencephalon, from which the dorsal pallium (cortex) and ventral subpallium will develop, and the caudal diencephalon which contains the prethalamus, thalamus, and the pretectum (Rubenstein and Beachy 1998; Wilson and Houart 2004).

Telencephalon structure

The vertebrate telencephalon is divided into two distinct regions: the pallium, which gives rise to the neocortex (NCX) and the subpallium (**Figure 1**), which is largely specified by expression of the *Dlx* gene family (Anderson, Eisenstat et al. 1997; Medina and Abellan 2009; Moreno, Gonzalez et al. 2009). The pallium is located dorsally in the telencephalon and contains the developing NCX, which is the area of the brain responsible for cognitive function and sensory perception. The NCX is a six layered structure which develops in an "inside out" fashion with the earliest born neurons

populating the deep layers of the NCX and the later born neurons migrating to the most superficial, pial region of the NCX (Caviness 1982; Allendoerfer and Shatz 1994; Gilmore and Herrup 1997). Lamination of the cortex commences when the earliest born cortical neurons establish the subpial preplate layer. Next, neurons that will establish the cortical plate migrate to the preplate and split this transient layer into the superficially localized marginal zone (layer I) and the subplate (layer VI). Following this step, successive layers of newborn cortical neurons migrate through the subplate and past the prior established layers, stopping beneath the marginal zone. Consequently, layer I and layer VI are the oldest neurons in terms of neuronal birthdate, and layers II-VI represent the newest to oldest generated cortical layers, respectively.

The subpallium, positioned ventrally to the pallium, is the birthplace of interneurons that use the inhibitory neurotransmitter γ aminobutryic acid (GABA), which migrate tangentially from the subpallial structures to populate the developing NCX (**Figure 1**) (Anderson, Eisenstat et al. 1997; Marin and Rubenstein 2003; Chedotal and Rijli 2009). GABAergic interneurons are born in the proliferative ventricular zone (VZ) and subventricular zone (SVZ) in the evaginated ganglionic eminences (GE) of the subpallium. These cortical GABAergic interneurons arise from both the medial ganglionic eminences (MGE) and the lateral ganglionic eminences (LGE) (Anderson, Eisenstat et al. 1997; Anderson, Marin et al. 2001; Le, Du et al. 2007). The MGE is the source of cells migrating tangentially from the subpallium to the cortex in early and mid-embryonic stages (E11.5-14.5) of telencephalonic development (Lavdas, Grigoriou et al. 1999; Wichterle, Garcia-Verdugo et al. 1999; Anderson, Marin et al. 2001). During later stages of development (E14.5-E16.5), tangential migration of interneurons initiates from

both the MGE and the LGE (Anderson, Marin et al. 2001; Marin and Rubenstein 2003), resulting in a population of the NCX with inhibitory GABAergic interneurons

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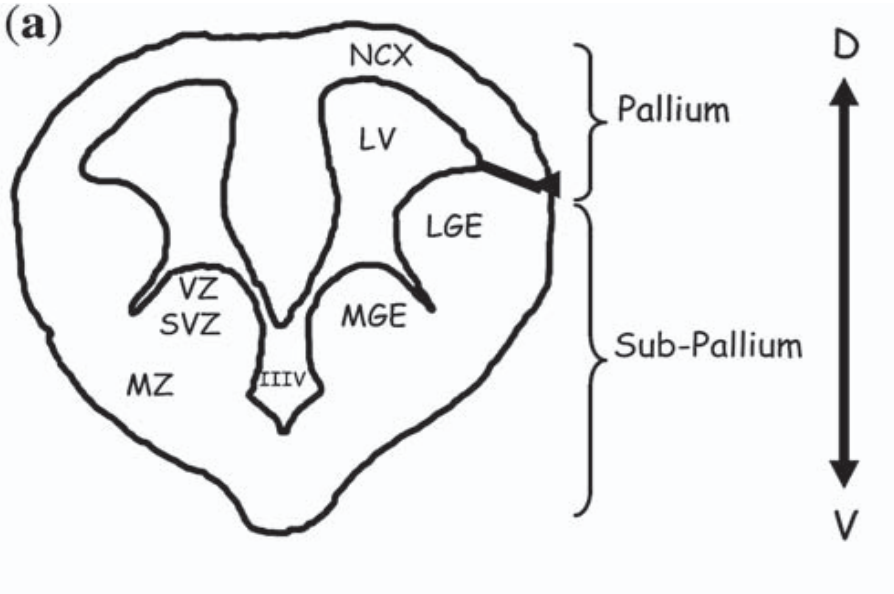

Figure 1: Schematic representation of the developing telencephalon. The pallium, located dorsally (D) in the developing telencephalon, contains the NCX. The ventrally (V) localized subpallium contains the LGE and the MGE. It is in these subpallial structures where GABAergic interneurons are born in the proliferative SVZ and VZ. Interneurons migrate to the SVZ and MZ followed by tangential migration to the pallium where they cross the pallial-subpallial boundary (arrow head) [LV = lateral ventricle, NCX = neocortex, LGE = lateral ganglionic eminence, MGE = medial ganglionic eminence, VZ = ventricular zone, SVZ = subventricular zone, MZ = mantle zone] (Wigle & Eisenstat, 2008, *Clinical Genetics*, John Wiley and Sons).

Neuronal migration patterns

In the developing forebrain, neurons are born in germinal ventricular zones and subsequently migrate to their final positions where they differentiate into mature neurons. Two modes of neural migration are observed during the development of the forebrain: radial and tangential migration (Hatten 1999; Nadarajah and Parnavelas 2002; Marin and Rubenstein 2003). Radial migration follows a pattern whereby postmitotic neurons follow the radial disposition of the neural tube to the brain surface. Radial migration has been most extensively studied during the process of cortical lamination and consequently, two types of radial migration patterns have been observed during cortical development. The first of these modes is somal translocation. Somal translocation is adopted by cortical neurons migrating during early stages of corticogenesis (Nadarajah and Parnavelas 2002; Marin and Rubenstein 2003). Cells undergoing somal translocation have long processes that are attached to the pial surface of the brain and short trailing processes. Cells will then lose their ventricular attachment and migrate toward the pial surface through a process called nucleokinesis. The second type of radial migration observed in corticogenesis is glial-guided migration. As the name implies, post-mitotic cells migrate from the ventricular zone to the pial surface during cortical development by attaching to, and migrating across glial cells that span the neural tube and are anchored into the pial surface (Nadarajah and Parnavelas 2002; Merkle, Mirzadeh et al. 2007).

Neurons that are born in the proliferative VZ of the ganglionic eminences in the basal telencephalon populate the developing NCX by undergoing tangential migration from the basal telencephalon to the NCX, crossing the pallial/subpallial barrier in the process (Anderson, Eisenstat et al. 1997; Nadarajah, Alifragis et al. 2002; Marin and Rubenstein

2003). Tangential migration is the migratory method adopted by GABAergic interneurons in order to populate the developing NCX. This tangential migration from the basal telencephalon to the cortex is reliant upon expression of *Dlx1* and *Dlx2* as the loss of expression of these genes in the developing forebrain results in interneurons remaining aberrantly in the SVZ of MGE (Anderson, Eisenstat et al. 1997).

Retinal development

Early retinal development

The retina is considered a part of the central nervous system (CNS) as the prospective retina arises from the neuroepithelium as an out-pouching of the diencephalon during early development (Barnstable 1987; Graw 2010). The first major step in early retinal development occurs at E9.5, when the central eye field splits to give rise to two optic vesicles in addition to concomitant formation of the lens placode. At E10.5, the lens placode invaginates and begins to form the lens pit and the prospective neural retina and retinal epithelium. The optic cup arises at E11.5 when the lens pit closes to form the lens vesicle and differentiation of the neural retina begins (Barnstable 1987; Graw 2010).

Retinal structure

The fully developed adult retina is a trilaminar structure (**Figure 2**), composed of seven retinal cells types; six neural and one glial cell type, which all arise from a single retinal progenitor cell (RPC) (Dowling and Boycott 1966; Dowling 1970). The innermost layer of the retina is the ganglion cell layer (GCL), named for the presence of the retinal ganglion cells (RGC). In addition to RGC, the GCL contains displaced amacrine cells.

The inner nuclear layer (INL) is the middle layer of the retina, which contains the cell bodies of amacrine cells, horizontal cells, bipolar cells, and the Müller glial cells. The outermost layer is termed the outer nuclear layer (ONL) or photoreceptor layer due to the presence of the photosensitive rods and cones. Between these cellular layers lie the plexiform layers where axonal and dendritic connections are established. When synaptic connections between neural cells in the retina are made, the main connections for processing visual information is a tricellular chain from photoreceptors synapsing on to bipolar cells then finally to the RGC, the axons of which bundle to form the optic nerve sending visual information to the brain (Dowling and Boycott 1966; Dowling 1970).

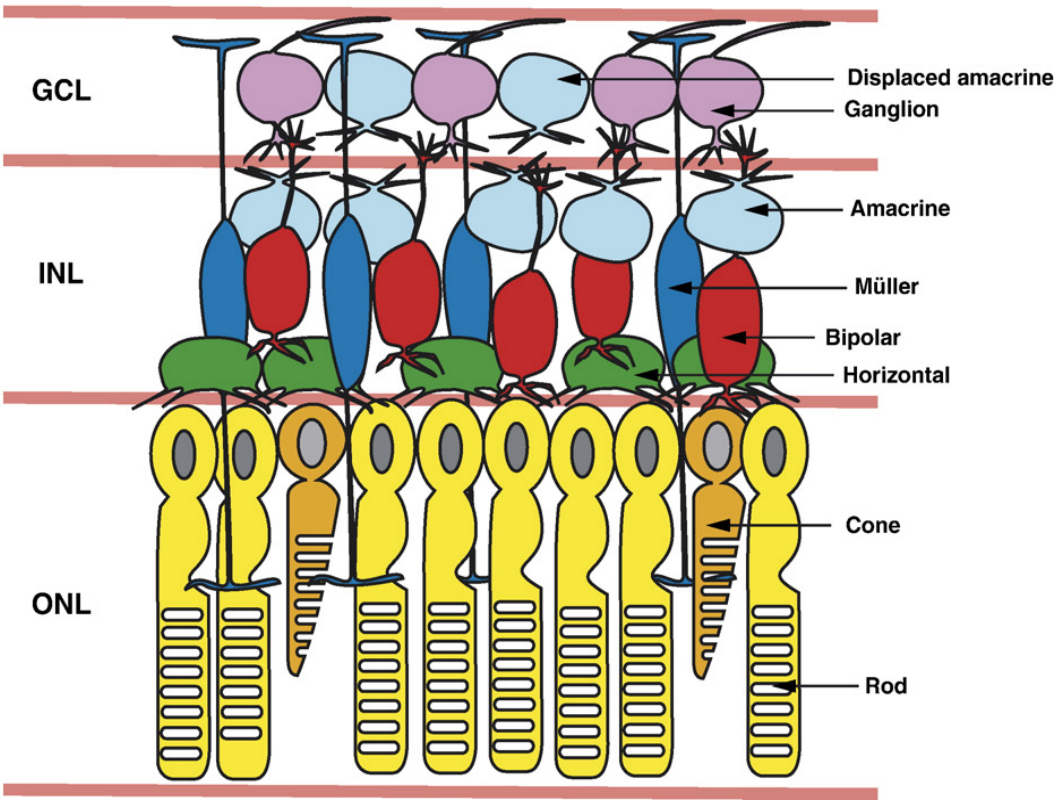

Figure 2: Lamination of the vertebrate retina. The mature, adult retina is a trilaminar structure made up of seven retinal cell types. The GCL, located adjacent to the vitreous, contains retinal ganglion cells and displaced amacrine cells. The INL contains the cell bodies of the horizontal, bipolar, amacrine and the Müller glial cells. The ONL or photoreceptor layer contains the photosensitive rods and cones. Between the GCL and INL lies the inner plexiform layer (IPL), and located between the INL and ONL is the outer plexiform layer (OPL). It is these plexiform layers where axonal dendritic connections are established. [GCL = ganglion cell layer, INL = inner nuclear layer, ONL = outer nuclear layer] (Ohsawa & Kageyama, 2008, *Brain Research*, Elsevier).

The competency model for retinal development

As mentioned previously, the mature retina is comprised of seven cell types which all arise from a single RPC. In order for this single, multipotent RPC to form all cell types in a uniform direction, it follows the retina competency model (**Figure 3**) (Cepko, Austin et al. 1996; Livesey and Cepko 2001; Reese 2011). This model proposes that RPC progress through stages of competency that are intrinsically determined. As development progresses, RPC competency changes through intrinsic transcription factor expression coupled with extrinsic cues enabling the production of subsets of retinal cell types. This model allows for a unidirectional formation of all retinal cell types in a conserved order. During early retinal development, the RPC pool increases by symmetric division, giving rise to two equivalent daughter cells. As proneural gene expression is initiated, subsets of the RPC exit the cell cycle and begin to differentiate into the earliest born retinal cells. The remainder of RPC continue symmetric division due to Notch-Delta mediated lateral inhibition in order to maintain the progenitor pool for the production of later born retinal cell types. As development progresses, again, signals from the environment, differentiated cell types and intrinsic transcription factor expression restrict the competency of the RPC so they give rise to later born retinal cells.

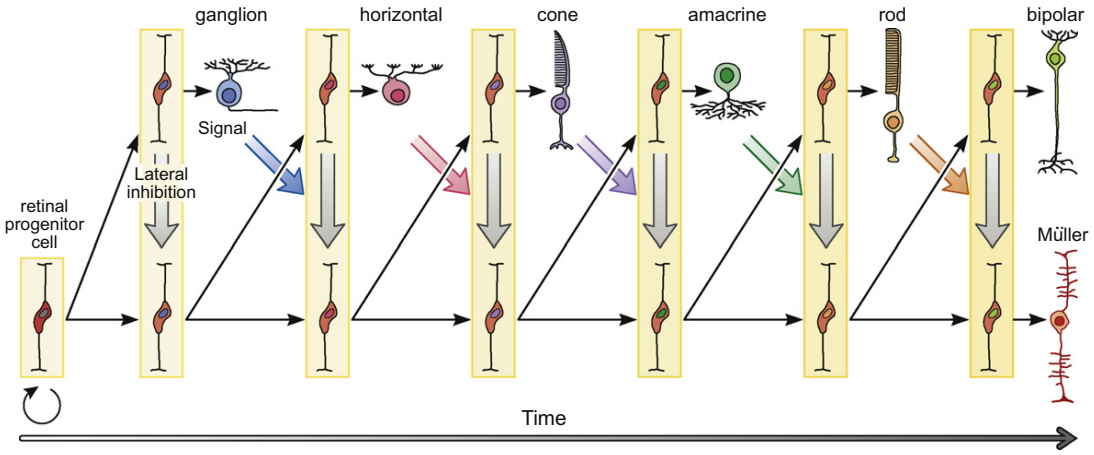

Figure 3: Retinal competency model. Competence of retinal progenitors gradually becomes restricted as times progresses, allowing for unidirectional production of all mature vertebrate retinal cell types. The retinal progenitor pool increases due to symmetric division (figure left, circular arrow). Proneural gene expression results in birth of early retinal cell types (top of figure) coupled with lateral inhibition (grey vertical arrows) that allows only a subset of progenitors to exit the cell cycle, maintaining the progenitor pool. Newborn neural cells change the extrinsic environment, which restricts production of cells of the same type (coloured arrows). The extrinsic and intrinsic changes induce expression of transcription factors by the progenitors, restricting progenitor competence, giving rise to later born retinal cell types. This process continues in a unidirectional manner until the mature retina is fully formed (Reese, 2010, *Vision Research*, Elsevier).

Vertebrate retinogenesis

As RPC divide to produce mature retinal cells, they occupy specific regions of the retina as they progress through the cell cycle. RPC undergo M (mitotic) phase at the ventricular surface in the neuroblastic layer (NBL) of the retina (Young 1985; Young 1985; Chen, Livne-bar et al. 2004). Here, cells will exit the cell cycle and then migrate to their final location where they differentiate into mature retinal cells. Alternatively, RPC that have divided symmetrically re-enter the cell cycle. RPC that re-enter the cell cycle progress to G_1 (first gap) phase and begin migration to the vitreal surface where they undergo S (synthesis) phase. As these cells progress to G_2 (second gap) phase they migrate to the ventricular surface where they will again enter M phase. In both murine and human retinal development, cell birth order of the seven retinal cell types follows a highly conserved temporal sequence with RGC being the first of the cell types to become postmitotic. The cell birth order that follows is amacrine cells, cones, horizontal cells, bipolar cells, rods and Müller glia (Livesey and Cepko 2001; Marquardt and Gruss 2002; Wang, Mu et al. 2002). Unlike human retinal development, the murine retina is not fully developed at the time of birth. At birth (P0), the murine retina is comprised of a differentiated GCL and the NBL, which consists of RPC of restricted competency and postmitotic cells. It is not until the second postnatal week that the retina has completed differentiating and is fully functional in the mouse.

Dlx homeobox genes

The *Dlx* gene family are a class of homeobox genes, which play important roles in the development of a number of vertebrate tissues, including the forebrain, retina, craniofacial structures and developing limbs (Panganiban and Rubenstein 2002). *Dlx* genes are the vertebrate homologs of the *Drosophila* distal-less (*Dll*) homeobox genes, which as the name implies, are responsible for development of distal appendages. Like all homeobox genes, *Dlx* genes encode a 60 amino acid homeodomain containing transcription factor. Through this homeodomain, these transcription factors bind to tetranucleotide motifs ATTA/TAAT in the major groove of DNA at target gene regulatory regions, usually promoters (Wigle and Eisenstat 2008). This binding enables either transcriptional repression or activation of target gene expression.

Dlx structure and organization

In both mice and humans, there are six *Dlx* genes. They include *Dlx1* through *Dlx6,* with *Dlx1* and *Dlx2*, *Dlx3* and *Dlx4,* and *Dlx5* and *Dlx6* arranged in bigenic clusters, respectively. Each bigenic cluster is linked to a *Hox* cluster (McGuinness, Porteus et al. 1996; Panganiban and Rubenstein 2002). The *Dlx1/Dlx2* pair is linked to *Hoxd*, *Dlx3/Dlx4* is linked to *Hoxb* and *Dlx5/Dlx6* is linked to *Hoxa*. *Dlx1* and *Dlx2* are only 10 kb apart, located on mouse chromosome 2 (McGuinness, Porteus et al. 1996). Each of the *Dlx* genes are composed of 3 exons and 2 introns with the homeobox contained within both exons 2 and exon 3.

Dlx gene function in the CNS

The function of *Dlx* genes during development of the CNS has largely been determined through knockout (KO) studies of one *Dlx* gene or *Dlx* gene pairs (Panganiban and Rubenstein 2002). Independent single KO of *Dlx1*, *Dlx2* and *Dlx5* have been generated and the phenotypes were analyzed. Heterozygotes are all viable, fertile and phenotypically normal (Panganiban and Rubenstein 2002). Independent single KOs of *Dlx1*, *Dlx2* and *Dlx5* die at birth, demonstrating only subtle forebrain defects. Loss of both *Dlx1* and *Dlx2* expression results in a blockage of lateral tangential migration of inhibitory GABAergic interneurons to the developing NCX (Anderson, Eisenstat et al. 1997; Marin and Rubenstein 2003). *Dlx1/Dlx2* double knockout (DKO) mice also die at birth. These results suggest partial redundancy between *Dlx* genes during development, which can be explained by the nearly identical homeodomains whereas the unique functions are perhaps due to divergence of their N- or C-terminal sequences and other domains (Liu, Ghattas et al. 1997; Eisenstat, Liu et al. 1999).

Dlx genes in forebrain and retinal development

Dlx gene expression patterns in the developing forebrain

Of the six known *Dlx* genes, four are expressed in the developing CNS: specifically, *Dlx1*, *Dlx2*, *Dlx5* and *Dlx6* (Liu, Ghattas et al. 1997; Eisenstat, Liu et al. 1999; Panganiban and Rubenstein 2002). During early subpallial development, the *Dlx* genes follow a temporal expression order with *Dlx2* being expressed first, followed by *Dlx1*, *Dlx5* and finally, *Dlx6*. These genes are expressed in the GE of the developing basal telencephalon with differing but overlapping patterns of expression. *Dlx2* and *Dlx1* are

largely expressed in progenitor cell populations in the VZ and SVZ of the subpallium, whereas *Dlx5* and *Dlx6* are restricted to postmitotic, differentiating neurons located in the SVZ and the mantle zone (MZ).

Dlx gene function in the developing forebrain

The loss of both *Dlx1* and *Dlx2* in the developing forebrain results in a time dependent block in striatal neurogenesis (Anderson, Qiu et al. 1997). Whereas early neurogenesis appears normal despite the loss of *Dlx1* and *Dlx2* expression, differentiation of cells born after E12.5 is largely aborted (Anderson, Qiu et al. 1997; Panganiban and Rubenstein 2002). These cells fail to migrate and remain in the SVZ, which in the absence of *Dlx1* and *Dlx2*, develops abnormally. The primary proliferative population (PPP), located in the VZ appears normal, whereas the secondary proliferative population (SPP), located in the SVZ, fails to mature. In the SPP of the mutant SVZ, there is aberrant expression of *Lhx2* which is a transcription factor associated with the proliferative properties of the PPP. There is also increased expression of Notch1 and its ligand, Delta1 in the SPP, which could block differentiation due to increased expression of *Hes5,* a basic-helix-loop-helix (bHLH) transcription factor which inhibits differentiation by repressing the expression of pro-neural genes such as *NeuroD*, *Mash1* and *Math1* (Panganiban and Rubenstein 2002; Vanderluit, Wylie et al. 2007). Additionally, failure of the SPP to mature in the absence of *Dlx1/Dlx2* is demonstrated by the lack of expression of SPP markers, such as *Dlx5*, *Dlx6* and *Oct6* (Anderson, Qiu et al. 1997).

Tangential migration of GABAergic interneurons from the subpallial structures to the developing NCX is reliant on expression of the *Dlx1* and *Dlx2*. In the absence of *Dlx1/Dlx2*, there is complete loss of tangential migration of GABAergic interneurons from the basal telencephalon to the developing NCX (Anderson, Eisenstat et al. 1997; Anderson, Qiu et al. 1997; Eisenstat, Liu et al. 1999; Le, Du et al. 2007). The accumulation of GABAergic interneurons in the *Dlx1/Dlx2* DKO GE may in part be due to loss of *Dlx* mediated repression of *neuropilin-2 (nrp-2)* (Marin, Yaron et al. 2001; Le, Du et al. 2007). The neuropilin proteins are receptors for the chemorepellant semaphorins, which are expressed in the striatum of the ventral telencephalon, resulting in repressive cues from the striatum, directing migrating interneurons to the developing NCX. There is increased, aberrant expression of NRP2 in the GABAergic interneurons that accumulate in the *Dlx1/Dlx2* DKO GE, resulting in responsiveness of interneurons to semaphorin signalling and blockage of tangential migration to the NCX (Marin, Yaron et al. 2001; Marin and Rubenstein 2003; Le, Du et al. 2007).

Dlx genes have also been directly linked to GABA synthesis in the developing forebrain. Ectopic expression of *Dlx2* in embryonic cerebral cortical slice cultures induces the expression of *Gad65* and *Gad67*, whose enzymatic protein products are responsible for the conversion of L-glutamic acid to GABA (Stuhmer, Anderson et al. 2002). Additionally, in our laboratory, we have determined that *Dlx1* and *Dlx2* bind *Gad65* (renamed *Gad2*) and *Gad67* (*Gad1*) promoters, and in the absence of *Dlx1* and *Dlx2*, there is reduced expression of GAD65 and GAD67 in the basal telencephalon (Le et al, in preparation). These studies establish a requirement for *Dlx* gene function for the proper development of the cortical GABAergic system.

Dlx expression patterns in the developing retina

DLX2 expression has been observed in the developing dorsal murine retina as early as E11.5. By E12.5, both *Dlx1* and *Dlx2* are expressed in retinal neuroepithelial progenitors (de Melo, Zhou et al. 2008). At P0, DLX2 expression is observed in the NBL, which consists of cycling RPC of restricted competence and postmitotic retinal cells, and also throughout the differentiated GCL where it is expressed in both RGC and displaced amacrine cells. Expression of DLX1 strongly resembles DLX2 expression up to P0 and then decreases dramatically postnatally. During the cell cycle, *Dlx2* is expressed in both cycling and postmitotic retinal cells, whereas *Dlx1* is exclusively expressed in postmitotic cells (Eisenstat, Liu et al. 1999). In the adult retina, DLX2 expression has been identified in RGC, amacrine cells, horizontal cells and a subset of bipolar cells through co-expression studies with specific markers for the above cell types (de Melo, Qiu et al. 2003).

Dlx gene function in the developing retina

Assessment of the *Dlx1/Dlx2* DKO mouse retinal phenotype has demonstrated a role for the *Dlx* homeobox genes in survival and terminal differentiation of late-born RGC (de Melo, Du et al. 2005). In the absence of *Dlx1/Dlx2* expression, the GCL of the developing retina at P0 shows a 33% decrease in cellularity due to increased apoptosis of differentiated RGC, which results in the concomitant thinning of the optic nerve by 25%. BrdU birth-dating experiments, coupled with BRN3B co-labelling to identify RGC, demonstrate significant reduction in RGC born after E13.5 in *Dlx1/Dlx2* DKO retinas. This supports a requirement for *Dlx1* and *Dlx2* for the terminal differentiation of late-

born RGC (de Melo, Du et al. 2005). Expression of the neurotrophin receptor, TrkB, has been demonstrated to inhibit death of RGC during retinal development (Ma, Hsieh et al. 1998; Pollock, Robichon et al. 2003). TrkB has been identified as a DLX2 target *in vivo* (de Melo, Zhou et al. 2008). Additionally, loss of *Dlx1/Dlx2* results in significantly reduced expression of TrkB at E16.5 in the developing retina. Deregulation of TrkB expression may partially explain the RGC death that is observed in the *Dlx1/Dlx2* DKO retina.

Cell cycle regulator, *p107*, belongs to the Retinoblastoma protein family

The retinoblastoma gene, *Rb*, was the first tumour suppressor gene identified and *Rb* mutations were determined to be responsible for the development of the childhood ocular carcinoma, retinoblastoma (Friend, Bernards et al. 1986; Lee, Bookstein et al. 1987; Weinberg 1991). Retinoblastoma develops either somatically or in a hereditary fashion. In hereditary retinoblastoma, a child inherits a mutated *Rb* allele from their parent and then subsequently sustains a mutation in the remaining wild-type (WT) allele in somatic retinal cells. Hereditary retinoblastoma typically results in formation of bilateral retinoblastoma where the child develops tumours in both eyes. Sporadic retinoblastoma arises when *de novo* mutations occur in both *Rb* alleles in somatic retinal cells and typically results in unilateral retinoblastomas where one eye is affected.

Retinoblastoma family of pocket proteins

During the initial discovery of *Rb*, it was determined that the protein product, pRb, interacted with the SV40 large T antigen of the SV40 DNA tumour viruses, the adenoviral E1A protein and the papilloma E7 proteins (Yee and Branton 1985; Dyson,

Guida et al. 1992). Further examination of these interactions revealed that the regions responsible for pRb binding to these viral oncoproteins also bound the two related proteins: P107 and P130. Both P107 and P130 were determined to be similar to pRb, particularly in their C terminal domain where a bipartite 'pocket' mediates the interaction between the retinoblastoma proteins and viral oncoproteins as well as interaction with the E2F transcription factors (Classon and Dyson 2001). It is the presence of this shared domain in pRb, P107, and P130 that resulted in naming this family the pocket proteins.

In the pocket protein family, P107 and P130 are more closely related to each other based on protein structure, than they are to pRb (**Figure 4**). In the N-terminal domain of P107 and P130, a region of homology exists which is absent in pRb, and is thought to enable both P107 and P130 to act as cyclin-dependant kinase inhibitors (CDKI) (Woo, Sanchez et al. 1997; Castano, Kleyner et al. 1998; Classon and Dyson 2001). In addition, the pocket domains of P107 and P130 share more similarity with each other than the pocket domain of pRb. The pocket domains of P107 and P130 have an insertion in the "B" half (refer to **Figure 4**) of the pocket, located at the C terminal region of the protein which is not observed in pRb (Classon and Dyson 2001). Also, there is additional sequence located in the pocket domain of P107 and P130 between the A and B subdomains of the pocket that is absent in pRb. This homologous motif enables formation of stable complexes with cyclin/cyclin dependant kinase (CDK) complexes, particularly, the cyclin A/CDK 2 and cyclin E/CDK2 complexes (Hannon, Demetrick et al. 1993; Li, Graham et al. 1993; Classon and Dyson 2001).

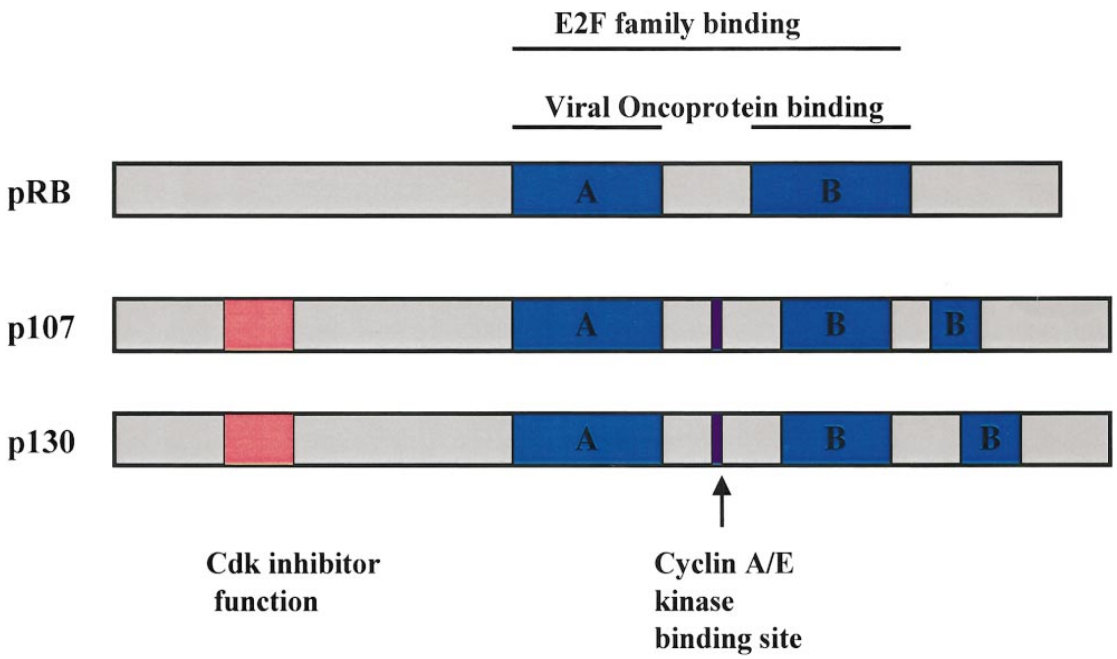

Figure 4: Schematic representation of the pocket proteins. Members of the pocket protein family are structurally and functionally related. The retinoblastoma protein, pRb and related members, P107 and P130 contain a "pocket" domain through which they interact with the E2f transcription factors and viral oncoproteins. P107 and P130 contain a unique region in the N terminal domain that is responsible for CDK inhibitor function. In addition, P107 and P130 contain a cyclin A/E binding site in the pocket region and an insertion adjacent to the "B" half of the pocket which is not observed in pRb [CDK = cyclin dependant kinase] (Classon & Dyson, 2001, *Experimental Cell Research*, Elsevier).

Regulation of cell cycle progression by the pocket proteins

The role of pRb in cell cycle regulation was initially outlined during its discovery as a tumour suppressor (Classon and Dyson 2001). Since this discovery, P107 and P130 have also been shown to play a role in negative regulation of cell cycle progression, in particular, G_1-S phase transition. During early G_1, the pocket proteins are in a hypophosphorylated state and bind to members of the E2F transcription factor family, preventing the transactivation of a number of genes required for progression of the cell cycle (**Figure 5**) (Flemington, Speck et al. 1993; Helin, Harlow et al. 1993). Repression of E2F mediated transcription initiation is accomplished though Rb family/E2F complex recruitment of chromatin remodelling enzymes such as histone deacetylases (HDAC), co-repressors and methyltransferases (Brehm, Miska et al. 1998; Frolov and Dyson 2004). As the cell cycle progresses, levels of cyclin D/Cdk4/6 rise, consequently resulting in phosphorylation of P107 and other pocket proteins. This phosphorylation results in the release of the E2Fs by the pocket proteins, where E2Fs then upregulate transcription of genes required for G_1 to S phase progression (Beijersbergen, Carlee et al. 1995; Xiao, Ginsberg et al. 1996). P107 remains phosphorylated until the next G1 phase where it is then presumed to be rapidly dephosphorylated by protein phosphatase 2 (PP2A) (Kolupaeva, Laplantine et al. 2008).

P107 and P130 differ from pRb in terms of their binding preference for E2f transcription factors and their expression patterns. The E2F family of transcription factors can be divided into two groups: the activator E2Fs and the repressor E2Fs. Activator E2Fs, which include E2F1, E2F2 and E2F3a, are preferentially bound by pRb during G_1-S phase regulation. pRb is thought to bind to activator E2Fs at, or away from the E2F

responsive promoters where they recruit chromatin remodelling proteins which result in the silencing of E2F responsive genes (Takahashi, Rayman et al. 2000; Cobrinik 2005). The repressor E2Fs on the other hand, are preferentially bound by P107 and P130, forming repressor complexes that are thought to bind directly to E2f responsive promoters. Similar to the activator E2F/pRb complexes, repressor E2F complexes recruit chromatin-remodelling enzymes, which inhibit transcription of the E2F responsive genes. As cells progress to S phase and the pocket proteins become hyperphosphorylated, activator E2Fs are released by the pocket proteins, bind to their responsive promoters and recruit histone acteyltransferases allowing upregulation of genes required for cell cycle progression.

In addition to the differences in binding affinity for E2F transcription factors, the pocket proteins also differ in expression patterns observed throughout the cell cycle (Classon and Dyson 2001; Wirt and Sage 2010). *Rb* expression is observed in moderate levels in all cell types, with a slight increase in expression in proliferative cells. In quiescent and differentiated cells, *p130* expression is highest, perhaps playing a role in maintaining the differentiated state of cells by preventing re-entry into the cell cycle. *p107* expression levels are low in quiescent cells and highest in S-phase proliferative cells; a time when P107 is phosphorylated and thereby functionally deactivated.

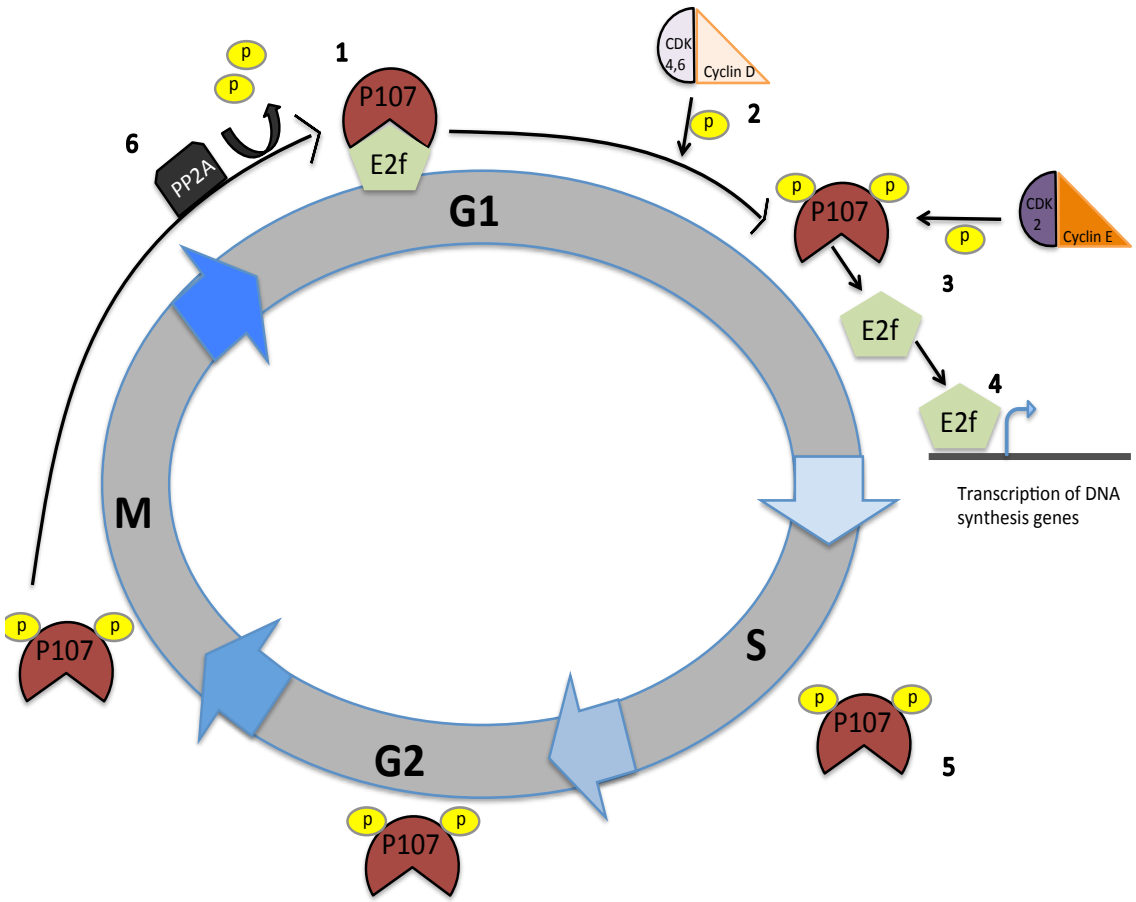

Figure 5: P107 mediated regulation of cell cycle progression. P107, like pRb and P130, is involved in inhibiting the progression of G_1 to S phase of the cell cycle. **1)** In early G_1 phase, P107 is hypophosphorylated and binds E2f transcription factors, restricting upregulation of E2f target genes. **2)** As G_1 progresses, levels of cyclin D/CDK4, 6 rise, phosphorylating P107. **3)** Phosphorylation of P107 results in the release of E2f. **4)** E2f is free to bind responsive promoters, upregulating genes involved in DNA synthesis. **5)** P107 is phosphorylated and thereby unable to restrict cell cycle progression from S to M phase. **6)** As cells enter G_1, PP2A dephosphorylates P107, which can again bind and restrict transcriptional activity of E2f [CDK = cyclin dependant kinase, PP2A = protein phosphatase 2].

Unique functions of *p107*

Compensation by p107 for loss of Rb

As regulation of the cell cycle is a critical process, functional redundancy and compensation exists between the different members of the retinoblastoma protein family, making it difficult to determine unique functions of these proteins. In the murine retina, *p107* has been shown to have a unique role in preventing the formation of retinoblastoma in the absence of *Rb* expression (Robanus-Maandag, Dekker et al. 1998). Thereby, *p107* has tumour suppressor function in the context of *Rb* deficiency in the retina. In humans, the loss of *Rb* expression alone is sufficient to initiate retinoblastoma tumour development. In mice however, the loss of *Rb* coupled with loss of *p107* expression is required for the formation of retinoblastoma, suggesting that in the mouse, there is compensation for the loss of *Rb* by *p107* (Lee, Williams et al. 1996). Donovan *et al* have shown that *p107* and *Rb* are expressed in largely non-overlapping patterns during retinal development (Donovan, Schweers et al. 2006). *p107* is most highly expressed in embryonic RPC with downregulation of expression following cell cycle exit and differentiation. At birth, *p107* expression tapers off and *Rb* expression increases in postnatal RPC and expression remains high in post-mitotic neurons and glia. The expression pattern of *p107* in embryonic progenitors is consistent with the absence of a retinal tumour phenotype in $Rb^{-/-}$ mice. Acute and chronic inactivation of *Rb*, in the developing retina leads to upregulation of *p107* expression, demonstrating compensation for *Rb* loss by *p107*, preventing the formation of retinoblastoma in mice. In addition, reciprocal upregulation of *Rb* expression is observed in the absence of *p107* expression in the mouse retina. The reason for the absence of compensation for *Rb* loss by *p107* in the

developing human retina is currently unknown. It is possible that the chromatin remodelling complexes at the *p107* promoter in human retinal cells prevent transcriptional upregulation. Alternatively, there are two E2F binding sites in both the human and murine promoters of *p107*. There is a single base pair (bp) mutation in the proximal E2F binding site in the murine promoter which may affect binding of E2F transcription factors and subsequent upregulation and therefore, could plausibly play a role in the absence of upregulation of *p107* expression in human retinal cells (Zhu, Xie et al. 1995; Wirt and Sage 2010).

p107 regulates neural progenitor renewal and commitment

The use of $p107^{-/-}$ mice has led to identification of novel roles for *p107* in neurogenesis. The expression of *p107* in the developing murine brain is restricted to cycling neural progenitor cells (Jiang, Zacksenhaus et al. 1997). In the absence of *p107* expression in the developing murine brain, an elevation in the number of proliferating neural progenitors is observed (Vanderluit, Ferguson et al. 2004; Vanderluit, Wylie et al. 2007). This is thought to be due to elevation of the basic HLH transcription factor HES1 in $p107^{-/-}$ neural progenitors cells. HES1, part of the Notch-Hes pathway, is involved in neural progenitor self renewal by repressing the expression of proneural genes such as *Mash1*, *Math1* and *NeuroD* (Sasai, Kageyama et al. 1992; Ishibashi, Ang et al. 1995). In addition to controlling the size of the neural progenitor population, *p107* has also been demonstrated to promote progenitor commitment to a neuronal fate (Vanderluit, Wylie et al. 2007). BrdU birth-dating experiments show a two-fold reduction in the number of cortical neurons born at E13.5 in *p107* deficient murine brains. Double labelling of BrdU positive cells 24 hours post-injection with doublecortin, a marker for migrating

neuroblasts, and TuJ1, a pan-neuronal marker induced after terminal mitosis, shows a significant reduction in the double labelled cells in the absence of *p107* (Vanderluit, Wylie et al. 2007). This neuronal differentiation defect has been attributed to deregulated expression of *Hes1* as loss of one *Hes1* allele in the absence of *p107* expression restores the number of neurons born at E13.5 to levels comparable to WT brains.

DLX and cell cycle regulation

DLX1 and DLX2 are both found to be expressed in subsets of cells in S phase, as determined by BrdU staining, in both the VZ and SVZ of the developing telencephalon and the NBL of the developing retina (Eisenstat, Liu et al. 1999). As mentioned previously, P107 expression levels are highest during S phase of the cell cycle and localized to both the ventral telencephalon and the NBL during forebrain and retinal development, respectively (Jiang, Zacksenhaus et al. 1997; Donovan, Schweers et al. 2006; Wirt and Sage 2010). In addition, embryonic forebrain chromatin immunoprecipitation (ChIP) coupled to a CpG island DNA microarray (ChIP-on-chip) identified *p107* as being a potential target for transcriptional regulation by DLX2. These observations suggest a role for DLX2 in regulation of cell cycle progression by regulating the expression of *p107* during development of the forebrain and retina.

The role of the *Dlx* homeobox genes in the development of the murine forebrain and retina has been widely studied in our laboratory through examining genetic regulation of a number of target genes. This is the first study to examine the transcriptional regulation of *p107* in a murine CNS model. The central goal of this proposal is to determine the role of the *Dlx* homeobox genes in commitment and differentiation of neural cells by

examining the genetic regulation of the cell cycle protein, P107 in CNS development.

HYPOTHESIS & RESEARCH AIMS

Hypothesis

Our embryonic forebrain ChIP-on-chip experiments identified *p107* as being a potential target for transcriptional regulation by DLX2. We hypothesize that DLX2 directly occupies the promoter of *p107*, resulting in activation of *p107* expression during forebrain and retinal development.

Specific research aim 1: Examine temporal and spatial expression patterns of DLX2 and P107 during forebrain and retinal development

In order to determine the temporal and spatial expression patterns of DLX2 and P107 and also determine if the co-expression of these two proteins is observed, double immunofluorescence using antibodies specific to DLX2 and P107 was carried out on forebrain and retinal tissue at multiple time points during embryonic development.

Specific research aim 2: Determine the specific interaction between the DLX2 and the *p107* promoter during forebrain and retinal development

Occupancy of DLX2 on the *p107* promoter *in situ* was examined by ChIP on embryonic forebrain and retinal tissues. While ChIP determines occupancy of DLX2, electrophoretic mobility shift assays (EMSAs) were subsequently carried out to determine specific binding of DLX2 to labelled *p107* promoter probes using recombinant DLX2 protein.

Specific research aim 3: Identify the functional consequence of *Dlx1/Dlx2* loss on *p107* expression *in vivo* and *in vitro*

The consequence *of Dlx1/Dlx2* regulation of *p107* expression was examined by comparing *p107* expression in *Dlx1/Dlx2* wild-type (WT) embryonic mice and *Dlx1/Dlx2* double knockout (DKO) mice. RNA was isolated from *Dlx1/Dlx2* WT and DKO embryonic forebrain and retina and *p107* transcript levels were measured semi-quantitatively. Immunofluorescence microscopy using a P107 specific antibody was carried out on cryopreserved WT and DKO forebrain and retina sections to examine differences in P107 expression. *In vitro* reporter gene assays were used to determine transcriptional activity of DLX2 by co-expression of *Dlx2* expression plasmids and reporter vectors under *p107* promoter control in HEK293 cells followed by quantification of luciferase expression.

Specific research aim 4: Identifying DLX2 binding sites critical for activation and direct binding of the *p107* promoter *in vitro*

Site directed mutagenesis was carried out on putative DLX2 binding sites in the ChIP positive *p107* promoter regions to identify critical sites for DLX2 binding and *p107* activation *in vitro*. Mutagenesis was carried out by PCR based methods, by introducing a single bp mutation in putative DLX2 binding sites. Mutated regions were then cloned into pGL3 reporter vectors for luciferase reporter gene assays and EMSA to examine expression and binding patterns, respectively.

MATERIALS & METHODS

Animals and tissue collection

Tissues utilized in this study were isolated from mouse (*Mus musculus*) during multiple embryonic stages. For the purposes of ChIP, CD-1 Swiss albino mice were used. To examine transcriptional regulation by *Dlx* genes, *Dlx1/Dlx2* DKO mice first developed in, and kindly provided by J. Rubenstein's laboratory at UCSF, San Francisco, CA (Qiu et al., 1997; Anderson et al., 1997a), were utilized where *Dlx1/Dlx2* DKO tissues were compared to WT littermates. To obtain embryonic tissues at various developmental time points, timed-pregnant mice were utilized. Pregnancy was determined visually by presence of a vaginal plug marking 0.5 days pregnancy. Pregnant dams were euthanized by cervical dislocation and embryos removed. Embryos were sacrificed by decapitation. Following euthanization, embryos were washed thoroughly in cold 1X phosphate buffered saline (PBS). Embryonic tails or limbs were retained for genotyping. Genomic DNA (gDNA) isolation was completed using Extract-N-Amp kit (Sigma Catalogue# XNAT2R) followed by PCR amplification using primer sets specific to *Dlx1* and *Dlx2*. All animal protocols were conducted in accordance to the guidelines set by the Canadian Council on Animal Care and the University of Manitoba.

Tissue fixation, embedding and sectioning

Following extraction and euthanization of embryos, embryonic forebrains and retinas were dissected. Tissues were fixed in 4% paraformaldehyde (PFA) at 4°C with rotation at the time points indicated in Table 1.

Table 1: Fixation times for embryonic forebrain and retinal tissues

Tissue & age	Time in 4% PFA
E13.5 heads (forebrain and retina)	4 hours
E16.5 retina	25 minutes
E16.5 forebrain	2 hours
E18.5 retina	45 minutes
E18.5 forebrain	3 hours

Subsequent to fixation, tissues were placed in 10% sucrose and incubated with rotation at 4°C until tissues sediment to the bottom of the 2 ml centrifuge tubes. This step was then repeated with 15% and 20% sucrose solutions for cryopreservation. Tissues were stored in 20% sucrose solution at 4°C until embedding. Prior to embedding, the tissue was incubated for 30 minutes in 1:1 ratio of 20% sucrose and Optimal Cutting Temperatures (O.C.T.) compound (Sakura Finetek, USA) at room temperature (RT) with rotation. Tissue was then quickly frozen in O.C.T. in plastic embedding moulds over dry ice. Embedded tissues were stored at -80°C. Frozen tissues were cut at 12 µm on a Leica CM-3050S cryostat and placed on Fisher Scientific Superfrost Plus slides. Tissue slides were maintained at -80ºC.

Immunofluorescence

Protein expression in forebrain and retinal tissue was analyzed by immunofluorescence (IF) microscopy. Tissue was first incubated in blocking buffer (0.1% Bovine Serum Albumin (BSA), 0.2% Triton-X 100, 0.2% Sodium Azide, 5% Horse/Goat Serum in

1XPBS pH 7.4) for 2 hours at room temperature to prevent non-specific antibody binding. Following tissue blocking, the blocking buffer was removed and the primary antibody, specific to the protein of interest, was diluted in blocking buffer then applied to the tissue. Tissue was incubated with the primary antibody at 4ºC overnight (O/N) (see **Table 2** lists primary and secondary antibodies used for IF). The following day, tissues were washed three times with 1X PBS + 0.05% Triton at 5 minute intervals. The appropriate secondary antibody conjugated to a fluorophore was applied to the tissue and incubated for two hours at RT. Washing was repeated as above and tissues were then mounted with Vectashield fluorescence mounting medium (Vector Laboratories). Negative controls received no primary antibody and were treated with secondary antibody along with experimental slides on day two of IF assays.

Table 2: Antibodies for immunofluorescence analysis in forebrain and retina tissue sections

Name of antibody	**Primary or Secondary**	**Source**	**Working Dilution**
Rabbit polyclonal α-DLX2	Primary	Antisera from Dr. Rubenstein, purified in house by Dr. Eisenstat	1/400
Rabbit polyclonal α-P107	Primary	Santa Cruz Biotechnologies (catalog number: sc-318)	1/200
Alexa 488 conjugated goat α-rabbit IgG	Secondary	Invitrogen (catalog number: A-11008)	1/200
Alexa 594 conjugated goat α-rabbit IgG	Secondary	Invitrogen (catalog number: A-21207)	1/200
Alexa 488 conjugated donkey α- goat IgG	Secondary	Invitrogen (catalog number: A-11055	1/200

Double Immunofluorescence

Double immunofluorescence (DIF) microscopy was carried out to determine the co-expression patterns of two proteins. Following a 2 hour blocking step, the first primary antibody was applied to the tissue and incubated at 4°C O/N. On the second day, the first primary antibody was washed as described above. A conjugated secondary antibody was applied for 2 hours and washed as described above. Following washing of the first secondary antibody, the second primary antibody was applied to the tissue and incubated O/N at 4°C. The following day, the second primary antibody was washed and detected with a conjugated secondary antibody as described above.

Microscopy and imaging

Single IF slides were imaged using an Olympus BX51 Microscope, Olympus DP70 Digital Camera System and ImagePro Plus software (Media Cybernetics). DIF slides were then imaged using an Olympus IX81 platform.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were carried out on embryonic forebrains and retinas to examine DLX2 interaction at homeodomain binding sites at the *p107* promoter *in situ*. For forebrain ChIP, ganglionic eminences (GEs) were dissected from E13.5 mice. Retinas were extracted from E16.5 mouse eyes for retina ChIP assays. All tissues were obtained from timed pregnant CD-1 mice. Hindbrains were taken as a negative control since DLX2 is not expressed in developing murine forebrain. Following tissue extraction, cells were washed in 1X PBS and dissociated using a 9" glass Pasteur

pipette. Tissue was then fixed in 1% PFA plus protease inhibitor cocktail (PIC) (Roche) at RT with rotation for 90 minutes in embryonic forebrain and 30 minutes for retina. Tissue samples were washed twice in cold 1X PBS and centrifuged at 2500 rpm to collect tissue. Dissociated cells were then suspended in 400 µl of lysis buffer (1% SDS, 10 mM Tris-HCl pH 8.1, and 10 mM EDTA) plus PIC and sonicated 10-20 times on ice at 15-second intervals with 30-second rest time between sonication. Sonication was carried out on ice at 40% pulse strength with the output control set to 4 on a Brandon Cell Disruptor. Samples of the sonicated lysate were separated on a 1% agarose gel to determine size of sheered DNA fragments, which were desired to be 500-700 bp in length.

Protein A-sepharose beads (Promega) were prewashed with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 16 7mM NaCl) and PIC, and subsequently prepared in a 50% final solution of beads with dilution buffer. Next, 60 µl of beads were added to the lysate and incubated for 1 hour with rotation at 4ºC to remove IgGs from sample solution. The solution was then centrifuged for 2 minutes at 2500 rpm and the supernatant reserved. To the supernatant, 500 µg/ml of tRNA and BSA were added in addition to 1-2 µl of concentrated DLX2 antibody in order to precipitate DLX2/DNA complexes from solution. Negative controls received no antibody. Samples were incubated at 4ºC O/N with rotation.

On the following day, 60 µl of beads, incubated with BSA and tRNA at a final concentration of 500 µg/ml, were added to the samples and incubated for an additional 24 hours at 4ºC with rotation to allow antibody/DLX2/DNA complexes to bind to the beads. The solution was centrifuged at 2500 rpm for 5 minutes with the supernatant removed and saved as the total input samples, which contain any unbound protein/DNA

complexes. The beads were then washed as follows: low salt wash solution (0.1% SDS, 1% Triton-X 100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl) for 5 minutes, high salt wash solution (0.1% SDS, 1% Triton-X 100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl) for 30 minutes, LiCl wash solution (0.25 M LiCl 1% deoxycholate, 1mM EDTA, 10 mM Tris-HCl pH 8.1, 1% NP-40) for 30 minutes and finally, commercial TE buffer (Promega) pH 8.0, washed twice for 5 minutes each.

Following the final wash, 250 µl of freshly prepared elution buffer (1% SDS, 0.1M NaHCO3), preheated at 65ºC was applied to the washed beads. Samples are incubated at RT with agitation for 15 minutes followed by centrifugation at 13 000rpm for 5 minutes at 4ºC. Supernatant was collected and transferred to a fresh tube. Incubation in preheated elution buffer with agitation was repeated and supernatant was again collected as above and pooled with previous collection for a total volume of 500 µl. 1 µl of RNaseA and 25 µl of 5M NaCl were added to pooled samples and incubated at 65ºC O/N to reverse crosslinking.

The following day, 20 µl 1M Tris-HCl (pH 6.5), 10 µl of 0.5M EDTA and 2 µl proteinase K (Invitrogen), were added to sample and incubated for 2 hours at 65ºC. DNA samples were then purified using QIAquick PCR purification kit (Qiagen). Purified DNA samples were then amplified using primers designed to flank potential homeodomain binding sites in the 3kb promoter of *p107* (**Table 3** and **Figure 6**). PCR amplification of ChIP output DNA was carried out with Phusion High-Fidelity polymerase (New England BioLabs).

Table 3: Primers for PCR amplification of p107 promoter regions for ChIP

Region	Promoter location	Primer Sequence (F=forward, R=reverse)
Region 1	48-340	F: 5' TAC TGA GCC TCT ACT ATT TTA TC 3'
		R: 5' CGT CAC AAC TGC TGG AAT ATA G 3'
Region 2	401-725	F: 5' CTG GTA TAC AGC AAG ATT CTC 3'
		R: 5' GAG GTA GTT CAG TAA ATG GCA 3'
Region 3	668-990	F: 5'ATA GAA TGA AGT ACA TAC ACG C3'
		R: 5'GAT ACC TTC TTC AAG TCT CAG 3'
Region 4	970-1223	F: 5' CTG AGA CTT GAA GAA GGT ATC 3'
		R: 5' GAG AGG CCT GGA AGG TGA AGA 3'
Region 5	1490-1690	F: 5' CCT GCT ACT CCT CTA TGT AAC 3'
		R: 5' CTT ACA TGA CGT ATC GGA AGG 3'
Region 6	1670-1821	F: 5' CCT TCC GAT ACG TCA TGT AAG 3'
		R: 5' CTA AAG CCT CAT GGT ATA CTC 3'
Region 7	1801-2000	F: 5' GAG TAT ACC ATG AGG CTT TAG 3'
		R: 5' TGG TAG GAT AGA ATC GTT TG 3'
Region 8	2073-2319	F: 5' GAA GTT AGG TGT CAG CTT ATA G 3'
		R: 5' CAC TCA GAA TCC TGC ACG AG 3'

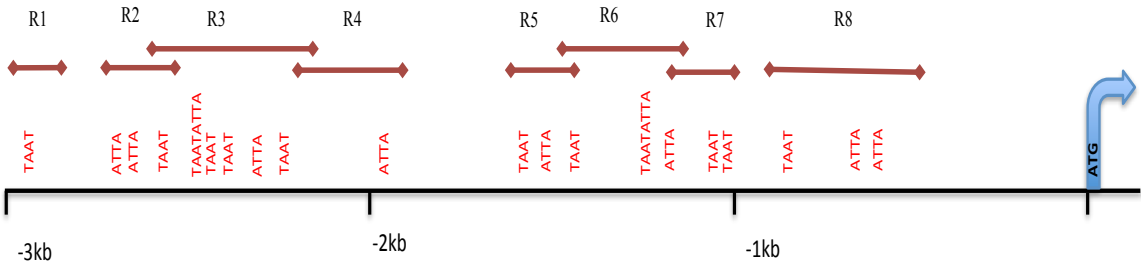

Figure 6: Schematic representation of the *p107* promoter with putative DLX2 binding sites. DLX2 binds tetranucleotide motifs ATTA/TAAT in target promoters. The promoter was arbitrarily divided into eight regions (R1-R8) for ChIP analysis using primers designed to flank promoter regions containing one or more potential DLX2 binding site(s) (a minimum of 1 putative binding site and a maximum of six putative sites). Putative DLX binding sites located in each region are indicated as tetranucleotide motifs. The promoter regions amplified by the PCR primers described above is indicated above the tetranucleotide motifs. The blue arrow at the 3' end indicates the *p107* translational start site.

Electrophoretic mobility shift assay

To determine specificity of DLX2 binding to *p107*, EMSA was carried out. DNA that is directly bound by protein will migrate slower through an acrylamide gel compared to unbound (free) labelled probe due to the higher molecular weight of the DLX2/DNA complex. Promoter regions of *p107* which were determined to occupy the *p107* promoter *in situ* by ChIP were restriction digested O/N from pGL3 basic vectors (see molecular cloning below) using *MluI* and *XhoI* restriction enzymes according to manufacturer

protocols (New England Biolabs). Digested plasmids were separated on a 1% agarose gel and linear promoter regions were gel purified using QIAquick Gel Extraction Kit (Qiagen). Radiolabelling of promoter regions was accomplished by 3'end labelling using α-P^{32}-GTP (Perkin Elmer) and DNA Polymerase I, Large (Klenow) Fragment polymerase (Invitrogen). Labelled probes were then purified by passing them though GE HealthCare Illustra Micro-spin G-25 spin columns. Radioactivity of probes was subsequently measured using a Beckman Coulter scintillation counter. Samples were prepared by first incubating 5x Binding buffer (Promega), poly(dI-dC) •poly(dI-dC), and 200 ng of DLX2 recombinant protein (rDLX2) (see DLX2 recombinant protein production below) for 30 minutes at RT. Radiolabelled probes at 100,000 cpm were then added to samples and incubated for a further 30 minutes at RT. A 4% acrylamide, 60:1 acrylamide:bisacrylamide gel was cast 24 hours prior to use, using the following protocol (Promega): 32.4 ml ddH_20, 2 ml 10X Tris/Borate/EDTA buffer (TBE), 2.5 ml 37.5:1 acrylamide/bisacrylamide (40% w/v), 1.5 ml acrylamide (w/v), 1.25 ml 80% glycerol, and 300 μl 10X Ammonium Persulfate (freshly prepared in dH_20). Following mixing of these items, 20 μl of N,N,N',N'-Tetramethylethylenediamine (TEMED) was added and mixed for 1 minute to polymerize the gel. During incubation of the labelled probes and rDLX2 protein, the non-denaturing polyacrylamide gel was pre-run at 350V for 15 minutes. Samples were then loaded in the gel and run for 1 hour at 300 Volts in 0.5X TBE. The gel was then placed on filter paper and dried under vacuum in a Biorad Gel dryer. The dried gel was then exposed to Kodak autoradiography film for 2 hours at RT and developed using an AGFA CP 1000 automatic film processor.

DLX2 recombinant proteins production

To generate and affinity purify rDLX2 protein, the nickel (Ni2+)-charged HisTrap FF crude Kit column chromatography from GE Healthcare was used. Recombinant DLX2 C-terminal domain (199 amino acids, including the homeodomain) was sub-cloned into pET11d (Novagen) and used to transform BL21DE3pLysS *Escherichia coli (E. coli*). To generate rDLX2, an O/N culture of the above transformed *E. coli* was used to inoculate Lysogeny Broth (LB) containing carbenicillin and chloramphenicol (each 50 mg/ml). Induction of rDLX2 was carried out by the addition of 1 mM of isopropyl-β-thiogalactopyranoside (IPTG), followed by 6 hour incubation at 25 ºC. Affinity purification was carried out as described previously (Porteus, Bulfone et al. 1994; Eisenstat, Liu et al. 1999)

Quantitative Real-Time Polymerase Chain Reaction

RNA extraction

Eyes from E18.5 mice and GE from E13.5 mice were extracted from both WT and *Dlx1/Dlx2* DKO embryos. Tissues were placed in RNA Later at -20 ºC (Qiagen) until RNA extraction was carried out. To extract RNA, tissues were flash frozen in liquid nitrogen then ground to a fine powder using a mortar and pestle. RNA isolation was conducted using QiaShredders and RNEasy Mini Kit (Qiagen).

Reverse transcription

To generate cDNA for quantitative real-time polymerase chain reaction (qRT-PCR), reverse transcription of RNA extracted from WT and DKO tissue was accomplished

using a number of reagents acquired from Invitrogen. 1µg of RNA was added to 1µl of Oligo d(T)50 and 1µl 10 mM dNTPs, and the volume was brought to 13 µl with ddH_20 heated to 65ºC for 5 minutes. Immediately following heating, samples were placed on ice for 1 minute. Samples received 4 µl 5x First Strand Buffer, 1 µl RNase Out, 1 µl 0.1M Dithiothreitol (DTT) and 1µl SuperScript III. Samples were then incubated at 50ºC for 1 hour, followed by incubation at 70ºC for 15 minutes. The resulting cDNA was stored at -20ºC.

Quantitative Real-Time Polymerase Chain Reaction

To measure changes in *p107* transcript levels in the absence of *Dlx* regulation, qRT-PCR was performed using primers designed to amplify cDNA corresponding to the first exon of *p107* (**Table 4**) and the Qiagen QuantiTech SYBR green RT PCR kit. qRT-PCR was performed on a Applied Biosystem 7500 Real Time PCR System with the following cycling conditions: initial denaturation for 15 minutes at 95ºC followed by 40 cycles of 15 second denaturation at 95ºC, annealing at 60ºC for 30 seconds, and extension at 72ºC for 33 seconds. A dissociation step was carried out following amplification. All output cycle threshold (Ct) values were normalized to GAPDH. Analysis was completed using Applied Biosystems Sequence Detection Software (SDS) version 1.4. Significance was determined with a Student's paired t-test in Graph Prism 5.

Table 4: Primers used for qRT-PCR, quantifying *p107* transcript

Primer Name	**Primer sequence 5'to 3'**
p107 forward	GGC AAC TAC AGC CTA GAG G
p107 reverse	GCC TTT CTA TCC GCT CAC G
GAPDH forward	CTC ATG ACC ACA GTC CAT GC
GAPDH reverse	CAC ATT GGG GGG TAG GAA CAC

Luciferase Reporter Gene Analysis

Molecular Cloning

Regions of the *p107* promoter determined to be occupied by DLX2 *in situ* in ChIP were amplified using Phusion High-Fidelity polymerase (New England BioLabs) and primers which add *MluI* and *XhoI* restriction sites to the 5' and 3' ends (**Table 5**). The whole promoter of *p107* (here, the "whole" *p107* promoter is defined as 3 kb upstream of the translational start site) was also amplified with the addition of *MluI* and *XhoI* restrictions sites using HotStar HiFidelity Polymerase (Qiagen). Amplified regions with restriction sites were gel purified using the Qiagen QIAquick Gel Extraction Kit. 1µg of amplified *p107* promoter regions and *p107* whole promoter were digested using 1 unit of *XhoI* and *MluI*, 4 µl of NEB buffer 3, 4 µl 10x BSA and topped to 50 µl total reaction volume with ddH_2O. Empty pGL3 basic reporter vectors were also restriction digested using *XhoI* and *MluI* to generate compatible ends with restriction digested promoter regions. Digestion reactions were incubated at 37ºC O/N. Digested plasmids and promoter regions were then resolved on a 1% agarose gel, followed by excision of desired band from the agrose gel

and gel purification using a QIAquick Gel Extraction Kit (Qiagen) to isolate digested fragments and vector. Restriction digested *p107* promoter regions were ligated O/N at 16ºC into the restriction digested multiple cloning site in pGL3 basic vector using T4 DNA Ligase (New England Biolabs).

The following day, ligated reporter plasmids were used to transform chemically competent DH5α cells (Invitrogen) by first adding 3 µl of ligated plasmid to 30 µl of DH5α cells, followed by incubating on ice for 30 minutes. Next, samples were heat shocked at 42ºC for 1 minute then immediately incubated on ice. Transformed cells were incubated in LB with no antibiotics at 37ºC, 250 rpm in a New Brunswick Scientific Laboratory Shaker for 2 hours. Next, 20, 50, 100 and 200 µl aliquots were spread on LB agar + carbenicillin plates and incubated O/N at 37ºC. The next day, isolated colonies from each plate were picked and used to inoculate 4 ml LB + 2 µl carbenicillin (50 mg/ml). Inoculated broth was then incubated at 37ºC O/N at 250 rpm. Purification of plasmids was carried out using a Sigma mini-prep kit. Plasmids were then restriction digested with MluI and XhoI for 2 hours to verify correct insertion of promoter regions into pGL3 basic reporter vector. Successfully ligated plasmids were then sent for sequence verification (University of Calgary).

Table 5: Primers for cloning *p107* promoter regions into pGL3 basic reporter vectors (restriction sites indicated in red text)

Region Cloned	Primer sequence 5' to 3'
p107 promoter (3 kb upstream of translational start)	F: GAC ACG CGT TAC TGA GCC TCT ACT ATT TTA TC
	R: GAC CTC GAG CAC TCA GAA TCC TGC ACG AG
p107 region 1	F: GAC ACG CGT TAC TGA GCC TCT ACT ATT TTA TC
	R: GAC CTC GAG CGT CAC AAC TGC TGG AAT ATA G
p107 region 8	F: GAC ACG CGT GAA GTT AGG TGT CAG CTT ATA G
	R: GAC CTC GAG CAC TCA GAA TCC TGC ACG AG

Luciferase reporter gene assay

The functional effect of DLX2 binding at the *p107* promoter *in vitro* was analysed by luciferase reporter assays where the *p107* promoter DNA was sub-cloned directly upstream of a reporter gene. The transcriptional activity of DLX2 was then measured by quantifying bioluminescence of luciferase reporter protein. HEK293 cells were first seeded on 75cm^2 cell culture flasks (Nalgene) and grown to confluency. Cells were trypsinized to detach cells from culture plates. Trypsinization was stopped by adding equal amounts of DMEM (Gibco) + 10% Fetal Bovine Serum (FBS) (Invitrogen). Cells were counted using a haemocytometer and 1.0 x 10^5 cells were plated in each well of 12 well cell culture plates (Nalgene) with 1 ml of DMEM+10% FBS to reach approximately 80% confluence for transfection. Plates were incubated for 24 hours at 37ºC, 5% CO_2. Transfection was carried out the following day using Lipofectamine 2000 (Invitrogen) and 0.5 µg of pCDNA3-DLX2 plus *p107*-R1, *p107*-R8 or *p107*-whole promoter. Transfection with 0.5 µg of empty pGL3 basic and pCDNA3 was conducted as a control. To each well, 0.1 µg of β-galactosidase expression plasmid was added to measure

transfection efficiency. Plasmid mixtures were then incubated in OPTIMEM (Gibco-Invitrogen) for 5 minutes, followed by addition of a solution of lipofectamine and OPTIMEM and incubated for a further 20 minutes at RT. The plasmid mixture was added slowly to cells and incubated O/N at 37ºC, 5% CO_2. The transfection mixture was removed 4 hours following transfection and replaced with fresh DMEM+10%FBS. Cells were harvested 48 hours after transfection by first washing cells gently with cold 1X PBS followed by addition of 200 µl of 1X Lysis Reporter Buffer (Protégé). Cell lysis was carried out for 30 minutes on ice. Cells were scraped from the plates, collected in centrifuge tubes and pelleted at 3000 rpm for 5 minutes. 20 µl of supernatant was added to both white bottomed and clear 96-well plates for measurement of both luciferase activity and β-galactosidase activity, respectively. Luciferase activity was measured using an Lmax luminometer (Molecular Devices) subsequent to adding Luciferase assay substrate buffer (Promega) to 20 µl of cell lysate. Transfection efficiency was determined by measuring β-galactosidase activity at 420 nm using the SpectraMax 190 Instrument (Molecular Devices) and after addition 20 µl Assay 2x buffer from the β-galactosidase enzyme Assay System (Promega) to 20 µl of cell lysate.

Site directed mutagenesis

In order to determine critical binding sites for DLX2 binding and activation, site directed mutagenesis (SDM) of putative DLX2 binding sites located within ChIP positive *p107* promoter regions was performed and direct binding and alterations in *p107* expression *in vitro* was examined. Mutagenic primers were designed which introduced a single bp change in the putative homeodomain binding sites located within ChIP positive *p107* promoter regions upon PCR amplification (**Table 6**). Three separate PCR reactions were

carried out. First, 5' and 3' ends of *p107* promoter regions were amplified in separate reactions using mutagenic primers and primers used in molecular cloning which contain *XhoI* and *MluI* restriction sites (see primers for *p107*-R1 and *p107*-R8 in Table 5). The products of these reactions resulted in a region of overlap containing the mutated DLX2 binding site and served as the template for a third PCR reaction where the regions of overlap were first extended, followed by amplification of the mutagenic promoter region using the outside flanking primers with restriction sites. Mutagenic *p107* promoter regions were subsequently cloned into pGL3 basic reporter vectors as described above for luciferase reporter gene assays and EMSAs to analyze consequence of mutating homeodomain binding sites on activation of *p107* expression and direct binding of DLX2 to *p107,* respectively. Mutagenesis of putative DLX2 binding sites was sequence verified (University of Calgary).

Table 6: Primers for site directed mutagenesis of DNA binding sites in *p107* promoter regions (mutation site indicated by red text)

Primer name	Base pair mutation made	Primer design 5' to 3'
p107-mR1	TAAT →TAAC	F: 5' GGT CCC TCC TAT ACT GTA AAG ATA ACG 3'
		R: 5' CTT CGT TAT CTT TAC AGT ATA GGA GGG ACC 3'
p107-mR8S1	TAAT→TAAG	F: ACT GCT CAG TAA GGG CCT G
		R: C AGG CCC TTA CTG AGC AGT
p107-mR8S2	ATTA →ATTG	F: GAA GGT TAA AAT TGG ACT TAA ATG
		R: CAT TTA AGT CCA ATT TTA ACC TTC
p107-mR8S3	ATTA→ATTG	F: ATC TGA ATG CTA TTG TTT TTC TG
		R: CA GAA AAA CAA TAG CAT TCA GAT

RESULTS

DLX2 and P107 co-expression is observed throughout forebrain and retinal development

Temporal and spatial expression patterns of DLX2 and P107 were examined at developmental time points E13.5, E16.5 and E18.5 in the developing forebrain and retina by double immunofluorescence (DIF), using antibodies specific to both DLX2 and P107. Co-expression of P107 and DLX2 was determined by the presence of a fluorescent signal from each protein in the same cell. DLX2 localized to the nuclei of cells in the NBL in early retinal development (E13.5) and becomes localized to the GCL in addition to the NBL as development progresses (E16.5 – E18.5). These cells have previously been established to be RGC and displaced amacrine cells (de Melo, Qiu et al. 2003). P107 is localized to the cytoplasm and is co-localized with DLX2 throughout retinal development (**Figure 7**). In early forebrain development (E13.5), DLX2 expressing cells localize to the proliferative VZ and SVZ of the GE in the ventral telencephalon (**Figure 8**). The majority of these DLX2 expressing cells are GABAergic interneurons (Anderson, Eisenstat et al. 1997; Panganiban and Rubenstein 2002). As development progresses to E16.5, DLX2 expressing GABAergic interneurons have initiated tangential migration to the developing neocortex. Similar to the developing retina, P107 was localized to the cytoplasm and found to be co-expressed with DLX2 throughout development of the forebrain.

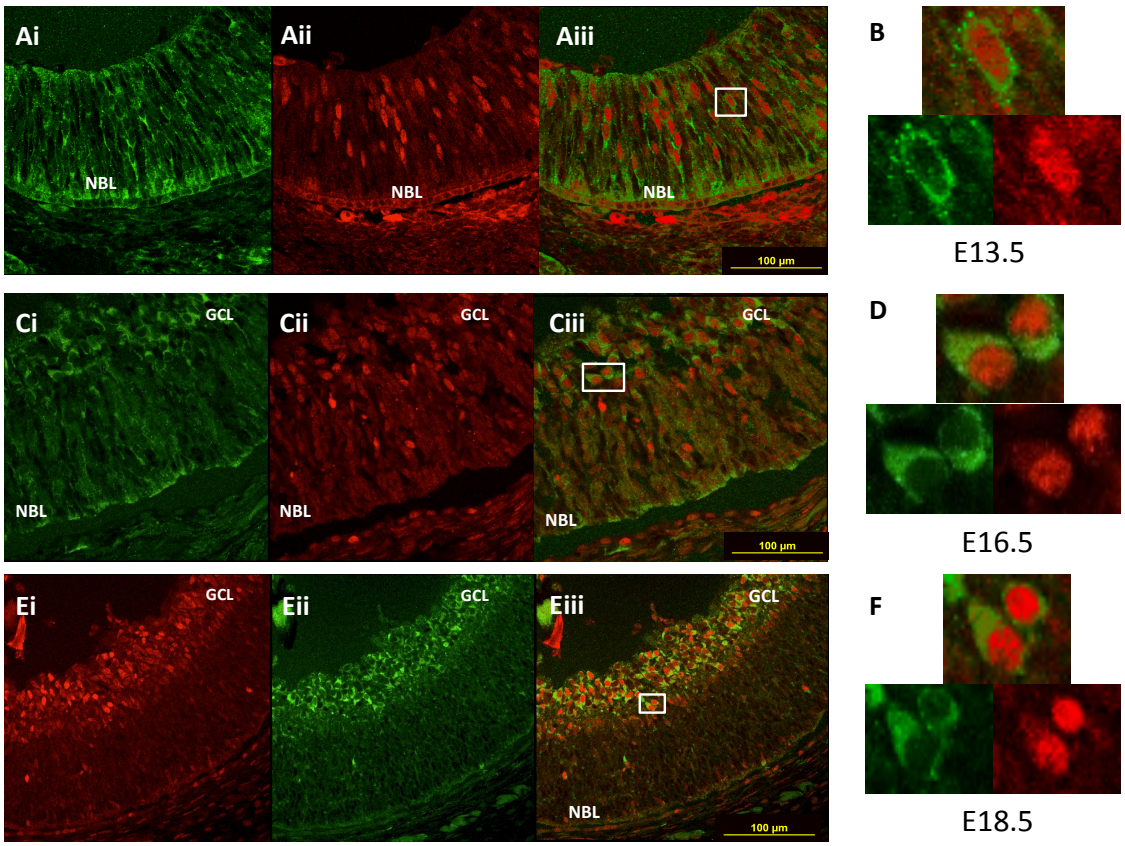
Ai
Aii
Aiii
NBL
NBL
NBL
100 µm
B
E13.5
Ci
Cii
Ciii
GCL
GCL
GCL
NBL
NBL
NBL
100 µm
D
E16.5
Ei
Eii
Eiii
GCL
GCL
GCL
NBL
100 µm
F
E18.5

Figure 7: Temporal expression patterns of DLX2 and P107 during retinal development. DLX2 (red) is localized to the nucleus of both RGC and displaced amacrine cells in both the NBL and GCL of the developing retina. During early retinal development (A, B), DLX2 is localized to the nuclei of RCG and amacrine cells in the NBL (Aii). P107 (green) is localized to the cytoplasm (Ai) of cells located in the NBL and is co-localized with DLX2 (Aiii). A DLX2-P107 co-expressing cell located in the boxed region in Aiii is indicated in B in addition to DLX2 and P107 single staining. During late retinal development (C-F), DLX2 is localized to the nuclei of RGC and amacrine cells in the GCL and NBL (Cii, Eii). P107 is localized to the cytoplasm of cells in the NBL and GCL (Ci, Ei) and is co-localized with DLX2. Individual DLX2-P107 co-expressing cells in late retinal development, located in the boxed regions in Ciii and Eii, are indicated in D and F along with single DLX2 and P107 stained cells. Images were taken at 40x magnification [RGC = retinal ganglion cells, GCL = ganglion cell layer, NBL = neuroblastic layer].

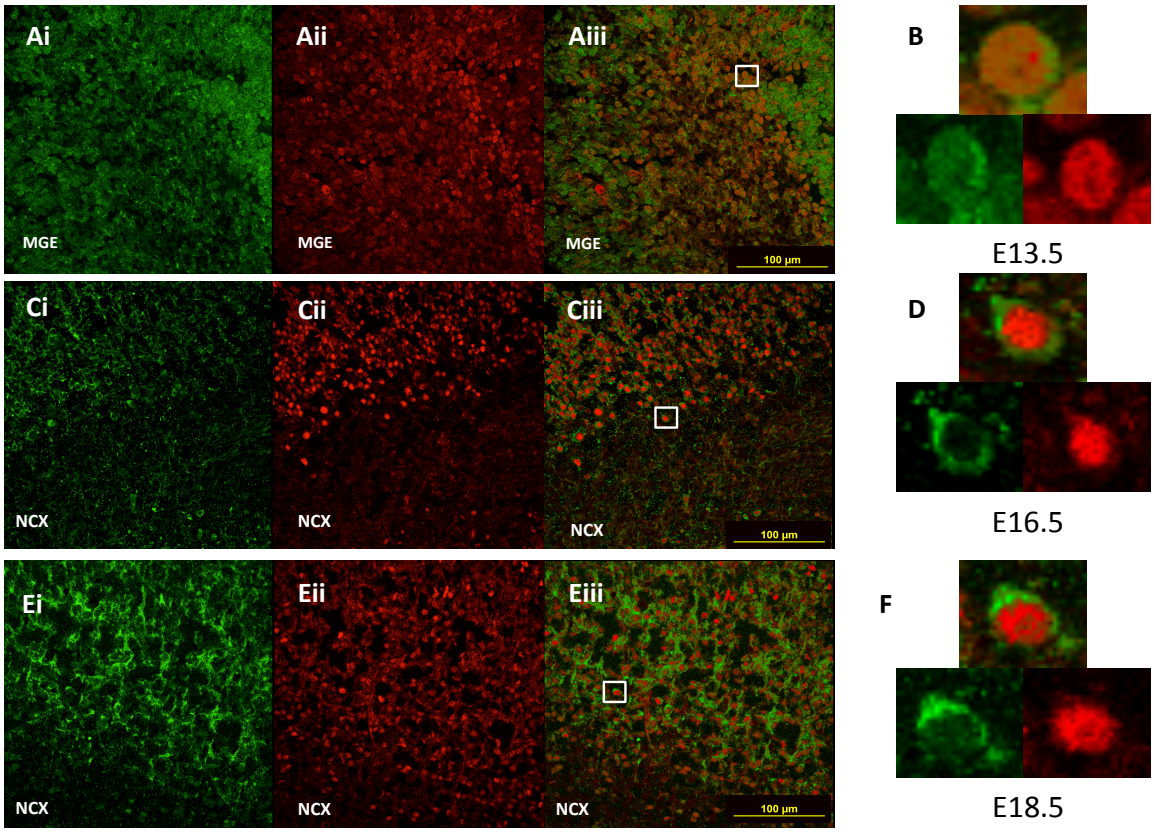
Ai
Aii
Aiii
MGE
MGE
MGE
100 µm
Ci
Cii
Ciii
NCX
NCX
NCX
100 µm
Ei
Eii
Eiii
NCX
NCX
NCX
100 µm
B
E13.5
D
E16.5
F
E18.5

Figure 8: Temporal expression patterns of DLX2 and P107 during forebrain development. DLX2 (red) is localized to the nucleus of GABAergic interneurons of the developing forebrain. During early forebrain development (A, B), DLX2 is localized to the nuclei of GABAergic interneurons in the GE of the developing ventral telencephalon (Aii). P107 (green) is localized to the cytoplasm (Ai) and is co-localized with DLX2 (Aiii). A DLX2-P107 co-expressing cell located in the boxed region in Aiii is indicated in B in addition to DLX2 and P107 single staining. During late forebrain development (C-F), DLX2 is localized to the nuclei of GABAergic interneurons, which have migrated from the ventral telencephalon to the developing NCX (Cii, Eii). P107 is localized to the cytoplasm (Ci, Ei) in late forebrain development and is co-localized with DLX2. Individual DLX2-P107 co-expressing cells found in late forebrain development, located in the boxed regions in Ciii and Eii, are indicated in D and F along with single DLX2 and P107 stained cells. Images were taken at 40x magnification [GE = ganglionic eminences, NCX = neocortex].

DLX2 directly binds to the promoter of *p107 in situ* and *in vitro.*

We have previously demonstrated that *p107* is a potential target for transcriptional regulation by DLX2 (Pind & Eisenstat, unpublished). Examination of a 3 kb promoter sequence 5' to the translational start site of *p107* led to the identification of 22 potential DLX2 binding sites (**Figure 6**). We arbitrarily divided the promoter of *p107* into eight regions ranging from 151 bp to 324 bp in size using PCR primers designed to flank regions containing at least one putative DLX2 binding site and a maximum of six putative binding sites. To determine specific regions of DLX2 occupancy at the *p107* promoter *in situ*, ChIP assays were performed on E13.5 GE of the murine forebrain and E16.5 murine retinal tissues where DLX2 expression is enriched. DLX2 was cross-linked at regulatory regions on target genes, followed by precipitation of DLX2/DNA complexes using an antibody specific to DLX2. Complexes were reverse cross-linked, resulting in enrichment of gDNA fragments bound by DLX2 in the forebrain and retina. PCR analysis on the enriched gDNA fragments using primers described above demonstrated that in the developing forebrain, DLX2 occupies region 1 (*p107*-R1) and region 8 (*p107*-R8) of the *p107* promoter *in situ* (**Figure 9** and **Figure 10**). Of the candidate *p107* promoter sites that have been tested in the developing retina (R1, R6, R7, R8), we have demonstrated that DLX2 occupies *p107*-R1 *in situ*. *p107* promoter regions 2-5 have yet to be tested in the developing retina. Also, enrichment for DLX2 binding to the *p107* promoter appeared increased in the developing forebrain than that observed in the developing retina *in situ*, relative to the gDNA-derived bands. Hindbrain ChIP was used as a negative tissue control for ChIP on *p107*-R1 – *p107*-R8, as DLX2 is not expressed in the developing hindbrain (Zhou, Le et al. 2004).

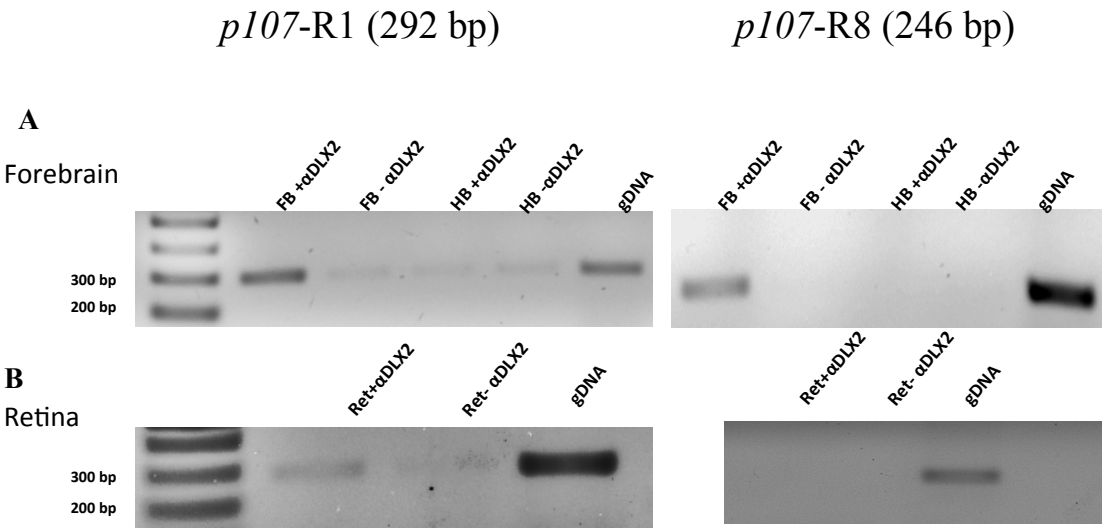

Figure 9: DLX2 binds to the *p107* promoter in the developing forebrain and retina *in situ*. Embryonic forebrain ChIP shows DLX2 occupies both *p107* region 1 (*p107*-R1), and *p107* region 8 (*p107*-R8) in the developing forebrain (A). DLX2 occupies *p107*-R1 in the developing retina (B). *p107*-R8 in the developing retina is negative for binding by DLX2. ChIP was carried out with high affinity purified antibody to DLX2. Controls included a negative antibody control where no αDLX2 is added, along with negative HB tissue controls as DLX2 is not expressed in the developing HB [ChIP = chromatin immunoprecipitation, FB = forebrain, HB = hindbrain, Ret = retina, gDNA = genomic DNA, αDLX2 = DLX2 high affinity antibody].

p107-R1 292 bp (48-340)

TACTGAGCCTCTACTATTTTATCAAATTCATAGACTGGCCACT
TCATTCCCAAAGTTAGTATACTGTACATTTTTGGTCCCTCCTA
TACTGTAAAGA**TAAT**GAAGTTATATAAAAACTGGTATTTATTT
GTGAACCTTTTTTCAGTCCTTGATTGATTTCATGAATTGTTCC
ATTCTCAGGGTAGAAACAAACCTATAGGGAAAGATATTCATC
TTTAAATTTCCGTACTTAAAATGTGAGAAAACAAGCCAGGCT
TGGTGTCTTATGCCTATATTCCAGCAGTTGTGACG

p107-R8 246 bp (2073-2319)

GAAGTTAGGTGTCAGCTTATAGATGTACTGCTCAG**TAAT**GGC
CTGAGAAGGTTAAA**ATTA**GACTTAAATGATGAAGAGCAAGG
TATGCAAAGATATGCACAATAGCATTTTCACACACGGAGAAA
AGCCTCCGACTGAGAACTAGCTTGATATTCCCAAGGTTACGA
GTTACCTGGTGCATCTGAATGCT**ATTA**TTTTTCTGTATTTTAA
AGACAAAAAATGATCTCGTGCAGGATTCTGAGTG

Figure 10: Regions of the *p107* promoter bound by DLX2 as assessed by ChIP. Both *p107*-R1 and *p107*-R8 are occupied by DLX2 in embryonic forebrain *in situ*. Sequence, amplification size and location within the *p107* promoter for *p107*-R1 and *p107*-R8 are indicated above. Promoter locations are indicated in brackets above with 1 being the most distal location from the translational start site of the *p107* promoter. Putative DLX2 homeodomain binding sites are indicated in red.

Although ChIP is a powerful technique that demonstrated interaction of DLX2 at the promoter of *p107 in situ*, it alone does not signify whether DLX2 is directly and/or specifically bound to the *p107* promoter DNA. In order to determine specific binding of DLX2 to *p107*, electrophoretic mobility shift assays (EMSA) were utilized. Here, *p107*-R1 and *p107*-R8, which were occupied by DLX2 *in situ,* were radiolabelled with αP^{32}-GTP and incubated with rDLX2. Direct binding by DLX2 to radiolabelled probes resulted in a shift of DLX2/*p107* complexes in a polyacrylamide gel due to the formation of larger molecular weight complexes that migrate more slowly in a gel as compared to labelled probe alone. We demonstrated specific binding of rDLX2 to *p107*-R1 and *p107*-R8 of the promoter of *p107* (**Figure 11**). Cold competitions using 100-fold excess unlabelled probes competed for binding by rDLX2 with radiolabelled *p107* promoter regions. Supershifts were observed when DLX2/*p107* probe complexes were incubated with a DLX2 antibody, confirming the specificity of DLX2 binding to labelled *p107* probes *in vitro*. Incubation of DLX2/*p107* probe complexes with an irrelevant antibody, specific to the Adenomatous polyposis coli (APC) protein, did not induce the formation of supershift complexes.

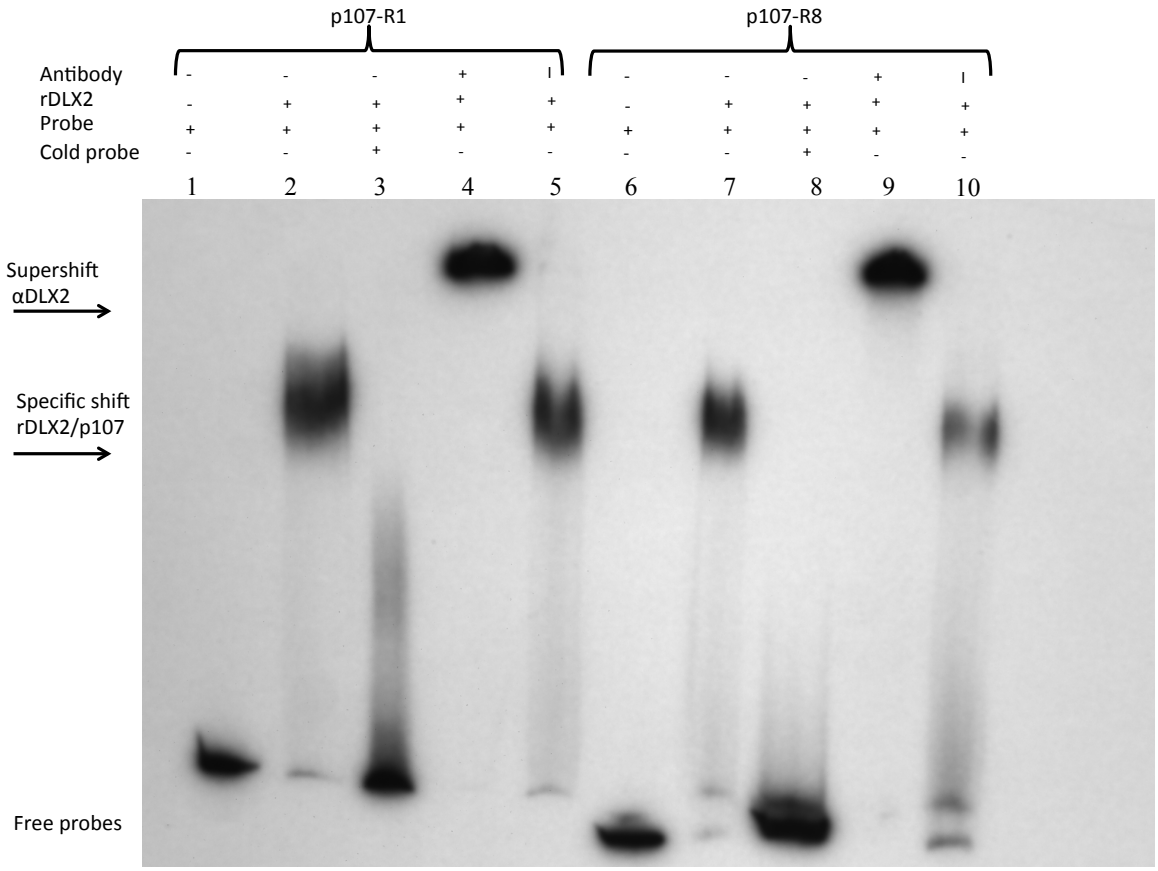

Figure 11: DLX2 directly binds *p107*-R1 and *p107*-R8 *in vitro*. EMSA carried out on radiolabelled ChIP *p107* promoter regions demonstrated specific binding of rDLX2 to *p107*-R1 (lanes 1-5) and *p107*-R8 (lanes 6-10) *in vitro* resulting in a shift of DLX2/*p107* complexes in a non-denaturing polyacrylamide gel (lanes 2 & 7) compared to free labelled DNA probes (lanes 1 & 6). Cold competition (lanes 3 & 8) with 100-fold unlabelled probe out competed labelled *p107* probes for DNA binding. Specificity of rDLX2 binding to labelled probes was demonstrated by supershift of complexes treated with αDLX2 (lanes 4 & 9). As a control, incubation with an irrelevant antibody (lanes 5 & 10) did not demonstrate a supershift. [rDLX2 = recombinant DLX2 protein, αDLX2 = DLX2 antibody, I = irrelevant antibody]

Loss of *Dlx1/Dlx2* results in decreased *p107* expression in the developing forebrain and retina

To examine the functional effect of DLX binding to the *p107* promoter in the developing forebrain and retina *in vivo*, we utilized *Dlx1/Dlx2* DKO forebrain and retinal tissues and compared the *p107* expression patterns observed in WT and DKO forebrain and retina at developmental time points E13.5 and E18.5, respectively. We first conducted IF on cryopreserved tissue sections using an antibody specific to P107. In the absence of *Dlx1/Dlx2* regulation, we observed reduced *p107* expression in the NBL of the developing retina (**Figure 12**) and in the SVZ of the GE in the developing ventral forebrain (**Figure 13**), both of which are the proliferative regions of these tissues.

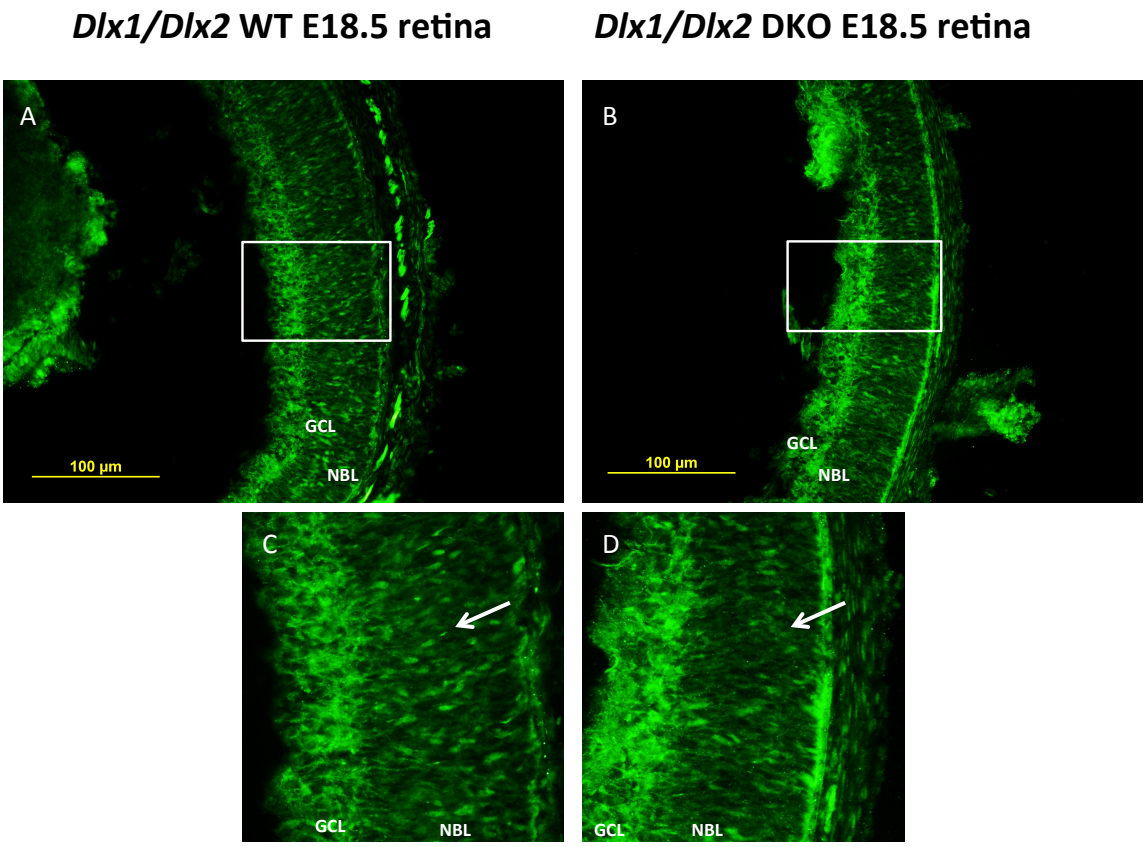

Figure 12: *p107* expression is reduced in *Dlx1/Dlx2* DKO retina. In the absence of *Dlx1/Dlx2* (B and D), *p107* expression is reduced in the NBL of the developing retina (arrows) compared to WT (A and C). Images A and B were taken at 20x magnification. C and D are a magnification of A and B, respectively. Areas of the E18.5 retina which have been magnified in C and D are indicated in the boxed area in A and B. Magnification of images was completed using Adobe Photoshop Elements 9 software where magnification of the selected area is 75% larger then the original image. Arrows in C and D indicated the reduction in P107 expression that is observed in the DKO NBL compared to WT. [DKO = double knockout, GCL = ganglion cell layer, NBL = neuroblastic layer].

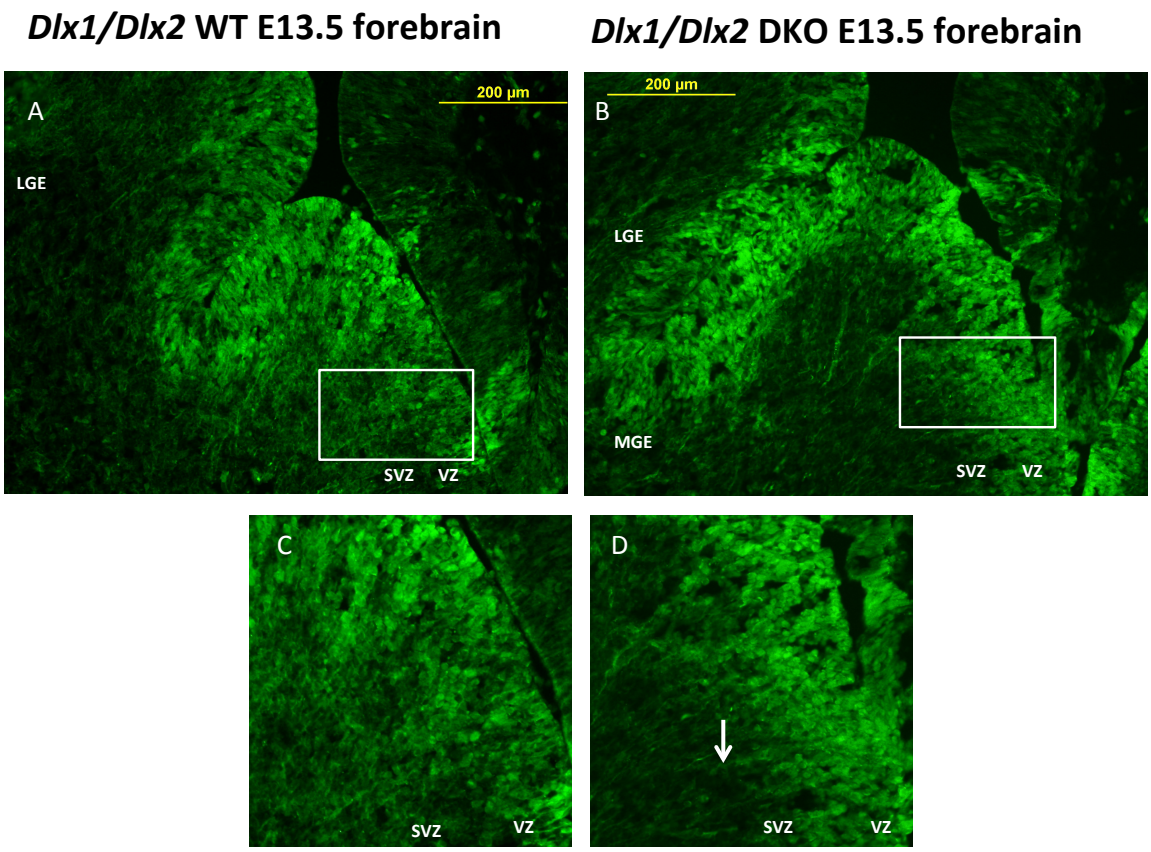

Figure 13: *p107* expression is reduced in *Dlx1/Dlx2* DKO forebrain. In the absence of *Dlx* expression (B and D), *p107* expression is reduced in the SVZ of the GE in the developing forebrain compared to WT (A and C) Images A & B were taken at 20x magnification. C and D are a magnification of A and D, respectively. Magnification of images was completed using Adobe Photoshop Elements 9 software where magnification of the selected area is 75% larger then the original image. The arrow in D indicates the decrease in P107 expression observed in the SVZ MGE. [LGE = lateral ganglionic eminence, MGE = medial ganglionic eminence, SVZ = subventricular zone, VZ = ventricular zone].

Semi-quantification of *p107* transcript levels in murine tissues was examined *in vivo* by quantitative real-time PCR (qRT-PCR) analysis. In both the developing forebrain and retina at E13.5 and E18.5, respectively, the absence of *Dlx1/Dlx2* resulted in significant decreases in *p107* transcript levels **(Figure 14)**. Preliminary analysis indicates that DLX2 regulation is specific to *p107*, not having any regulatory role on expression of the remaining members of the Rb family, which include *Rb* and *p130* (data not shown).

DLX2 significantly activates *p107* expression *in vitro*

To support our findings of *Dlx* mediated regulation of *p107* expression *in vivo*, the functional significance of DLX2 binding on the *p107* promoter was examined *in vitro* by luciferase reporter gene assays. Here, regions of the *p107* promoter identified to be occupied by DLX2 *in situ* and the 3 kb region upstream of the translational start site of the *p107* promoter (herein referred to as the whole promoter) were cloned into pGL3 reporter vectors (Promega), placing the reporter gene directly downstream and under transcriptional control of the *p107* promoter. The functional effect of DLX2 binding was then determined by quantifying bioluminescence of luciferase reporter protein product. Empty pcDNA3 plasmids (lacking *Dlx2*) were used as controls. Upon co-transfection of *p107* reporter vectors with a *Dlx2* expression plasmid, significant activation of luciferase expression was observed with reporter vectors *p107*-R1 (3.78 fold), *p107*-R8 (1.71 fold) and the whole promoter of *p107* [(*p107*-wp), 2.16 fold] compared to controls (**Figure 15**).

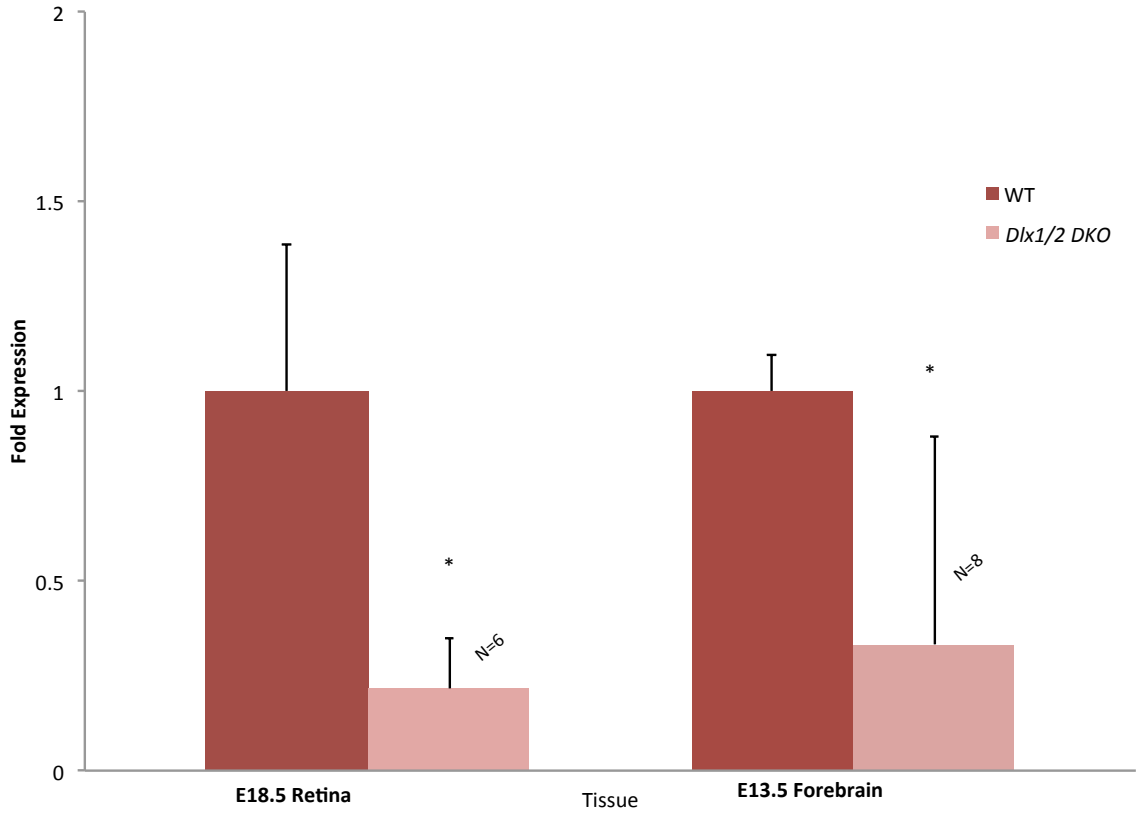

Figure 14: *p107* transcript levels are reduced in the *Dlx1/Dlx2* DKO. RNA was extracted from whole embryonic retinas and GEs of the ventral telencephalon at E18.5 and E13.5, respectively. qRT-PCR results demonstrated a significant reduction in *p107* expression *in vivo* in the absence of *Dlx1/Dlx2* in the developing retina and forebrain. Error bars represent standard error of the mean (SEM) [P = <0.05, retina: n = 6 embryonic retinas. Forebrain: n = 8 ganglionic eminences, GEs = ganglionic eminences, SEM = standard error of the mean].

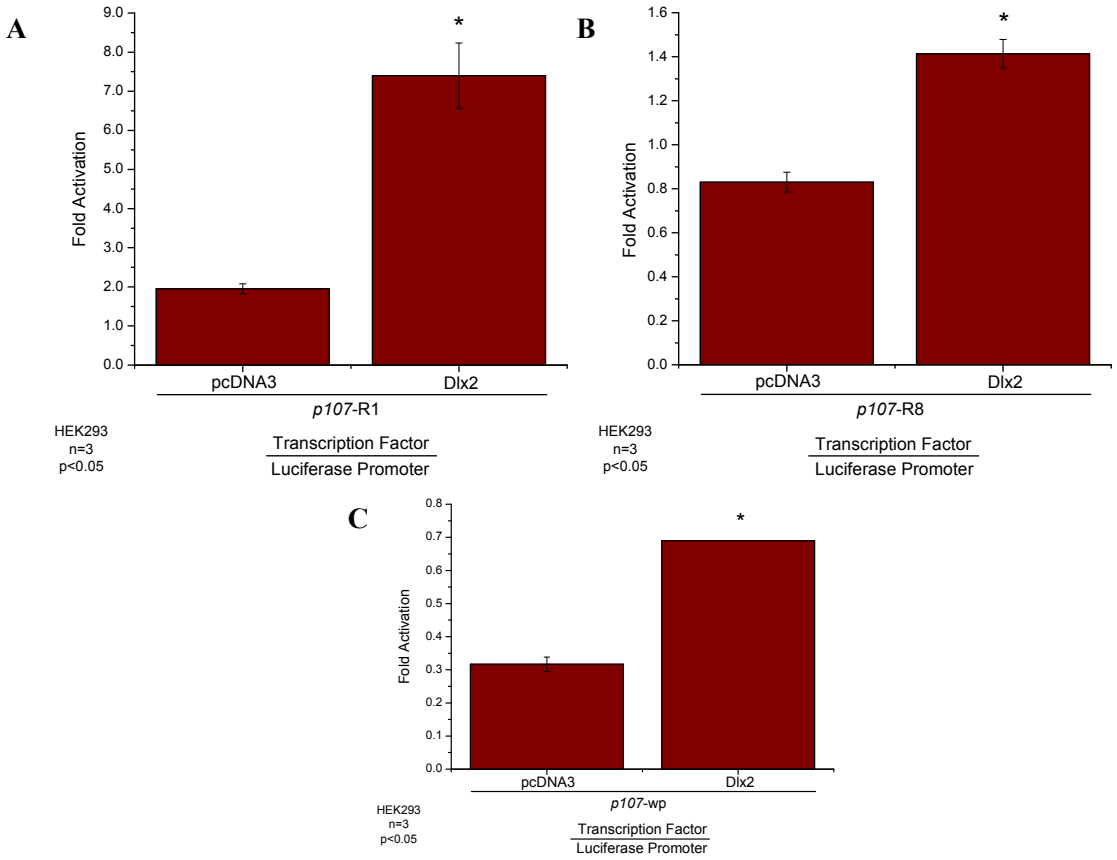

Figure 15. *p107* is activated upon DLX2 co-expression *in vitro*. *p107*-R1 (A) , *p107*-R8 (B) and the whole promoter of *p107* (*p107*-wp) (C) were sub-cloned into pGL3 reporter vectors and co-transfected into HEK 293 cells with a *Dlx2* expression plasmid, resulting in significant activation of luciferase reporter gene expression *in vitro*. All luciferase activities were normalized to β-galactosidase activity [p107-R1 = 3.78 fold activation. p107-R8 = 1.71 fold activation. p107-wp = 2.16 fold activation. All P values <0.05].

Mutagenesis of DLX2 binding sites in the *p107* promoter results in loss of *p107* activation *in vitro*

As previously mentioned, DLX2 binds to tetranucleotide motifs ATTA/TAAT in target gene promoters. In the two specific regions of the *p107* promoter that we identified to be occupied by DLX2 *in situ*, there are a total of four potential DLX2 binding sites. SDM, which introduces single bp mutations in putative DLX2 binding sites, was performed to identify critical sites for DLX2 activation of *p107* expression *in vitro*. *p107*-R1 contains one putative DLX2 binding site, whereas *p107*-R8 contains three putative DLX2 binding sites. Mutant promoters were generated for *p107*-R1 (*p107*-mR1) and each putative binding site in *p107*-R8 individually, resulting in three mutant constructs for *p107*-R8: *p107*-mR8S1, *p107*-mR8S2 and *p107*-mR8S3 (**Figure 16**). Mutant promoters were cloned into pGL3 reporter vectors and analyzed for their ability to activate luciferase expression *in vitro* as described above. Activation of luciferase expression, which was observed in the WT promoter regions of *p107*, was abrogated with the introduction of mutations in DLX2 binding sites of promoters *p107*-mR1 and *p107*-mR8S2 (**Figure 17**). Luciferase reporter gene assays with *p107*-mR8S1 and *p107*-mR8S3 remain to be carried out.

***p107*-mR1**

TACTGAGCCTCTACTATTTTATCAAATTCATAGACTGGCCACTTCATTCCCA
AAGTTAGTATACTGTACATTTTTGGTCCCTCCTATACTGTAAAGATAAGGA
AGTTATATAAAAACTGGTATTTATTTGTGAACCTTTTTTCAGTCCTTGATTG
ATTTCATGAATTGTTCCATTCTCAGGGTAGAAACAAACCTATAGGGAAAG
ATATTCATCTTTAAATTTCCGTACTTAAAATGTGAGAAAACAAGCCAGGCT
TGGTGTCTTATGCCTATATTCCAGCAGTTGTGACG

***p107*-mR8S1**

GAAGTTAGGTGTCAGCTTATAGATGTACTGCTCAGTAAGGGCCTGAGAAG
GTTAAAATTAGACTTAAATGATGAAGAGCAAGGTATGCAAAGATATGCAC
AATAGCATTTTCACACACGGAGAAAAGCCTCCGACTGAGAACTAGCTTG
ATATTCCCAAGGTTACGAGTTACCTGGTGCATCTGAATGCTATTATTTTTCT
GTATTTTAAAGACAAAAAATGATCTCGTGCAGGATTCTGAGTG

***p107*-mR8S2**

GAAGTTAGGTGTCAGCTTATAGATGTACTGCTCAGTAATGGCCTGAGAAG
GTTAAAATTGGACTTAAATGATGAAGAGCAAGGTATGCAAAGATATGCAC
AATAGCATTTTCACACACGGAGAAAAGCCTCCGACTGAGAACTAGCTTG
ATATTCCCAAGGTTACGAGTTACCTGGTGCATCTGAATGCTATTATTTTTCT
GTATTTTAAAGACAAAAAATGATCTCGTGCAGGATTCTGAGTG

***p107*-mR8S3**

GAAGTTAGGTGTCAGCTTATAGATGTACTGCTCAGTAATGGCCTGAGAAG
GTTAAAATTAGACTTAAATGATGAAGAGCAAGGTATGCAAAGATATGCAC
AATAGCATTTTCACACACGGAGAAAAGCCTCCGACTGAGAACTAGCTTG
ATATTCCCAAGGTTACGAGTTACCTGGTGCATCTGAATGCTATTGTTTTTCT
GTATTTTAAAGACAAAAAATGATCTCGTGCAGGATTCTGAGTG

Figure 16: Site directed mutagenesis of putative DLX2 binding sites. Mutations were introduced in putative homeodomain DNA binding motifs located in ChIP positive *p107* promoter regions in order to identify critical binding sites for activation and direct binding of *p107 in vitro*. Blue-labelled nucleotides indicate single bp mutations

introduced in putative DLX2 binding sites. Red font indicates putative DLX2 binding motifs.

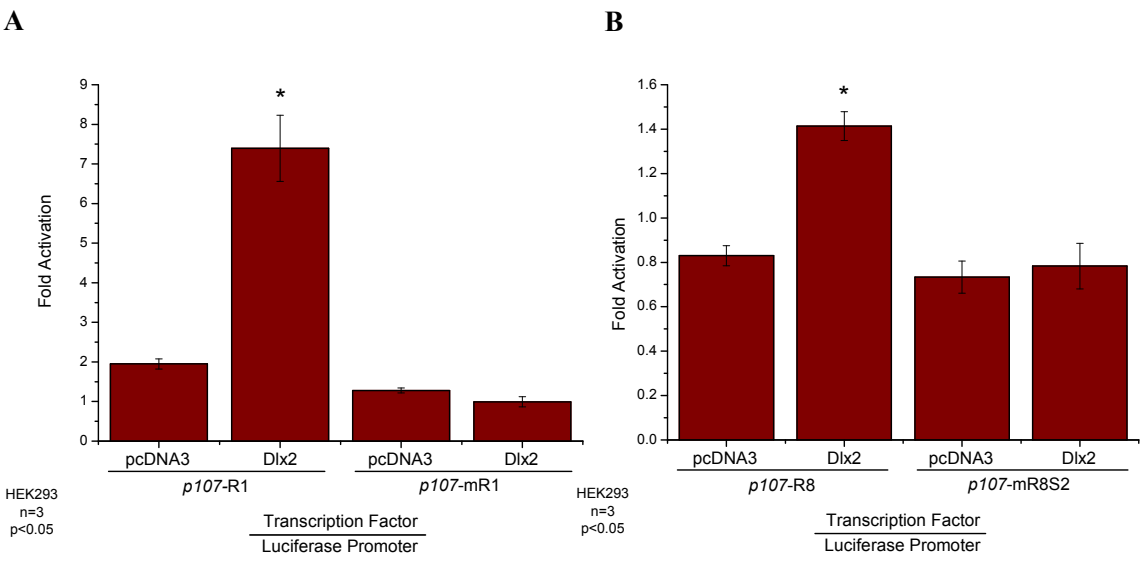

Figure 17: Activation of *p107* promoter is abrogated with introduction of mutations in putative DLX2 binding sites. SDM of the lone DLX2 binding site in A) *p107*-R1 (*p107*-mR1) and the second putative homeodomain DNA binding motif in B) *p107*-R8 (*p107*-mR8S2) results in loss of activation of luciferase reporter gene expression observed in WT *p107* promoter regions. All luciferase activities are normalized to β-galactosidase activity [P = <0.05, N=3, SDM = site directed mutagenesis].

Direct binding to *p107* is abrogated with the introduction of mutations in putative DLX2 binding motifs

We have demonstrated that rDLX2 directly binds both *p107*-R1 and *p107*-R8 *in vitro* through shift of rDLX2/*p107* complexes in polyacrylamide gel electrophoresis (**Figure 11**). To identify DNA binding sites that are critical for direct interaction with *p107* promoter DNA, further EMSA were carried out using mutant *p107* promoter probes for *p107*-R1 and *p107*-R8, which were previously described. Mutant probes were examined for the ability of rDLX2 to bind putative DNA binding motifs *in vitro*. Direct binding of rDLX2 to *p107*-R1 was abrogated with the introduction of a mutation in the putative DLX2 binding site (**Figure 18**). Direct binding to all three mutant *p107*-R8 constructs was still observed despite the introduction of individual mutations in the three putative binding sites located within this promoter region.

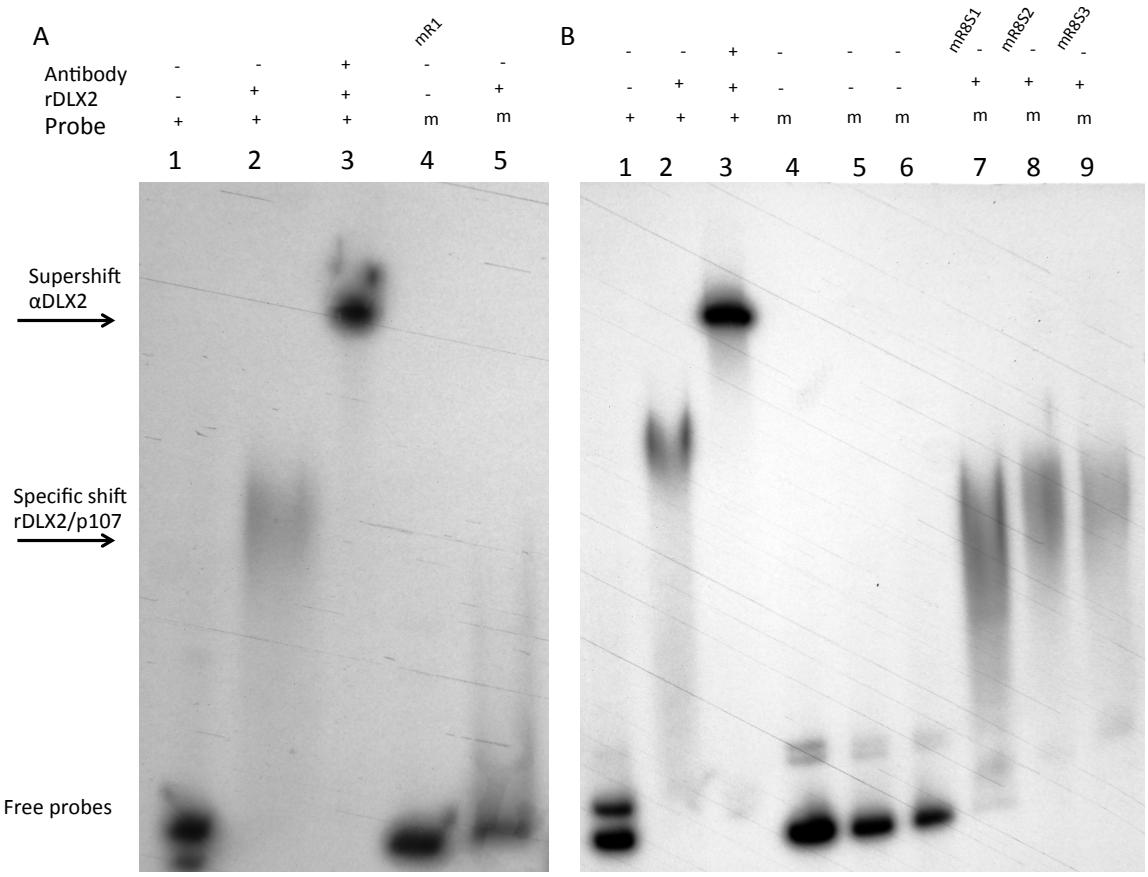

Figure 18: The homeodomain binding site in *p107*-R1 is critical for specific *p107* binding *in vitro*. Specific binding of rDLX2 to *p107*-R1 is abrogated upon mutation of the putative DLX2 binding site (*p107*-mR1) in this promoter region (compare 2, *p107*-R1 & 5, *p107*-mR1) (A). In *p107*-R8, direct binding to mutant constructs is still observed when each putative DLX2 binding site is individually mutated (compare 2, *p107*-R8 & 7 = *p107*-mR8S1, 8 = *p107*-mR8S2, and 9 = *p107*-mR8S3) (B). Lane 3 of A & B demonstrates specificity of rDLX2 binding to *p107*-R1 and *p107*-R8 by supershift of DLX2/DNA complexes in the polyacrylamide gel [m = mutant probes, rDLX2 = recombinant DLX2].

DISCUSSION

Strict regulation of the cell cycle is critical for the proper development of the CNS. Careful cell cycle control maintains the neural progenitor population, regulates appropriate cell cycle exit, and neural differentiation in the developing CNS (Ferguson, Vanderluit et al. 2002; Ferguson, McClellan et al. 2005). *Dlx* genes have been demonstrated to play a key role in neural progenitor cell fate specification, differentiation and neuronal migration in the developing CNS (Anderson, Eisenstat et al. 1997; Anderson, Qiu et al. 1997; Eisenstat, Liu et al. 1999; de Melo, Du et al. 2005; Le, Du et al. 2007). This project assessed the role of the *Dlx* homeobox genes in cell cycle regulation and neuronal development by examining the transcriptional control of the retinoblastoma family member, *p107,* during development of the CNS. By examining *Dlx* regulation of cell cycle progression, we may discover novel roles for *Dlx* in tumourogenesis as well as expand on our understanding of *Dlx* mediated regulation of neural cell fate specification, neural differentiation and migration.

DLX2 and P107 are co-expressed throughout CNS development

DLX2 expression in the developing retina initiates at E11.5 and is localized to the dorsal NBL (de Melo, Zhou et al. 2008). Expression of DLX2 precedes that of P107 in the retina which at the earliest, is expressed at E14.5 in the NBL (Donovan, Schweers et al. 2006). In the vertebrate forebrain, *Dlx2* expression starts at E9.5, followed by *p107* expression at E10.5 in the proliferative ventricular zones of the GE (Jiang, Zacksenhaus et al. 1997; Liu, Ghattas et al. 1997; Eisenstat, Liu et al. 1999). Given the known temporal expression patterns of these proteins, we wanted to examine concomitant temporal and spatial

patterns of DLX2 and P107 in the developing forebrain and retina to identify cellular co-expression of these proteins during development. By establishing co-expression, this demonstrates the potential for DLX2 to regulate expression of *p107.* DLX2 expression in both the forebrain and retina has been well established (Eisenstat, Liu et al. 1999; de Melo, Qiu et al. 2003; de Melo, Du et al. 2005; Le, Du et al. 2007). During retinal development, DLX2 is expressed in both RGC and displaced amacrine cells in the NBL during early development, and the GCL and NBL at later developmental time points (de Melo, Qiu et al. 2003). During development of the forebrain, DLX2 expression is observed in GABAergic interneurons localized to the GE of the basal telencephalon. In later stages of forebrain development, these GABAergic interneurons begin migrating tangentially to the developing NCX (Anderson, Eisenstat et al. 1997; Panganiban and Rubenstein 2002). To our knowledge, IF analysis of P107 expression in the developing forebrain has not previously been demonstrated, whereas Donovan *et al* (Donovan, Schweers et al. 2006) have shown P107 expression patterns in embryonic retinal tissue. DIF analysis carried out on forebrain and retinal tissue sections at developmental time points E13.5, E16.5 and E18.5 show that DLX2 and P107 are co-expressed throughout development of the forebrain and retina (**Figure 7 and 8**). Our DIF analysis demonstrates nuclear expression of DLX2 and cytoplasmic expression patterns for P107 throughout forebrain and retinal development. Donovan *et al* however, demonstrate nuclear expression of P107 during retinal development in the NBL. P107 can be detected in both the nucleus as well as the cytoplasm. It is thought that P107 binds repressor E2Fs in the cytoplasm and carries these repressor complexes to the nucleus where they bind to and inhibit the expression of E2F target genes resulting in cessation of cell cycle

progression (Verona, Moberg et al. 1997; Puri, Cimino et al. 1998). Therefore, nuclear expression of P107 should also be observed during forebrain and retinal development. Further optimization of our DIF, including use of other commercial and proprietary p107 antibodies is required in order to establish the temporal and spatial expression pattern of P107 and subsequent co-expression with DLX2 expressing cells during forebrain and retinal development. Alternatively, *in situ* hybridization (ISH) has successfully been conducted for *p107* expression in both the forebrain and retina in previous studies (Jiang, Zacksenhaus et al. 1997; Vanderluit, Ferguson et al. 2004; Donovan, Schweers et al. 2006). ISH could help establish the proper expression pattern of *p107* during development of the CNS since it has been shown that *p107* transcript levels are strongly correlated with *p107* protein levels (Smith, Leone et al. 1998; Burkhart, Viatour et al. 2008).

DLX2 directly binds to the *p107* promoter during CNS development

Of the members of the retinoblastoma family, *p107* is thought to be the most highly regulated at the transcriptional level (Smith, Leone et al. 1998; Burkhart, Viatour et al. 2008). DLX2 regulates transcription by binding to promoter DNA at ATTA/TAAT motifs and either activates (*Dlx56ie*, *TrkB*) or represses (*Nrp2*) gene expression. We have identified *p107*-R1 and *p107*-R8 promoter regions to be occupied by DLX2 in the developing forebrain and *p107*-R1 to be occupied by DLX2 in the developing retina *in situ*. ChIP analysis on *p107*-R2 through *p107*-R5 remains to be carried out for retina. Enrichment for DLX2 binding to *p107* was observed to be stronger in the developing forebrain compared to the retina (compare A and B, **Figure 9**). This can be due to higher expression of DLX2 in the GE of the developing forebrain at E13.5 compared to that in

the embryonic retina at E18.5. Also, the presence of additional transcription factors interacting with DLX2 may vary between the forebrain and retina and lead to differences in DLX2 binding to *p107* between these two tissues, resulting in greater enrichment for DLX2 binding in the forebrain ChIP compared to the retina. Although *p107*-R2 through *p107*-R5 were negative for DLX2 interaction in the forebrain, transcription factor occupancy on a promoter is specific to the tissue being examined and thereby, these regions could potentially be occupied by DLX2 in the developing retina. In addition, DLX2 binding specificity may differ at varying developmental time points during tissue development. For instance, preference for DLX2 binding at homeodomain DNA binding motifs on the adenopolyposis coli (APC) promoter differs when comparing the embryonic and adult murine gastrointestinal tract. This suggests varying temporal requirements for *Dlx* mediated regulation of *Apc* expression throughout gastrointestinal development (Fonseca and Eisenstat, unpublished).

DLX2 occupancy on the *p107* promoter *in situ* as examined by ChIP is not indicative of direct and/or specific binding pattern of DLX2 at the promoter. DLX2 can bind either directly to homeodomain binding motifs on the promoter, through secondary protein-protein interactions or through a complex of proteins. EMSA was utilized to identify direct binding of DLX2 to the *p107* promoter *in vitro*. We have identified specific binding of affinity-purified rDLX2 to *p107*-R1 and *p107*-R8 of the *p107* promoter. *In vitro* supershift of the DLX2/*p107* probe complex confirms specific DLX2 binding to labelled *p107* probes. EMSA using nuclear extracts from embryonic forebrain and retinal tissues will further confirm specificity of DLX2 binding to the *p107* promoter using endogenous DLX2 protein from the developing forebrain and retina. Both through ChIP

and EMSA, we have demonstrated that DLX2 directly binds the *p107* promoter *in situ* and *in vitro* which is consistent with our hypothesis.

DLX2 activates *p107* expression in CNS development

The *Dlx* genes play an important role in the development of the vertebrate forebrain and retina by regulating the expression of a number of important target genes. KO mouse models of *Dlx* are useful tools to study *Dlx* transcriptional regulation by examining changes in expression of suspected targets of DLX. In our laboratory, *Dlx1/Dlx2* DKO mice were utilized to examine the consequence of *Dlx* loss on regulation of *p107* expression in the developing forebrain and retina. As the *Dlx1/Dlx2* DKO mouse dies at birth, the transcriptional effect of DLX2 was only examined during embryonic stages of brain and retina development. First, we carried out qualitative IF studies, where *p107* expression was examined and compared in WT and *Dlx1/Dlx2* DKO murine forebrain and retinal tissue. In the absence of *Dlx1/Dlx2*, P107 expression is reduced in the developing retina and forebrain at E18.5 and E13.5, respectively (**Figure 12** and **Figure 13**). In the developing retina, reduction in *p107* expression is observed in the NBL which contains the proliferating RPC and post-mitotic retinal cells. Similarly in the developing forebrain, reduction in P107 expression is observed in the basal telencephalon SVZ, which is the region of the developing forebrain containing the SPP. It is interesting to note that in the *Dlx1/Dlx2* DKO murine forebrain, the SVZ fails to mature, demonstrating increased expression of Notch1 and Delta1 which are involved in maintaining proliferation of neural progenitors (Anderson, Qiu et al. 1997; Panganiban and

Rubenstein 2002). As *p107* has been shown to be involved in negative regulation of neural progenitor renewal by repression of *Hes*, a downstream target of the Notch pathway, and also been shown to be required for progenitor commitment to neuronal fates (Vanderluit, Ferguson et al. 2004; Vanderluit, Wylie et al. 2007), it is plausible that *Dlx1* and *Dlx2* expression is required to activate *p107* expression in order for cells in the SPP to differentiate. Therefore, the reduction of P107 expression observed specifically in the SVZ in the *Dlx1/Dlx2* DKO forebrain (**Figure 13**) could partially explain the failure of the cells in the SPP to mature and subsequently migrate from the SVZ. This could also be the case in retinal development where *Dlx1* and *Dlx2* expression is required for terminal differentiation of late born RGC (de Melo, Du et al. 2005). Since *p107* is highly expressed in RPC in the developing retina (Donovan, Schweers et al. 2006), perhaps it plays a similar role in retinal development as in forebrain and is required for commitment to, and differentiation of neuronal RGC fates.

Changes in *p107* RNA expression in *Dlx1/Dlx2* DKO forebrain and retina *in vivo* were semi-quantified by quantitative real-time PCR (qRT-PCR). In both forebrain and retina, significant decreases in *p107* RNA expression were observed upon the loss of *Dlx1/Dlx2* expression. This decrease in transcript levels supports our IF data and our hypothesis that DLX2 activates expression of *p107* during forebrain and retinal development (**Figure 14**). We coupled our *in vivo* studies with *in vitro* luciferase reporter gene assays to identify the functional significance of DLX2 binding to the *p107* promoter *in vitro*. Our positive ChIP regions, *p107*-R1 and *p107*-R8, along with the *p107* whole promoter significantly activated expression of the luciferase reporter gene when co-transfected with a *Dlx2* expression plasmid (**Figure 15**).

Taken together, our results support a role for DLX2 as a transcriptional activator of *p107* expression during CNS development. In addition, we suggest DLX2 activation of *p107* expression mediates neuronal commitment and differentiation in forebrain and retinal development.

Homeodomain binding sites within the *p107* promoter are critical for activation and binding by DLX2 *in vitro*

DLX2 binds DNA at putative ATTA/TAAT tetranucleotide motifs. However, not all motifs located within a region of the *p107* promoter will necessarily be bound by DLX2. To identify critical sites for promoter binding and activation of *p107* expression *in vitro*, we mutated putative DLX2 binding sites in ChIP positive *p107*-R1 and *p107*-R8. SDM of the lone DLX2 binding site located in *p107*-R1 resulted in loss of activation of *p107* expression that we observed for WT *p107*-R1, indicating that this particular DNA binding motif is a critical site for activation of *p107* expression *in vitro* (**Figure 17a**). We also mutated all three putative DLX2 binding sites in *p107*-R8 individually, leaving two WT DNA binding sites in each *p107*-R8 reporter plasmid. To date, luciferase assays were only carried out using *p107*-mR8S2 and similar to *p107*-mR1, this mutation resulted in the abrogation of *p107*-R8 activation *in vitro* (**Figure 17b**). We have therefore established DLX2 mediated activation of *p107* expression to be due, in part, to specific interaction at these DNA binding motifs in *p107*-R1 and *p107*-R8 of the *p107* promoter. Luciferase using reporter vectors containing the remaining mutant DNA binding motifs

located in *p107*-R8 remain to be carried out and will further identify critical sites for *p107* activation *in vitro.*

Both *p107*-R1 and *p107*-R8 were identified by EMSA to be directly bound by rDLX2, which suggests that transcriptional regulation by DLX2 is partially due to direct binding of DLX2 to homeodomain binding motifs in the *p107* promoter. Direct binding of rDLX2 to *p107*-R1 was abrogated when incubated with mutant *p107*-mR1 labelled probes, further confirming specificity of direct binding of rDLX2 to the lone DNA binding motif in this region. However, EMSA carried out with mutant *p107*-R8 probes (*p107*-mR8S1, *p107*-R8S2 and *p107*-mR8S3) still showed direct binding of rDLX2 to these *p107* promoter probes *in vitro* (**Figure 18**). As there are two WT homeodomain binding sites in each mutated *p107*-R8 probe, the observed shift of this probe in vertical gel electrophoresis could be due to the direct binding of rDLX2 to one or both of the remaining DLX2 binding sites. Since we observed loss of *p107* activation upon mutation of *p107*-mR8S2, but with this mutation still observed direct binding by rDLX2, this may indicate that the second DNA binding site in *p107*-R8 is absolutely critical for activation of *p107* expression despite DLX2 binding at the remaining sites. Luciferase assays using *p107*-mR8S1 and *p107*-mR8S3 promoter regions cloned into pGL3 reporter vectors will determine if these remaining sites are also important for activation of *p107* expression *in vitro*. Future studies where mutations are created in all three putative DLX2 binding sites simultaneously, as well as in two of the three sites may further identify which of the putative DLX2 binding sites in *p107*-R8 are critical for activation of *p107* expression and direct binding to *p107 in vitro*.

Role for DLX in tumourogenesis

One of the hallmarks of cancer is the ability to maintain a constitutive proliferative state (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011). Disruptions in the Rb pathways have been demonstrated in a number of cancers. As mentioned previously, Rb and its related family member, *p107*, block proliferation by inhibiting the transcriptional activation of E2F target genes, which are responsible for progression from G_1 to S phase (Flemington, Speck et al. 1993; Helin, Harlow et al. 1993; Hanahan and Weinberg 2000; Hanahan and Weinberg 2011). Another hallmark of cancer cells is resistance to apoptosis (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011). DLX2 has recently been shown to protect cancer cells from both transforming growth factor β (TGF-β) induced cell cycle arrest and apoptosis (Yilmaz, Maass et al. 2011). DLX2 represses the expression of TGFβ receptor II (TGBβRII), resulting in reduced Smad-dependent TGFβ signalling, reduced expression of the cell cycle inhibitor $p21^{CIP1}$, and upregulation of c-Myc expression. Additionally, DLX2 promotes the expression of the epidermal growth factor (EGF) family member betaellulin, which results in tumour cell survival (Yilmaz, Maass et al. 2011). We have previously established DLX to have a role in promoting cell survival during retinal development (de Melo, Du et al. 2005), in part through activation of TrkB expression. By continuing our studies of DLX2 mediated regulation of *p107* in CNS development, we may uncover novel roles for the DLX family in tumourogenesis by controlling cell cycle progression and prevention of apoptosis.

CONCLUSIONS AND FUTURE DIRECTIONS

We have demonstrated that *p107* is a direct DLX2 transcriptional target during the development of the forebrain and retina *in vivo*. In addition, we have determined that direct binding of DLX2 to the *p107* promoter results in the activation of *p107* expression both *in vivo* and *in vitro.* Critical DNA binding sites for *p107* activation by DLX2 have been identified *in vitro* with a few sites remaining to be examined. These findings are consistent with our hypothesis. We have only begun to elucidate the role of *Dlx* mediated regulation of *p107* expression and a number of experiments could contribute greatly to our knowledge of *Dlx* regulation of cell cycle control.

To strengthen our *in vivo* findings, additional loss-of-function assays using siRNA/shRNA mediated knockdowns of *Dlx2* expression in primary forebrain and retinal cultures will further determine the functional significance of DLX2 transcriptional regulation of *p107* expression in the developing forebrain and retina. We expect that knocking down *Dlx2* expression will result in concomitant reduction in *p107* expression in primary cultures. Additionally, gain-of-function experiments can be carried out where *Dlx2* is ectopically expressed by retroviral transduction into forebrain and retinal explants and subsequently, examined for induction of *p107* expression.

Alterations in cell cycle progression during forebrain and retinal development will be examined by flow cytometry, coupled with tissue staining marking cells in various stages of the cell cylce such as BrdU for cells undergoing S phase and phospho-histone H3 for M phase. This will enable us to determine whether DLX mediated regulation of *p107* expression has an impact on progression of the cell cycle.

To further examine transcriptional regulation of *p107* expression by *Dlx* genes *in vivo*, GFP-*p107* reporter mice (Burkhart, Viatour et al. 2008) can be crossed into our *Dlx1/Dlx2* DKO colony. Here, *p107* expression in the absence of *Dlx1/Dlx2* is examined by analyzing GFP reporter expression. In addition to providing a model for examining *Dlx* mediated regulation of *p107* expression, this reporter model will also provide an excellent tool to examine temporal and spatial expression patterns of *p107* in the developing forebrain and retina *in vivo*. We can thereby conduct our DIF assays using this model to examine the co-expression patterns of DLX2 and P107, perhaps circumventing issues which were encountered in our current DIF studies.

Unfortunately, our *Dlx1/Dlx2* DKO mice die shortly after birth. Therefore, we have been unable to determine the functional significance of *Dlx* gene expression in postnatal retinal and forebrain development. This is particularly difficult in studying retinal development, as the murine retina is not fully developed until the second postnatal week. Production of a conditional knockout (CKO) mouse using the *Cre-lox* system would be a pivotal tool to examine the role of *Dlx* in postnatal forebrain and retinal development, and circumvent embryonic lethality observed in the current germline *Dlx* KO mouse models. Until recently, a CKO has not been available for studies. Dr. John Rubenstein at UCSF has very recently produced a floxed *Dlx2* mouse (J. Rubenstein personal communication with Dr. Eisenstat). Crossing this floxed transgenic mouse line with different *Cre* lines to delete *Dlx2* specifically in the forebrain and retina would be an important advancement for studies on the role of *Dlx2* in postnatal development of the murine retina and forebrain, and contribute to our understanding of the role that *Dlx2* plays in neuronal differentiation and migrations disorders.

Lastly, to determine critical DLX2 binding sites for activation of *p107* expression *in vivo*, mutations in the DLX2 binding sites that we identified to abrogate *p107* expression *in vitro*, can be knocked-in to the *p107* promoter to create germline mutations in the DNA binding sites. Our *in vivo* assays, including IF and qRT-PCR would be repeated to determine if mutation in these putative DLX2 binding sites results in reduction of *p107* activation, supporting our hypothesis that DLX2 activates *p107* expression in the developing forebrain and retina, and pinpointing sites which are critical for this activation *in vivo*.

Precise regulation of the cell cycle is imperative for proper development of the CNS. Ours is the first study to investigate the transcriptional regulation of the p107 pocket protein in any biological system. We thereby propose a model where DLX2 binds to the *p107* promoter and activates the expression of *p107*, enabling the commitment and differentiation of neuronal cells in the developing forebrain and retina (**Figure 19**). By continuing our studies of *Dlx* mediated regulation of the cell cycle progression, we may uncover novel targets for treatment of neuronal developmental disorders as well as neuronal migration disorders.

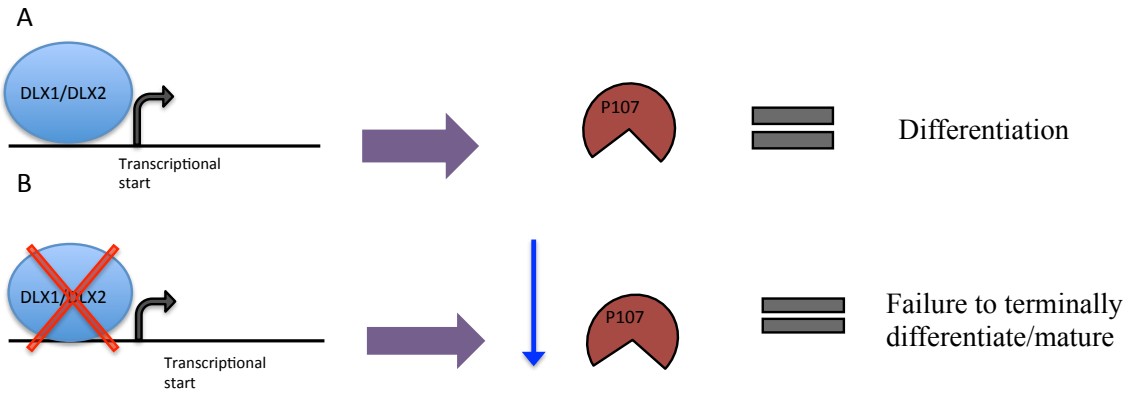

Figure 19: Proposed model for regulation of *p107* by DLX1/DLX2. DLX1/DLX2 bind to the *p107* promoter (A) and activate *p107* expression, which promotes neuronal differentiation during forebrain and retinal development. In the absence of DLX1/DLX2 (B) *p107* expression is reduced, resulting in the failure of neurons to terminally differentiate in the GE of the developing forebrain and the developing retina.

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