EFFECT OF TOP2B INHIBITION ON CADHERIN EXPRESSION IN DEVELOPING CHICKEN RETINA

By

RAMESHWARI RAYAJI

A thesis submitted to the

The Graduate School-New Brunswick

And

The Graduate School of Biomedical Sciences

Rutgers, the State University of New Jersey,

In Partial fulfillment of the requirements

For the degree of

Master of Sciences

Graduate program in Biomedical Engineering

Written under the direction of

Li Cai

And approved by

New Brunswick, New Jersey

October, 2016

ABSTRACT OF THE THESIS

EFFECT OF TOP2B INHIBITION ON CADHERIN EXPRESSION IN DEVELOPING CHICKEN RETINA

By

RAMESHWARI RAYAJI

Thesis Director:

Li Cai, Ph.D.

Retinal development involves defined guidance of neuronal axons to their destined cells through cell adhesion molecules, by compilation of cell-cell connections at synapses. Cell adhesion molecules promote oriented axonal outgrowth and help in target synaptic specificity to maintain histoarchitecture of retina. Cell adhesion molecules like cadherins are the products of expression of long genes >200 kilobases of genomic DNA. Topoisomerase II beta (Top2b) involved in modulating DNA supercoiling by catalyzing the double strand breaks and passing the stands through one another. Top2b is pervasively expressed in all terminally differentiated cells and participates in gene transcription. Recent studies have shown that Top2b facilitates the expression of long genes. However, its particular role in transcription of cadherins and effect on organization of retinal cells such as retinal ganglion cells and horizontal cells remain unclear. In current study, the role of Top2b in expression of cadherin genes was analyzed in

developing chick retina via immunohistochemistry and real-time PCR. Top2b function in developing retina was inhibited by injections of 500µM Top2b catalytic inhibitor (ICRF-193) into sub-retinal space at embryonic day 4 (E4). Retinal tissues were analyzed by immunohistochemistry using cell specific markers, e.g., Brn3a for ganglion cells, 4F2 for horizontal cells, and Tuj1 for neuronal processes at E6, E8, E10, and E12. We showed that inhibition of Top2b by ICRF-193 i) reduced the expression level of cadherin genes, e.g., N-cadherin (Cdh2), Cadherin-6B (Cad6B), Cadherin-7 (Cdh7), and Cadhrin-8 (Cdh8)in developing retina; ii)led to a disoriented cellular organization; and iii) delayed migration of RGCs and HCs. The results from immunohistochemistry were confirmed by quantitative real-time PCR (qRT-PCR). Results from qRT-PCR showed significant reduction in expression levels of cadherin genes, e.g., Cad6B and Cdh7 with an average fold reduction of 2.5 prominent at embryonic day 10 (E10) ($p < 0.02$, $p < 0.006$), respectively and Cdh8 with an average fold reduction of 4 prominent at embryonic day 8 (E8) ($p < 0.02$), however expression of Cdh2 was not effected by Top2b inhibition at E6-E12. This study helps to understand the molecular basis of vertebrate retinal development via calcium-dependent intercellular cell adhesion molecules and revealed the vital role of Top2b in expression of cadherin genes.

ACKNOWLEDGEMENTS

First, I would like to express my gratitude to my advisor, Dr. Li Cai for giving me the opportunity to be a part of the esteemed Cai Lab. I thank him for his constant guidance and support that helped me improve my calibre and work to my maximum potential. Without his patience and kindness, I could not have completed my thesis.

I would like to thank my committee members, Dr. Jay C. Sy, Dr. Rene Schloss and Dr. Kelvin Kwan for their guidance, support, and critical reviews on my thesis and their constant encouragement and advice.

I would also like to thank all the past and current members in the Cai lab during my time here. In particular, I want to thank Dr. Ying Li for helping me learning various new techniques and help me analyze my results. I would also like to thank Jeremy Anderson and Misaal Patel for useful scientific discussion and advices about this thesis.

Last but not the least; I would like to express my heartfelt gratitude to my family who had the faith in me and dared to send me abroad to pursue my dreams. I thank them for their constant love and support in me all through this time.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

CHAPTER 3

Chapter 4

LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
Cadherin-6B	Cad6B
Cadherin-7	Cdh7
Cadherin-8	Cdh8
CNS	Central nervous system
E	Embryonic day
EC	Extracellular cadherin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCL	Ganglion cell layer
GFP	Green fluorescent protein
HC	Horizontal cell
INBL	Inner neuroblastic layer
INL	Inner nuclear layer
IPL	Inner plexiform layer
N-Cadherin	Cdh2
NR	Neural retina
OLM	Outer limiting membrane

ONL	Outer nuclear layer
OPL	Outer plexiform layer
PBS	Phosphate buffered saline
PVR	Proliferative vitreoretinopathy
qRT-PCR	Quantitative real time-polymerase chain reaction
RGC	Retinal ganglion cell
RPE	Retinal pigmented epithelium
Top2b	Topoisomerase IIbeta
WHO	World health organization

Chapter I

1.1 Introduction

The number of people with blindness or visual impairment in the US is expected to cross 8 million by 2050, according to estimation based on the most recent census data by the National Eye Institute (part of NIH). Another 16.4 million Americans suffer refractive errors such as hyperopia (farsightedness) and myopia (nearsightedness). WHO estimates 19 million children below the age of 15 are visually impaired and need rehabilitation interventions for a psychological and personal development. Another common cause for visual impairment in current times is retinal detachment, which can occur at any age, usually caused by an eye injury, a family history of retinal problems or diabetes. However, 80% of all visual impairment can be treated and cured. A prime cause of visual impairment is age-related retinal degeneration that leads to huge social and economic impact.

The primary function of the eye is to capture light, transduce that data into neurological impulses, and process it into images in the brain (Coleman, 1969). Light first enters the eye along the pupil, whose size is controlled by quantity of light probing the eye. The size of the pupil is controlled by papillary sphincter muscles of iris. If it is very bright, the iris contracts and in the dark, the iris expands so. The light reaches the lens which is attached to muscles that help in changing its shape (Schmidt R 2011). This enables the eye to adapt to things that are closer up and further away. The lens focuses light onto the retina. The area between the retina and lens is filled with vitreous humor a transparent gel-like substance. Retina consists of sensory cells, which translates the light

into nerve impulses that are carried to the brain along the optic nerve, where they are processed to form the images we perceive (Menche N. 2012).

1.2 Vertebrate Retina

The retina is the neurosensory surface lining at the back of the eye. It plays a role in forming the visuals and helping us see things around. As the most accessible part of the vertebrate CNS, the neural retina has become a prime model to study key aspects of neurogenesis. The mechanisms underlying retinal architecture development and cell differentiation processes are highly conserved across vertebrates (Centanin and Wittbrodt 2014). A complex arrangement of diverse cell types gives the retina its unique functionality. Though, 50 plus types of retinal neuronal cells were discovered (Masland 2001), retina is primarily composed of six main types of neuronal cells and one type of glial cell (**Fig. 1.1**).

The vertebrate retina consists of seven main classes of cell groups (Masland and Raviola 2000). Retina comprises of three prominent cellular layers. The RGC layer consisting of all GCs and displaced amacrine cells. Followed by BCs, HCs, and amacrine cells, which make up the INL. While photoreceptors (rod and cone) cell bodies make up the ONL of retina. At the time of retinal development, entire population of mitotic progenitor cells is restricted to ONBL, while newborn neurons are contained in the INBL (Young 1985b, 1985a). The photoreceptor outer segments interdigitate with the apical microvilli of the RPE. The RPE is a monolayer of polarized cells that supports the retina. It absorbs light by virtue of its pigment, facilitates the exchange of nutrients and waste between the retina and blood vessels and participates in the regeneration of Vitamin A

moieties involved in visual phototransduction (Strauss 2005). The RPE between the retina and the choroid acts as a blood-brain barrier. Nutrients are supplied to the retina by the vascular choroid. The segmental distribution of the choroid is complementary to but distinct from that of the central retinal artery, necessitating both arterial sources for adequate oxygenation of the retina. When excited by light, photoreceptors transmit signals through short extending neurons of BCs and local circuit neurons consisting of HCs and amacrine cells to long projection neurons of ganglion cells located in the retinal GCL (Kaneko, 1979). From the GCL, these impulses carried through the optic nerve to the brain. Direct signaling pathway from photoreceptors to ganglion cells can be modulated by HCs and amacrine cells lateral connections in INL (Masland 1986). GCL consists significant proportion of amacrine cells specified to as displaced amacrine cells (Jeon, Strettoi, and Masland 1998). INL consists of müller glial cells and their processes span all three retinal cell layers, providing protection to the neurons (Dyer and Cepko 2001).

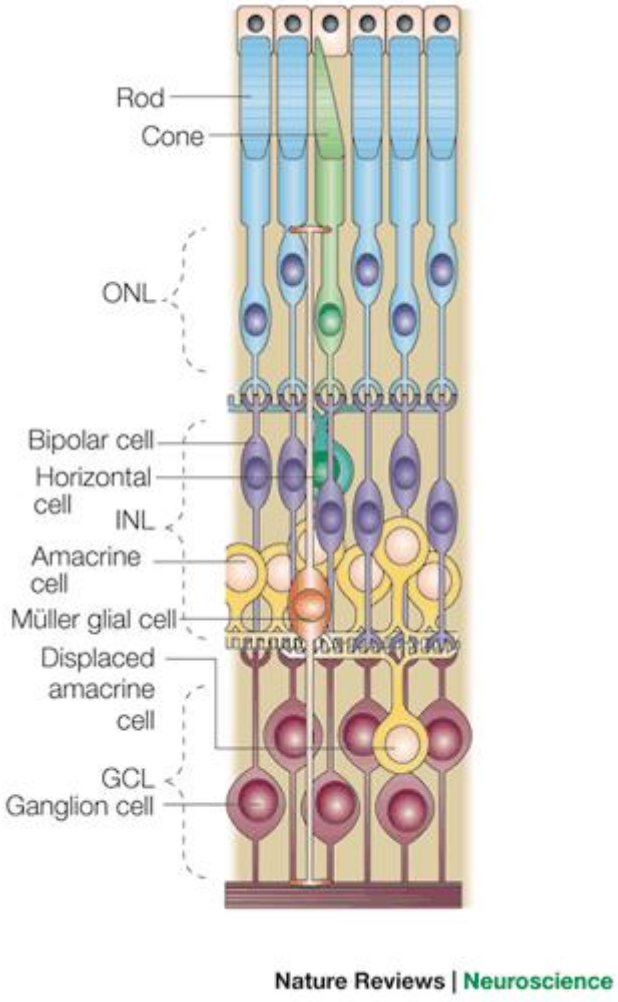

Figure 1.1.The laminar organization of vertebrate retina.

The vertebrate retina comprises of six main neuron types and one main type of glial cells.

Reproduced with permission from Connie Cepko: (Dyer and Cepko 2001)

1.3 Chick retina as a vertebrate model

The accessibility for visualization, ability of rapid development and experimental manipulation, planar structure and comparative large size during early developmental stages makes chicken the finest vertebrate model to study retinal development. It is also established as a crucial research model having a role significantly similar to the human embryo at cell and molecular anatomical levels (Vergara and Canto-Soler 2012).

Chief concepts of developmental biology like competence, induction, contact inhibition, and plasticity are due to research established on the chick model (Abercrombie M 1967). Chick embryo development imagining can be carried out through a small window, by breaking a piece of eggshell and cell marking techniques can be used as a tool to track cell migration and cell fates in real time (Kulesa PM 2011). Other advantages include array of established experimental methods, such as auto- and allografting, tissue excision, embryonic injections, rotation, *ex ovo* culturing of whole embryos, cell and explant culture systems, among many others (Darnell, Stark, and Schoenwolf 1999; Belecky-Adams et al. 2008). This model offers the possibilities of performing various manipulations at different embryonic stages and allowing the embryo to develop normally by sealing the window, followed by re-incubation of the experimentally manipulated egg. Re-incubation allows the retina to regenerate during development (Coulombre JL 1965). This adds a substantial advantage to study how the nervous system regenerates and compare it to early time points.

1.4 Cadherins

The capability of cells to stick to one another is a basic property in the evolution of multi-cellularity (Maitre and Heisenberg 2013). Cadherins are proteins constituting to

a large transmembrane glycoprotein family, assisting multi-cellularity. Cadherins are described based on their ability to carry out calcium-dependent, intercellular adhesions in tissues. Cadherins are characterized by extracellular cadherin (EC) domain repeats (Paulson et al. 2014). The EC domain part of each cadherin is similar to features with other cadherin family members (Ruan, Wedlich, and Koehler 2006) and mediate homophilic binding between same types of cadherins (Shibuya et al. 1995; Angst, Marcozzi, and Magee 2001). Cadherin proteins include the classical cadherin, protocadherin, desmosomes and cadherin-related protein families, among other members. Cadherin are conserved across species and play essential role in tissue morphogenesis and homeostasis (Arboleda-Estudillo et al. 2010), most are expressed in the CNS where they are involved in neurogenesis to cell migration and synapse formation (Basu, Taylor, and Williams 2015). Studies of overlapping genomic clones obtained from a genomic library have showed that cadherins are result of expression of long genes consisting of exons that disperse over greater than 200 kilobases of genomic DNA (Miyatani et al. 1992). During morphogenesis, they control cell–cell adhesion and cell signaling processes of developing tissues that help the tissues to change their shape and size, to form various cell layers. For instance, cadherins function in determining cell position and shape of drosophila melanogaster retina within the ommatidium (Hayashi and Carthew 2004). Cell fate specification is also mediated by cadherins (Lorthongpanich et al. 2012), cell polarity and cell proliferation (Schlegelmilch 2011). These characteristics make cadherins the molecular markers for establishment and maintainance of visual system architecture.

1.5 Role of cadherins in retinogenesis

Previously it has been proved that cadherins are expressed in neural circuits by particular brain nuclei in developing chick and mouse CNS (Ganzler and Redies 1995; Redies 1995). Tissue physiology and morphology are profoundly influenced by the distinctive organization of cells. Neuronal connectivity to target cells and synapse formation at cell-cell connection involves the precise guidance of axons (Fannon and Colman 1996)(Schuman and Murase 2003). Axon guidance and synaptic specificity is ingrained during embryonic development and maintained in the post-embryonic life. The challenge is to identify important aspects of tissue organization and mechanisms involved in morphogenesis (Niessen, Leckband, and Yap 2011). One such key determinant is cadherins whose function is required in establishment and maintenances of histoarchitecture. Cadherins are involved in regulation of axon-target interaction, formation of synapse and plasticity by pre and postsynaptic differentiation.

The development of a vertebrate eye involves localized changes in cell fate and cell shape achieved by conjunction with distinct changes in the patterns of cadherin gene expression (Lagunowich and Grunwald 1989; Ferreira-Cornwell et al. 2000). Studies have shown that changes in cadherin expression correspond to changes in morphogenesis events such as corneal endothelium differentiation, lens induction, lamination of retinal cell layers and RPE formation. Thus, cell fate determination and tissue specification during retinogenesis occurs synchronously with changes in cadherin expression (Xu, Overbeek, and Reneker 2002). Generally, each cadherin is distinctively expressed in a spatiotemporal pattern. In present study cadherins expression (Cdh2, Cad6B, Cdh7 and

Cdh8), was determined in the retinal development, in particular types of neurons and in a layer specific manner.

N-Cadherin

N-cadherin is also called as cadherin-2 in chicken (Cdh2) identified by (Hatta, Hosoda, and Takeichi 1986; Hatta and Takeichi 1986). N-cadherin functions in retinal axon outgrowth (Matsunaga, Hatta, and Takeichi 1988), laminar-specific connectivity in chick optic tectum and path finding of tecto-fugal axons (Treubert-Zimmermann, Heyers, and Redies 2002). The onset of N-cadherin expression differs between embryonic stage E2.5 and E9. N-cadherin expression is seen in undifferentiated retina of early embryos and is almost evenly distributed. At around E10 embryonic stage, N-cadherin was abundantly expressed in the plexiform layers, optic nerve fiber layer and the OLM. After that N-cadherin expression gradually reduced from most parts of the retina apart from photoreceptor cells of ONL and by horizontal cells of the INL. Expression of N-cadherin is maintained at least until E18. N-cadherin is uniformly expressed in early neural retina, as compared to other cadherins (Matsunaga et al. 1988; Matsunaga, Hatta, and Takeichi 1988) and is responsible for synaptic maturation and target recognition.

Cadherin-6B

Cadherin-6 is called cadherin-6B (Cad6B) in chicken(Wohrn et al. 1998; Wohrn et al. 1999). Studies show that progenitors with strong expression of Cad6B developed into RGCs which respond predominantly to single direction-selective vertical motion, during retinogenesis. Cad6Bpositive progenitors are also capable of develop into all major

retinal cell types, and are known for subtypes of amacrine and BCs generation (Wohrn et al. 1998)

Cad6B expression starts at early stages (E2.5–E4), and stagnates at around E18. Strong expression of Cad6B was first observed around E5 in ganglion cell layer in central retina. Strong expression of Cad6B is also seen in the neural crest (Lin, Wang, and Redies 2014). Immunostaining results showed prominent expression on cell surface and axo-dendritic trunk. At E9-E10 cells moving through the IPL to the GCL express Cad6B these cells include multipodal amacrine cells in the INL and epitomize a transient bush like display at the outer border of the IPL. By E11, the IPL has two prominent Cad6B expressing narrow sublaminae. Dimly stained dendrites are seen, extending into IPL; correspond to outermost sublamina of the IPL. A subpopulation of BCs expressing Cad6B in outer part of the INL, have some parts, of their neurites followed from the IPL to the OPL. Individual Cad6B-expressing processes are followed from the OLM to BCs in the INL.

Cadherin-7

Faint Cadherin-7 (Cdh7) expression was first seen in the central part of retina at early stage of E2.5 in densely spaced radial processes, in the differentiating ganglion cell layer and their neuritis in developing nerve fiber layer (Prada et al. 1991). Cdh7 expression within these structures increases and spreads peripherally at E7 and is seen to diminish by E11. Numerous Cdh7 positive amacrine cells are found in the INL. Also a subset of BCs in the INL, expressed Cdh7. The processes of bipolar cells were immunoreactive and projected to the IPL and OPL. These BCs show a relatively vast band in the outer one third of the INL (Wohrn et al. 1998). Studies have shown double labeling of Cad6B and

Cdh7 resulted in partial overlap of positive cells. Examples include rarely some displaced GCS, BCs and amacrine cells. Widespread expression of Cdh7 was seen in inner plexiform layer, but some sublaminae displayed high expression levels compared to others. These sublaminae were found to be a subset positive for Cad6B and Cdh7.

Cadherin-8

Cadherin-8 (Cdh8) is expressed in limited regions of the developing chick eye and brain (Lin, Luo, and Redies 2008). Cdh8 is selectively expressed by bipolar cells. Studies have shown that Cdh8 plays instructive roles in targeting bipolar cells to appropriate sublaminae for visually-evoked responses of direction-selective ganglion cells (Duan et al. 2014). Cdh8 expression was also seen subsets of cells in the outer portion of the INL. Cdh8 expression starts at early embryonic stages of E4 and increases at E8 and becomes relatively prominent from E10 to E20 (Lin, Luo, and Redies 2008). Cdh8 expression regions remain the same during the pre and postnatal periods of development. Cdh8 is expressed throughout the period of synaptogenesis and targets to bipolar cells (Korematsu and Redies 1997). The Cdh8 is prominent in the axon and dendrite arbors of the cells in INL. Therefore, the Cdh8 localization explains the possibility of its function in synapse formation (Duan et al. 2014).

1.6 Top 2b is required for proper retinal development

Topoisomerase IIb (Top2b) is a widely dispersed nuclear protein, which carries out chromatin modifications amid postmitotic development. DNA Top2b is an enzyme that resolves topological problems of DNA, encountered during cellular activities in an ATP-

dependent manner (Wang 2002; Nitiss 2009b) by introducing transient duplex strand break. Top2b introduces a double strand break, passes an uninterrupted strand through this transient break, followed by resealing the break (Li et al. 2013). Top2b is expressed in terminally differentiating post-mitotic neurons of CNS (Lyu and Wang 2003a; Tsutsui et al. 1993; Tsutsui, Sano, et al. 2001). Studies suggest that Top2b is associated with expression of long neuronal genes in humans and mouse, all genes >200 kb. Top2b is required for complete expression of long genes and is involved in regulating several genes involved in CNS development (Lyu and Wang 2003a). Disruption of Top2b function is related to neural degeneration disorders like autism spectrum disorder (King et al. 2013).

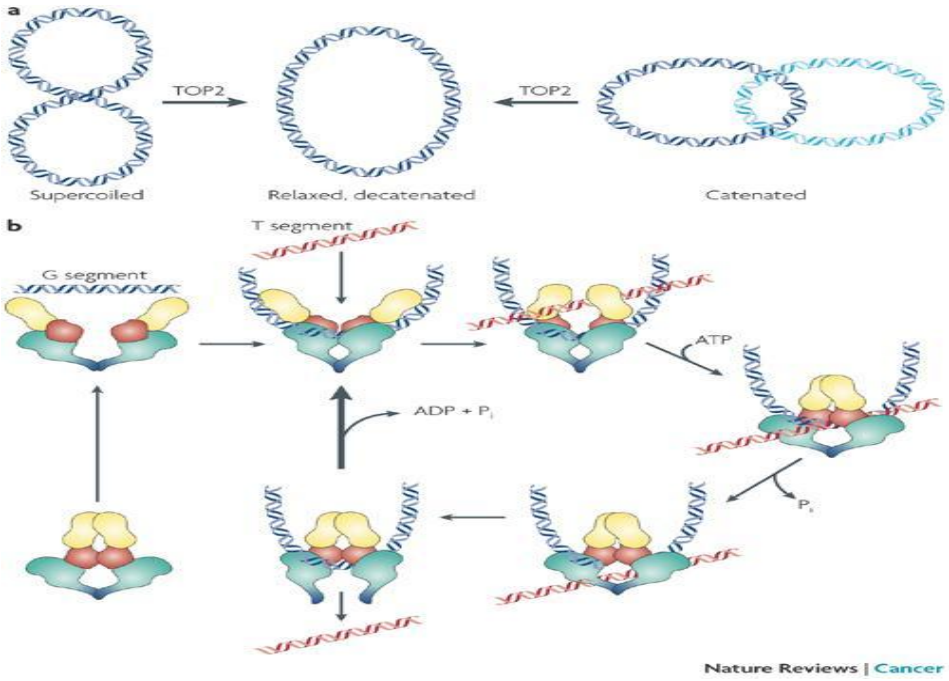

Figure 1.2. Function of Top2b

DNA Top2b is an enzyme that resolves topological problems encountered during cellular activities in an ATP-dependent manner.

Reproduced with permission from John Nitiss: (Nitiss 2009a)

Deletion of Top2b in mice leads to defective lamination of cerebral cortex, disrupted neurite out-growth and abnormal cerebral cortical neurons migration (Lyu and Wang 2003a; Yang et al. 2000; Nur et al. 2007). Top2b is required for laminar targeting of RGCs axons (Nevin et al. 2011a).

Top2b is expressed in all postmitotic cells. Top2b and DNA interactions are known to be involved in transcription of developmental genes such as cell adhesion molecules and axon guidance factors, but a specific role regulating these genes has not been previously described. Here in vivo chick retinal model was used for the study of neural development. This model was selected for the advantages of experimental manipulation, accessibility for visualization, ability of rapid development, comparative large sized eyes, planar structure during early developmental stages (Vergara and Canto-Soler 2012) and importantly easy reach for retinal site specific injections of Top2b inhibitor (ICRF-193).

The specific hypothesis to be tested in this study is the effect of Top2b inhibition on expression of cell adhesion molecules such as cadherins (Cdh2, Cad6B, Cdh7 andCdh8) which are required for proper retinal development.

1.7 Top2b inhibitor ICRF-193

ICRF-193 (meso-2, 3-bis(2,6-dioxopiperazin-4-yl) butane) inhibits Top2b by inducing a distinct type of Top2b clamps, which prevents the double-strand breaks. ICRF-193 act as a catalytic inhibitor, that inhibits Top2b by trapping it in a closed protein clamp called Topo II clamp (Adachi et al. 2003). Top2b is an ATP dependent enzyme which hydrolysis ATP to coordinate the passage of the DNA double strands, to repair

supercoiling (Jan G. Hengstler 2002). Hence, interrupting with ATPase enzyme function using low molecular weight inhibitors should affect cells that are committed to undergo mitosis (Chen et al. 2015).

Presence of the catalytic inhibitor ICRF-193 damages DNA by securing the Top2b-DNA complex and induces deterioration of the enzyme. ICRF-193 inhibits Top2b but, does not interfere with the DNA rejoining process, it rather binds to the closed clamp Top2b-ATP complex to contain the DNA strands topologically between the two subunits of the enzyme (Roca et al. 1994). Since ICRF-193 treated cells generated Top2b attached DNA strand breaks, which is toxic to cells, this reduces levels of Top2b. This reduced level of Top2b can be a form of cellular defense mechanism (Isik et al. 2003).

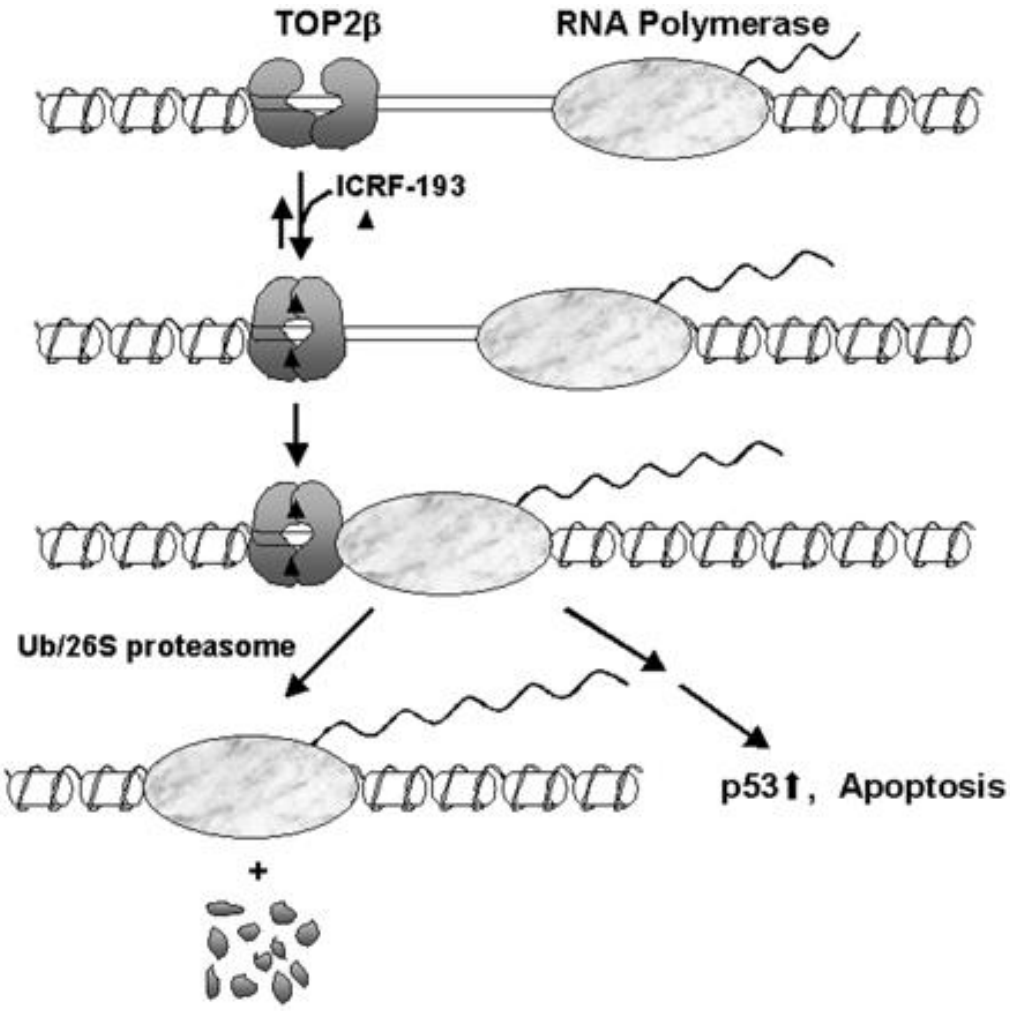

Figure 1.3. Effect of ICRF-193 on Top2b.

ICRF-193 traps Top2b in closed circular clamp. The clamp blocks the movement of the transcription-elongation complex, and triggers degradation of Top2b.

Reproduced with permission from PNAS, Copyright (2003) National Academy of Sciences, U.S.A.:(Xiao et al. 2003)

CHAPTER 2

2.1 RATIONALE

DNA topoisomeras is a nuclear enzyme inducing transient double strand break in the DNA allowing one strand to pass through the other. Top2b is known to catalyze unwinding of DNA tensioned supercoils in an ATP-dependent manner (Wang 2002; Nitiss 2009b). It is ubiquitously expressed in all post mitotic cells and is involved in releasing DNA, DNA condensation and DNA repair (Meyer-Ficca et al. 2011). Studies suggest that Top2b is involved in expression of long genes, >200 kb involved in brain and CNS development (Lyu and Wang 2003a). Disruption of Top2b causes neural degeneration disorders such as autism spectrum disorder (King et al. 2013).

Cadherins are described based on their ability to bring about calcium-dependent intercellular adhesion in tissues and play an important role CNS and retinal development, where they function in neurogenesis, cell migration to formation of synapse (Basu, Taylor, and Williams 2015) (Arboleda-Estudillo et al. 2010; Paulson et al. 2014). Their expression is are conserved across species. Studies of clones obtained from genomic library have showed that cadherins are result of expression of long genes consisting of exons that disperse over greater than 200 kilobases of genomic DNA (Miyatani et al. 1992).

Hence it can be speculated that Top2b is involved in regulation of different cadherins (Cdh2, Cad6B, Cdh7 and Cdh8) which are involved in axon path finding, cell specific adhesion, synapse formation, axon growth and laminal organization of retinal cell layers (Duan et al. 2014; Matsunaga, Hatta, and Takeichi 1988; Redies 1995; Wohrn

et al. 1998). An excellent way to understand of how cadherin expression is required for the above mentioned functions can be studied on the bases of their expression with the help of Top2b. Previously a genome-wide transcriptome RNA sequencing of retinal specific Top2b knockout mouse analysis revealed Top2b gene is involved in neuronal system survival and development during retinogenesis (Li et al. 2014). Among these genes cadherin-8 (Cdh8), cadherin-7 (Cdh7), were significantly down regulated by an approximate fold reduction of 7, 2 respectively ($p<0.05$) and cadherin-6 (Cad6B), N-cadherin (Cdh2) expression was not significantly affected in Top2b knockout mouse ($p< 0.05$). These down-regulated genes are speculated to cause delayed neuronal differentiation, migration, synaptic specificity, outer segment of photoreceptors, degeneration of the plexiform layers and reduced retinal cell number. Although Top2b deficiency down regulated the expression of cadherins (Cdh2, Cad6B, Cdh7 andCdh8) as per the RNA-Sequencing data, the transcriptional regulation mechanism for this remains unclear.

The hypothesis to be tested in this study is to test the role of Top2b in expression and regulation of cadherins during retinogenesis. To test this hypothesis,Top2b was inhibited in developing chick retina by injections of 500µM Top2b catalytic inhibitor (ICRF-193) into sub-retinal space at embryonic day 4 (E4). Subsequent retinal tissue from embryonic day 6 to 12 (E6-12) was examined for development, differentiation of different retinal cells. Immunohistochemistry assay was used to qualitatively study the proliferation, migration, differentiation, axon path-finding and dendrite growth of the retinal progenitor cells. The result from immunohistochemistry was quantified by real-time PCR (qRT-PCR) and $2^{-\Delta\Delta CT}$ method was used to calculate relative gene expression.

The analysis revealed that Top2b inhibition leads to delayed differentiation RGCs, HCs, degenerates the ganglion cell, inner nuclear and plexiform layer. In addition, the expression of cadherin genes was down regulated affecting retinogenesis in general and migration and laminar specificity in particular.

CHAPTER 3

MATERIALS AND METHODS

3.1 *Chicken embryos*

Premium fertilized pathogen-free (SPF) white leghorn chicken (*Gallus domesticus*) eggs were obtained from Charles River (North Franklin, CT). The eggs were kept at room temperature for 2 hours before placing them in the 37.5 ^{o}C incubator. The incubator was set to a 37.5 ^{o}C and 60% humidity for 96-100 hours (~ 4 days) to procure chick embryos at the development stage of E1-4 or HH12-22. All of the animal experiments were approved by the Institutional Animal Care and Facilities Committee at Rutgers University.

3.2 *In ovo retinal injections and electroporation*

In ovo electroporation is a popularly used to study gene function in developmental biology. In current study targeted retinal injections were performed according to the protocol set by (Doh et al. 2010). Embryos of around day 4, E-4 (stages 20–23, Hamburger and Hamilton, 1951) were chosen for *in vivo* electroporation (Momose et al. 1999). The amnion and vitelline membrane were torn off carefully with fine sterile forceps to expose the embryo. For retinal injection, capillary glass pipettes (diameter 1.4 mm; Hilgenberg, Germany) were pulled with a microelectrode puller to get a 20 mm taper tip. The tip was broken under a dissecting microscope with the help of a tweezer to obtain a tip opening about 0.1 μm in diameter. The microneedle was loaded with a

mixture of 500µM inhibitor ICRF-193 (Santa Cruz Biotechnology) dissolved in HIBERNATE® media (Gibco, Thermo Fisher Scientific) and reporter pCAG-GFP plasmid solution with a concentration ranging 3-6 µg/µl and 0.2 µl of fast green (0.025%) to visualize the solution delivery. The microneedle was attached to a 0.1 ml Hamilton Gastight 1710 syringe (Reno, NV) mounted on a WPI M3301-M3 micromanipulator (Sarasota, FL) and a small piece of masterplex silicone tube was used to attach the needle to the syringe(Islam, Doh, and Cai 2012). The mixture was injected directly into embryonic sub retinal space (**Figure 2.1**) and electroporated with 5 square pulses of 15V for 50 ms with 950 ms intervals using a pulse generator ECM 830 (BTX Harvard Apparatus). GFP expression was used as a marker to locate Top2b inhibitor ICRF-193 affected retinal tissue.

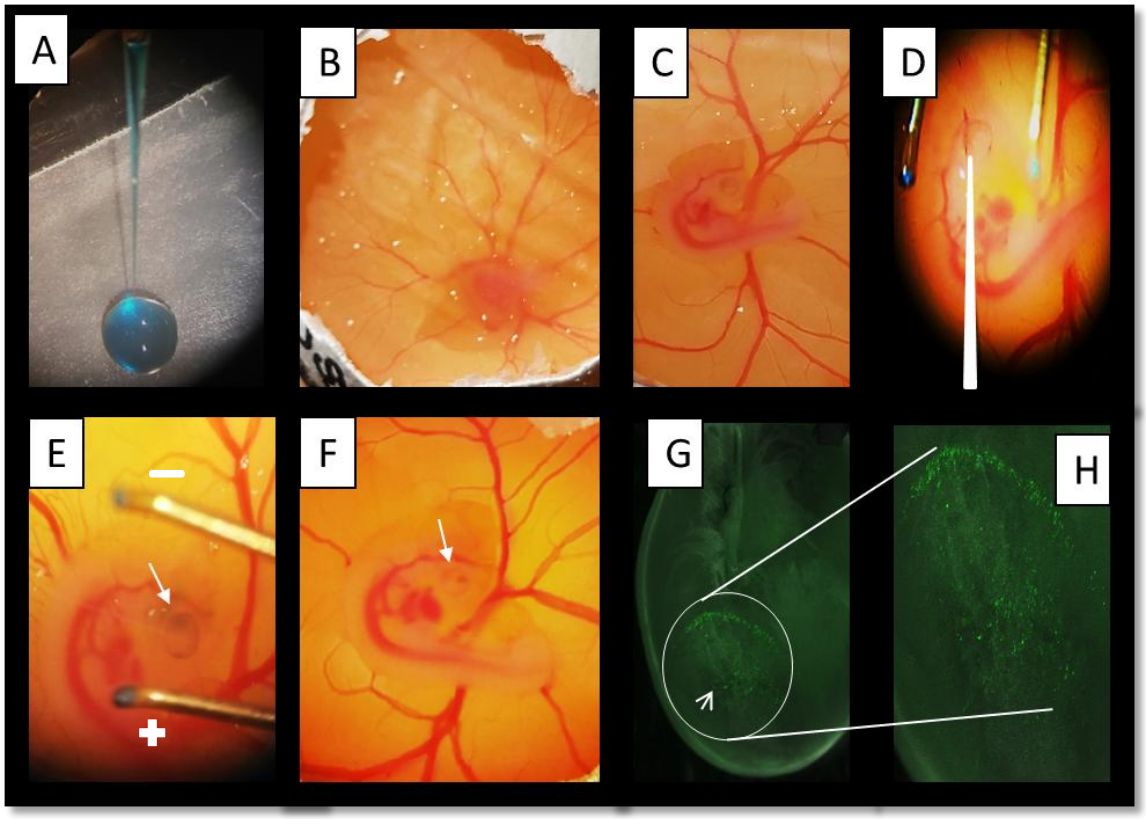

Figure 3.1. *In ovo*retinal injection and electroporation.

A:Loaded microneedle with a mixture of 500µM inhibitor ICRF-193 dissolved in Hibernate E and reporter pCAG-GFP plasmid solution with a concentration ranging 3-6 µg/µl and 0.2 µl of fast green (0.025%),

B: Embryo with vitelline and amnion membrane, **C:** Exposed E4 embryo **D:**Sub-retinal space injection of mixture

E: Electroporation with 5 square pulses of 15V for 50 ms with 950 ms intervals using a pulse generator.

F: E4 injected eye (arrow). **G:**Retinal GFP expression. **H:** Zoomed in image G.

3.3 *Tissue processing and sectioning*

Chick embryos after E4 retinal injections were harvested from the shell, euthanized by decapitation at various times periods E6, E8, E10, E12, E14 (stages 28, 32, 36, 38, and 40 respectively in HH system; at least three eyes for each stage). The eyes were harvested and transferred to a petri dish of cold 1X PBS (Phosphate buffered saline, Fischer Scientific). The RPE was carefully dissected using fine forceps, and the cornea, lens and vitreous were removed. The retinal tissue was then fixed in 4% PFA (paraformaldehyde in 1X PBS) overnight at 4°C, followed by 3 times cold PBS wash, for 10 minutes at 4°C and then infiltrated overnight in 30% sucrose (in 1X PBS). Embryos injected with GFP reporter plasmid constructs, (**Fig. 2.1, G**) was verified for successful transfection by inspecting the retinas under a fluorescent dissection microscope, Leica MZ16FA (Leica Microsystems, Germany) before embedding the retinal tissues in OCT (Electron Microscopy Sciences, Hatfield, PA). Tissues embedded in OCT were stored at -80°C until ready for sectioning. Retina tissue was sectioned using a cryostat to obtain sections of about 12μm (Thermo 0620E) and mounted on super-frost slides (Fisher Scientific), followed by air-drying. Immunohistochemistry using cell specific markers was performed immediately afterwards.

3.4 *Immunofluorescence*

Immunostaining was carried out using Shandon Slide Rack (Thermo Scientific, MA). The slides containing tissue sections were placed on warm plate for 15 minutes to get rid of the embedding OCT medium. Sections were then fixed with drops of 4% PFA,

followed by 1X PBS x3 wash for 10 min. Retina sections were incubated for 1 hour in blocking solution (0.05% Triton X-100, 3% BSA, 10% goat serum or donkey serum, in 1x PBS) at room temperature followed by application of primary antibody overnight. Primary antibodies and dilutions used were as follows: mouse anti-Brn3a (1:200, Millipore) mouse, mouse anti-Lim1+2 (1:20, 4F2 supernatant, DSHB), mouse Tuj-I (1:1000,Abcam), rat MNCD2 (1:100, DSHB), mouse Cad6B- CCD6B-c (1:200, DSHB), Cdh7 - CCD7-s (1:10, DSHB), Cdh8 - CAD-8-1 (1:200, DSHB), mouse Calretinin (1:2000, Chemicon) and mouse Parvalbumin (1:1000, Millipore).Control slides were not applied with any primary antibody. All slides were then washed with 1X PBS, followed by application of secondary antibody carrying fluorescence from the appropriate host (ms 549nm, 1:300 dilutions; Jackson Immuno Research, West Grove, PA) for an hour. The slides were washed with 1XPBS, air dried and placed with coverslip using cytoseal-60, for image analysis.

3.5 *Imaging*

Microscopic images were analyzed using an upright fluorescence microscope (Zeiss Axio Imager A1) with a monochrome digital camera Axiocam MRM (Zeiss, Germany). Images of immunofluorescence obtained from secondary antibody ms549and DAPI were taken separately using DsRed at549nm and DAPI at 461nm wavelengths filters, respectively. All images were taken maintaining the same exposure time, to make qualitative comparison. Images of DsRed and DAPI channels were then superimposed using Adobe Photoshop CS to generate pseudo colored double-labeled images.

3.6*Primer Design for qRT-PCR*

Designing primers to analyze the gene of interest is the most critical step in RT-PCR experiment. In this study we used the NCBI's Primer-BLAST software to design our gene specific primers. To design the primer FASTA sequence of desired species specific (*Gallus gallus*) mRNA (Cdh2, Cad6B, Cdh7 and Cdh8) was acquired from NCBI nucleotide database. Following this Primer-BLAST program was executed for species specific parameters with exon-exon junction span selection (**Table 1.1**). Primer pairs were selected based on PCR product size (200-300bp) and analyzed using IDT's oligo analyzer tool. Primers were examined for hairpin loop structures, high ΔG values and annealing temperature range Tm values for specificity and reliability.

Name	**PCR Product Size(bp)**	**Primer sequence**
N-Cad	426	Forward: AGCTGACCAACCTCCAACAG
		Reverse: TGTACTTTCTCTCTGTCGAGCC
Cad6B	269	Forward: CCCCCAGAGCACCTACCAAT
		Reverse: ATCGAGGGTCCACGTGAGTA
Cdh7	319	Forward: GACCCTTCAGCGACATGACA
		Reverse: TGTGCTGGATTCTGACTCTCC
Cdh8	289	Forward: TGGTGATTTGCGTAGCTTGTG
		Reverse: TCTGTATGTAACCGGCCAACT

Table 1.1 Design of chick specific primers for qRT-PCR

3.7*Quantitative RT-PCR*

Total RNA extraction of control and ICRF-193 injected GFP+ chick retinal tissues at E6, E8, E10 and E12 was carried out using Tri Reagent Solution (Ambion). cDNA template was obtained by reverse transcription using qScript cDNA SuperMix (Quanta Biosciences). qRT-PCR was performed on a Roche 480 Light Cycler using SYBR Green Fast Mix (Applied Biosystems) according to the manufacturer's instructions using primers specifically designed for Cdh2, Cad6B, Cdh7 and Cdh8 (**Table 1.1**), to generate a 45-cycle PCR products. For all qRT-PCR, data was analyzed using relative threshold cycles values (ΔCt). Gene expression was calculated by normalizing the threshold cycles based on the expression of reference gene GAPDH. Each data points contained at least three samples with three replicates.

QRT-PCR Efficiency Test

In this study qRT-PCR, data was analyzed using relative quantification. In relative quantification, the PCR signal of the target transcript in ICRF-193 treated group is related to that of control (untreated) sample. The $2^{-\Delta\Delta Ct}$ method is a convenient to evaluate the relative quantification. Relative quantification by $2^{-\Delta\Delta Ct}$ method follows certain assumptions, such as the amplification efficiencies of the target template (Cdh2, Cad6B, Cdh7 and Cdh8) and reference (GAPDH) must be approximately comparable. A sensitive method for assessing if two amplicons have the similar efficiency is to look at how ΔCT varies with template dilution over a 100-fold range. A plot of the log cDNA dilution vs ΔCT was established (**Fig.3.2**). If the slope value is near to zero, confirms similar efficiencies of the target and reference genes, and validated ΔΔCT method of

calculation for the relative quantification (Livak and Schmittgen 2001). The slope obtained was 0.04, hence the assumption holds and this method can be used to analyze qPCR data. This also proves the specificity of designed primers to the target genes.

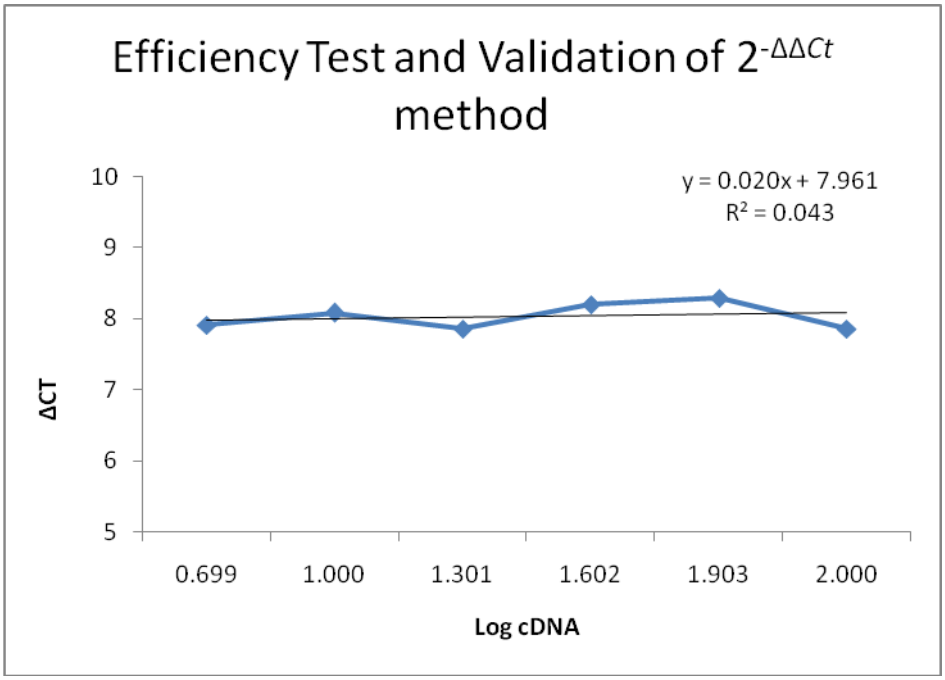

Figure 3.2 Efficiency test and validation of $2^{-\Delta\Delta Ct}$ method.

Representation of 100-fold dilution range of target template (Cdh7, Cdh8) in terms of log cDNA vs. ΔCT values. Slope 0.04 illustrates the efficiencies of the target and reference gene (GAPDH)to be similar, proving specificity of designed primers to the target genes and validation of ΔΔCT calculation for the relative quantification.(Livak and Schmittgen 2001).

CHAPTER 4

RESULTS

Top2b inhibition by ICRF-193 causes derangement of retinal ganglion cells, horizontal cell and processes

Retinal ganglion cells (RGC), horizontal cells (HC) and cell processes wereexamined using cell-specific markers such as Brn3a, 4F2 and Tuj1, respectively. Inhibition of Top2b in chick retina led to a disoriented organization, migration of retinal ganglion cells and horizontal cells, process formation and effected their survival. As it is easy to access chick retina at early embryonic stages around day 4 as compared mouse embryos, in this study *in ovo* embryonic retinal injection of 500 µM ICRF-193 (a catalytic inhibitor of Top2b) was used to observe its effect on gene expression of RGCs, HCs and cell processes. To define the cell specific expression pattern of RGCs and HCs in the chick retina, control retinal tissue were compared to 500 µM ICRF-193 treated retinal tissue via immunohistochemistry at E6, E8, E10 and E12. Retinal sections were stained with cell specific antibodies, such as Brn3a for retinal ganglion cells (Nadal-Nicolas et al. 2009; Badea et al. 2009; Huang et al. 2001; Liu et al. 2000), 4F2 for horizontal cells (Suga, Taira, and Nakagawa 2009; Boije, Edqvist, and Hallbook 2008; Margeta 2008; Poche et al. 2007; Edqvist, Myers, and Hallbook 2006) and Tuj1 for dendrites of RGCs (Lee et al. 2005; Robinson and Madison 2004; Chintalapudi et al. 2016) obtained at various time points of retinal development from E6 to E12.

The processes of cells in the INL such as bipolar, amacrine, horizontal and ganglion cells make up the IPL. Inhibition of Top2b affected the differentiation and final maturation of these cell types, although it did not affect INL and GCs specification. In

Top2b inhibited retinas, the expression of Brn3a in ganglion cells, Tuj1 for RGC processes and 4F2 in horizontal was detected (**Fig. 4.1, 4.2, 4.3, Control**), suggesting that laminar localization of these retinal cells was unaffected by Top2b inhibition. However, Brn3a+, Tuj1+ and 4F2+ cells were widely distributed in both the INL and ONL **(Fig. 4.1, 4.2, 4.3, Treated)** at E6 and E10 embryonic stages and seemed to be still enduring the migration in the INBL before reaching their final location in GCL, HC layer respectively (**Fig. 4.1C,D 4.3C,D, Treated**). These results suggest that terminal maturation and differentiation retinal cells requires Top2b.

In addition, reduced expression of Tuj1 in RGC processes of Top2b inhibited retina sections was seen compared to control sections **(Fig. 4.2A-D).** This dramatic reduced Tuj1 expression suggests that Top2b inhibition affected the emanation of process formation of the RGCs leading to slower migration of RGCs from IPL to retinal ganglion cell layer. Reduced number of Brn3a+ and 4F2+ cells, was observed along with cell cluster formation in Top2b inhibited retinas at E6 and E8 embryonic stages (**Fig. 4.1A,B, 4.3A,B, Treated**). Also the Top2b inhibited retinal sections were found to be more pervious compared to control. These results suggest that Top2b inhibition affects migration, terminal differentiation and survival of retinal cells during embryonic retinogenesis.

4.1: Top2b inhibition by ICRF-193 causes derangement of retinal ganglion cells

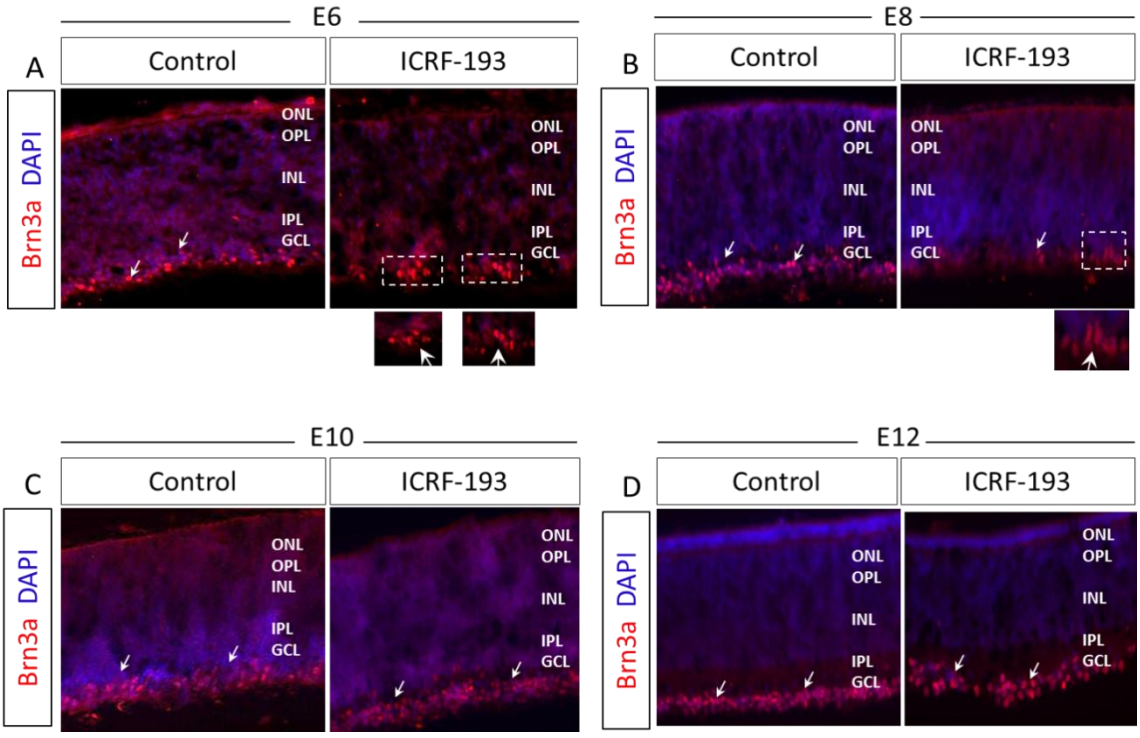

Figure 4.1: Top2b inhibition affects the expression of retinal specific marker Brn3a during chick retinal development. Sections of the control and ICRF-193 (500µM) treated retina at E6 (A), E8 (B), E10 (C) and E12 (D) were processed to reveal retinal ganglion cells using anti-Brn3a antibody staining and co-stained with DAPI. At E6 and E8, Brn3a+ cells were all located in the GCL in the control retinas, but were more widely dispersed and formed grouped cell masses in the INBL and ONBL (arrows, dashed box) in the ICRF-193 treated sections (Top2b inhibited) retinas (A, B). At E10 and E12,no significant differences in Brn3a+ cells (arrows) were observed, suggesting a reversible effect of ICRF-193 on later developmental stages (C, D). Scale bars = 50 µm.

4.2 Top2b inhibition by ICRF-193 affects processes of RGCs

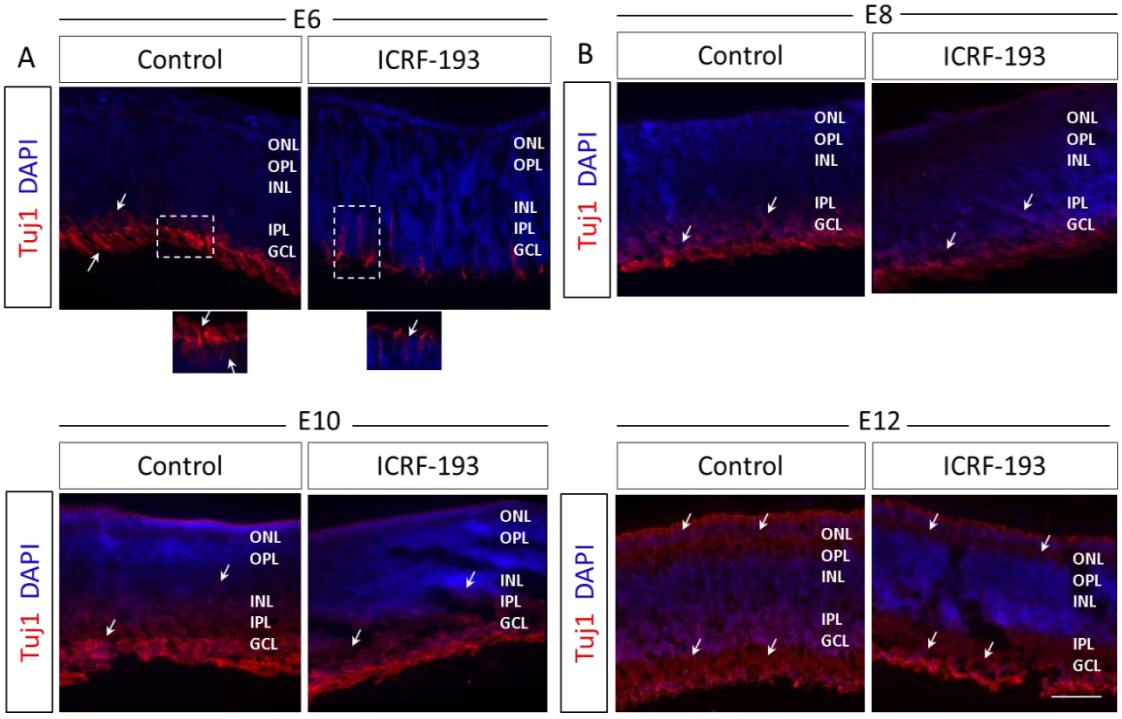

Figure 4.2: Top2b inhibition affects the expression of retinal specific marker Tuj1 during chick retinal development. Sections of the control and ICRF-193 (500µM) treated retina at E6 (A), E8 (B), E10 (C) and E12 (D) were processed to reveal neurofilaments of the IPL (arrows) and processes of RGCs (arrows, dashed box) using Tuj1antibody staining and co-stained with DAPI. At E6 and E8, Tuj1+ labeled processes of RGCs cells in the control retinas, but its expression was wide spread and reduced (arrows) in the ICRF-193 treated sections (Top2b inhibited) retinas (A, B). At E10 showed increased expression of Tuj1 in GCL and IPL (C) and E12,no significant differences in Tuj1+ expression were observed (D, arrows), suggesting a reversible effect of ICRF-193 on later developmental stages. Scale bars = 50 µm.

4.3: Top2b inhibition by ICRF-193 causesderangement of horizontal cells

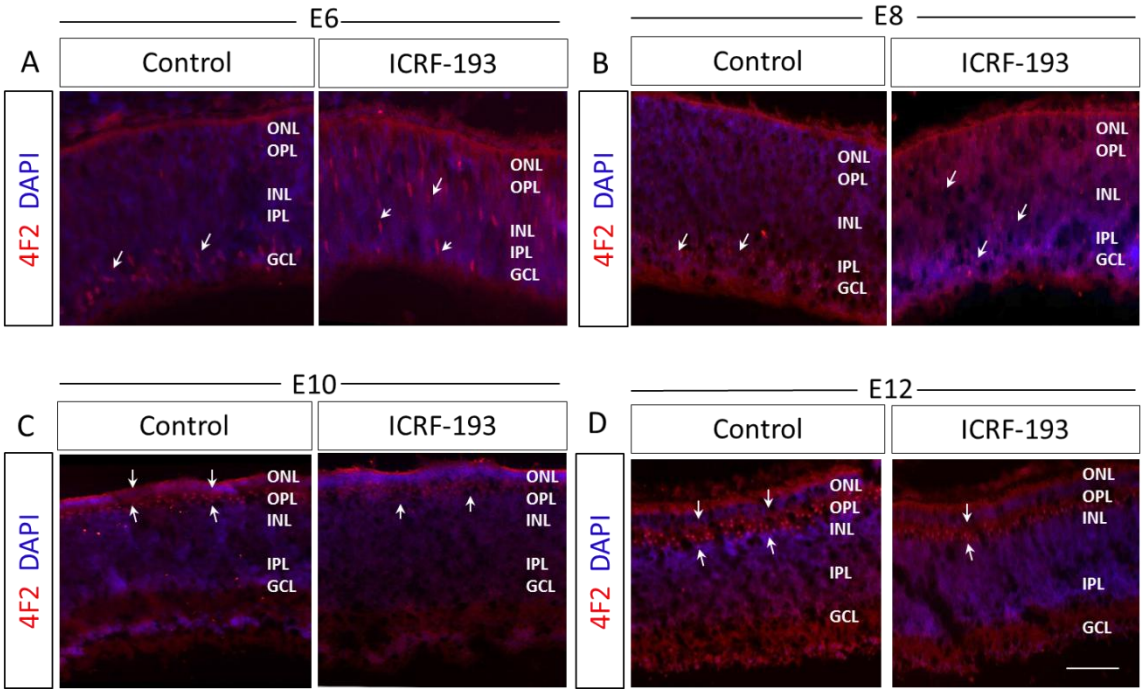

Figure 4.3: Top2b inhibition affects the expression of retinal specific marker 4F2 during chick retinal development. Sections of the control and ICRF-193 (500µM) treated retina at E6 (A), E8 (B), E10 (C) and E12 (D) were processed to reveal horizontal cells using 4F2antibody staining and co-stained with DAPI. At E6 and E8, 4F2+ cells were all located near the GCL in the control retinas, but they were more widely distributed in the INL and ONL (arrows) in the ICRF-193 treated sections (Top2b inhibited) retinas (A, B). At E10 and E12,no significant differences in 4F2+ cells were observed (arrows), suggesting a reversible effect of ICRF-193 on later developmental stages (C, D).

Scale bars = 50 µm.

Neurofilaments, dendrites, intracellular calcium-binding protein of RGC, HC and amacrine cells expression were labeled using cell-specific differentiation markers such as calretinin and parvalbumin. Chick retinas of embryonic day 4 treated with 500µM ICRF-193 and analyzed at E6-E12 stages. ICRF-193 treated sections showed dramatically reduced the number of cells expressing calretinin and parvalbumin at E6, E8, E10 stages and these cells were sparsely distributed as compared to control retinas.

4.4 Top2b inhibition by ICRF-193 affects expression of Calretinin

Calretinin stains the neurite plexuses of of RGCs, BCs and amacrine cells in the IPL. In the controls retina sections, Calretinin staining in the IPL was prominent from E6 (**Fig. 4.4A Control,arrow**). Also the stains revealed matured IPL contained three strata separated from each other by two plexuses at E10 and E12 (**Fig. 4.4C, 4.4D, Control**). However, Top2b inhibited retinas, when stained with Calretinin did not have these strata separation which may be due to disoriented and short processes and no plexus formation was seen until E10 stage (**Fig. 4.4A, B, C, ICRF-193**, **arrows**). These defects seemed to rectify by E12 referring to the reversible effect of ICRF-193 on later developmental stages (**Fig. 4.4D, Control, Treated**). These results suggest that neurite outgrowth and appropriated development of retinal plexiform layers requires Top2b function.

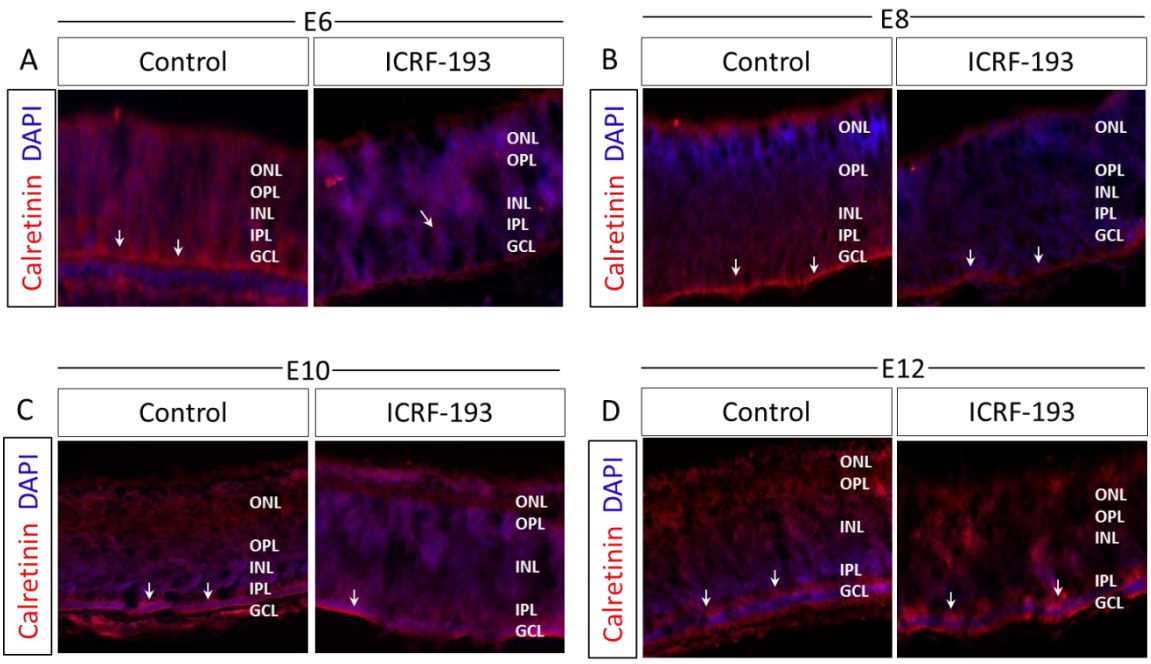

Figure 4.4: Top2b inhibition affects expression of retinal specific marker Calretinin during chick retinal development. Sections of the control and ICRF-193 (500µM) treated retina at E6 (A), E8 (B), E10 (C) and E12 (D) were processed to reveal neurofilaments using Calretinin antibody staining and co-stained with DAPI. At E6, E8 and E10 Calretinin labeled neurofilaments in the IPL, but its expression was dramatically reduced (arrows) in ICRF-193 treated (Top2b inhibited) retinas, (A, B, C). At E12 control and ICRF-193 treated showed no significant differences in Calretinin expression in IPL (arrows), suggesting to the reversible effect of ICRF-193 on later developmental stages (D, arrows). Scale bars = 50 µm.

4.5 Top2b inhibition by ICRF-193 affects expression of Parvalbumin

Parvalbumin labels the intracellular calcium binding proteins and appears as a faint band in the RGCs and at the INL-IPL boundary at early embryonic stages (**Fig. 4.5A, Control**). Parvalbumin expression was displayed by subpopulations of amacrine cells, long-distance projecting neurons like RGCs, involved in complex receptive fields. However, in Top2b inhibited retinas the expression of Parvalbumin was dramatically reduced, where the number of cells expressing parvalbumin were sparsely distributed (**Fig. 4.5A-C, ICRF-193, arrows**), suggesting that Top2b is required to achieve the intracellular calcium binding adhesion between retinal cells. It is also established that Parvalbumin expression peaks only at late embryonic stages, suggesting that its expression is required in establishment of synaptic contacts and organization of neuronal activity.

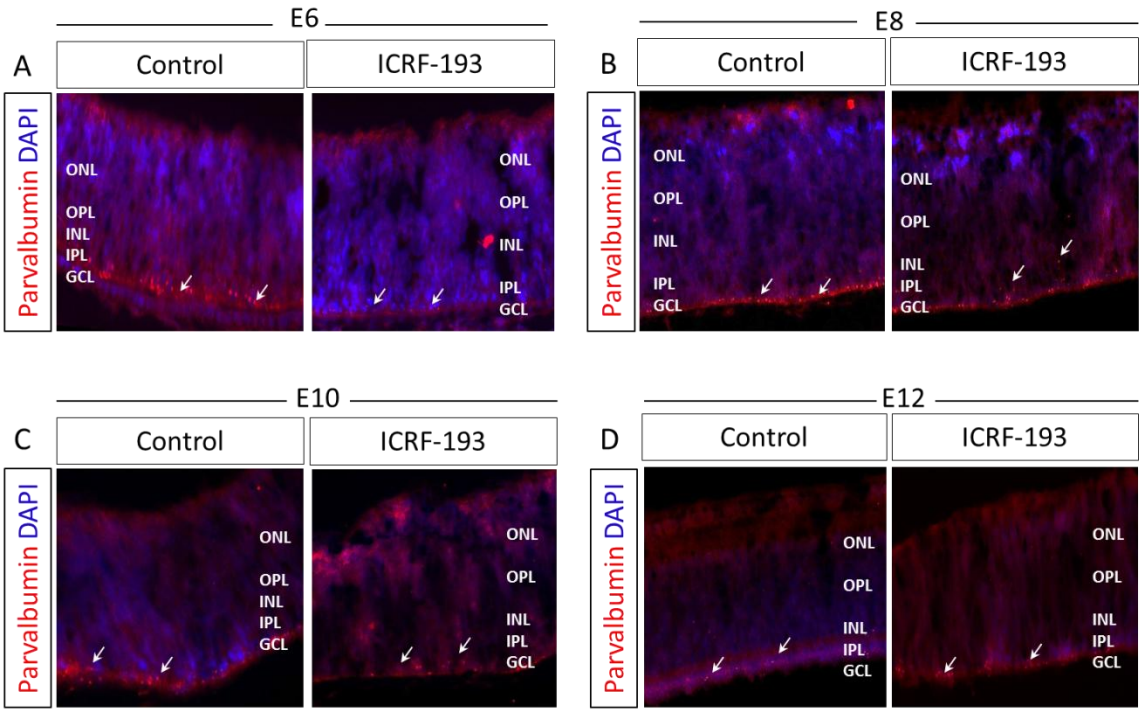

Figure 4.5: Top2b inhibition affects expression of retinal specific marker Parvalbumin during chick retinal development. Sections of the control and ICRF-193 (500µM) treated retina at E6 (A), E8 (B), E10 (C) and E12 (D) were processed to reveal intracellular calcium-binding protein using parvalbumin staining and co-stained with DAPI. At E6 and E8 Parvalbum was seen as faint bandin the RGCs and at the INL-IPL boundary, but in ICRF-193 treated (Top2b inhibited) retina the expression was sparsely distributed (A, B, C, arrows. At E12control and ICRF-193 treated showed no significant differences in Parvalbumin expression in RGC and IPL (arrows), suggesting to the reversible effect of ICRF-193 on later developmental stages (D). Scale bars = 50 µm.

4.6 Top2b inhibition by ICRF-193 affects expression of Cdh8

Cdh8 was labeled using cell-specific differentiation markers CAD-8-1. Cdh8 expression was seen in selectively expressed subsets of BCs in the outer portion of the INL. Expression of Cdh8 is prominent in axons and dendrite arbors of the bipolar cells.

Chick retinas of embryonic day 4 treated with 500μM ICRF-193 was analyzed at E6-E12 stages. Top2b inhibited retina sections revealed dramatically reduced Cdh8 expression compared to the control retina. By E8 IPL displays prominent expression of Cdh8 (**Fig. 4.6B-D Control, arrows**). However, its expression in Top2b inhibited retina was drastically reduced (**Fig. 4.6B-D, ICRF-193, arrows**), suggesting that ICRF-193 affected the expression of Cdh8 involved in promoting strong cell binding and axon extension of bipolar cells during retinogenesis. Application of ICRF-193 affected directing axonal targeting, maintaining lamina- restricted arbors after they were formed (**Fig. 4.6A-C, ICRF-193, arrows**). It was also seen that reduced Cdh8 expression selectively affected placement of BCs and most of these cells were monostratified with displaced arbors. For E12 retinas the expression of Cdh8 was equalized in both control and ICRF-193 treated sections. (**Fig. 4.6D, Control, ICRF-193, arrows**), referring to the reversible effect of ICRF-193 on later developmental stages. Thus suggests that Cdh8 activation may be important in stabilizing synaptic contacts during retinogenesis.

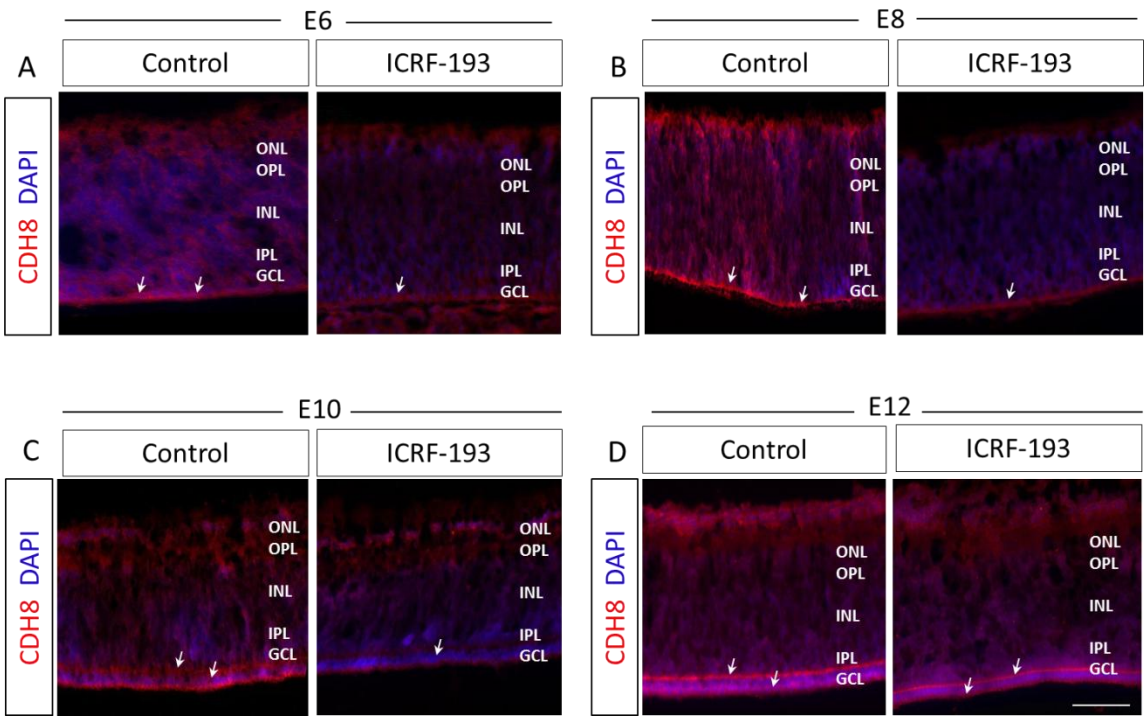

Figure 4.6: Top2b inhibition affects expression pattern of retinal specific marker CAD-8-1 during chick retinal development. Sections of the control and ICRF-193 (500µM) treated retina at E6 (A), E8 (B), E10 (C) and E12 (D) were processed to revealCdh8 in RGC layer and IPL (arrows) using CAD-8-1staining and co-stained with DAPI. At E6 and E8, CAD-8-1labeled Cdh8 in the control retinas, but its expression was dramatically reduced in ICRF-193 treated (Top2b inhibited) retinas (A, B). At E10 control retina sections, showed two prominent narrow sublaminae in IPL as compared to just one sublamina in ICRF-193 treated sections (C, arrows). At E12 control and E12 ICRF-193 treated sections showed two prominent narrow sublaminae in IPL (arrows), suggesting to the reversible effect of ICRF-193 on later developmental stages. Scale bars = 50 µm.

The qualitatively analyzed result from immunohistochemistry was confirmed quantitatively using qRT-PCR. Cdh8 expression in control and ICRF-193 treated retinas was quantified by qRT-PCR. Primers designed specifically tailored to chick retinal Cdh8 were used to amplify the mRNA by qPCR. The results from qPCR showed the dramatic reduction in expression levels of Cdh8 mRNA of ICRF-193 treated samples compared to Control samples from embryonic day 6 to 10 (E6-E10). A 4-fold reduction was seen at E8 in treated samples (**Fig. 4.7, $p<0.03$**), suggesting that Top2b is required for a healthy expression of Cdh8 in developing retina.

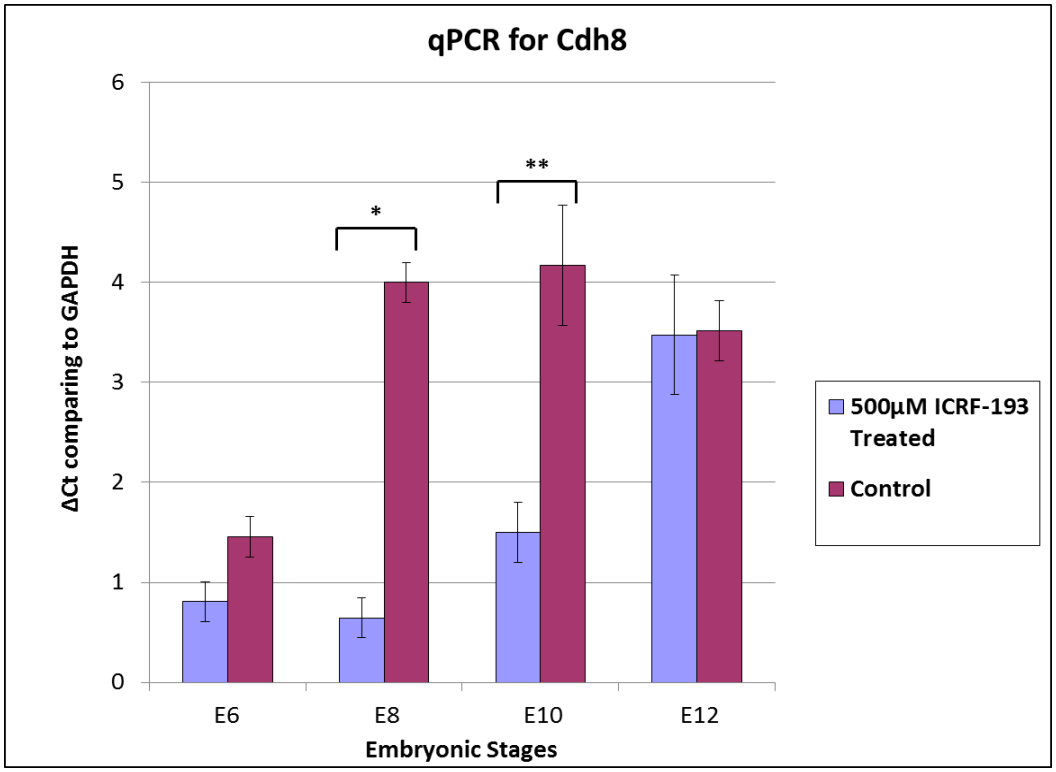

Figure 4.7:Top2b inhibition reduces the expression of Cdh8 at E8 and E10. The levels of Cdh8 mRNA at various embryonic stages (E6, E8, E10 and E12) normalized against levels of reference GAPDH were determined by qPCR and a sharp reduction in Cdh8 mRNA level was seen at E8 of ICRF-193 treated sample compared to control. * p-value < 0.03, ** p-value < 0.04, $n \geq 3$.

4.7 Top2b inhibition by ICRF-193 affects expression of Cad6B

Cad6Bis strongly expressed on the cell surface and on axodendritic trunk, labeled using cell-specific differentiation marker CCD6B-1. Cad6B+ progenitors are capable of developing all major retinal cells, such as sub-populations of BCs and amacrine cells and appear as a bushy outer border of the IPL. Cad6B expression helps in responding predominantly to single direction-selective vertical motion, during retinogenesis. Chick retinas of embryonic day 4 treated with 500µM ICRF-193 and analyzed at E6-E12 stages. Top2b inhibited retina sections revealed dramatically reduced Cad6B expression compared to the control retina. By E8 IPL displayed two prominent Cad6B+ narrow sublaminae (**Fig. 4.8B-D, Control, arrows**), representing stained dendrites extending into the IPL; corresponding to outermost sublamina of the IPL. However, only one sublaminae was seen in the Top2b inhibited retina (**Fig. 4.8B-D, ICRF-193, arrows**), suggesting that ICRF-193 affected the expression of Cad6B involved in direction selective dendritic extension during retinogenesis. For E12 retinas the expression of Cad6B was regained and both control and ICRF-193 treated sections showed two prominent narrow sublaminae in IPL (**Fig. 4.8D, control, ICRF-193, arrows**), referring to the reversible effect of ICRF-193 on later developmental stages.

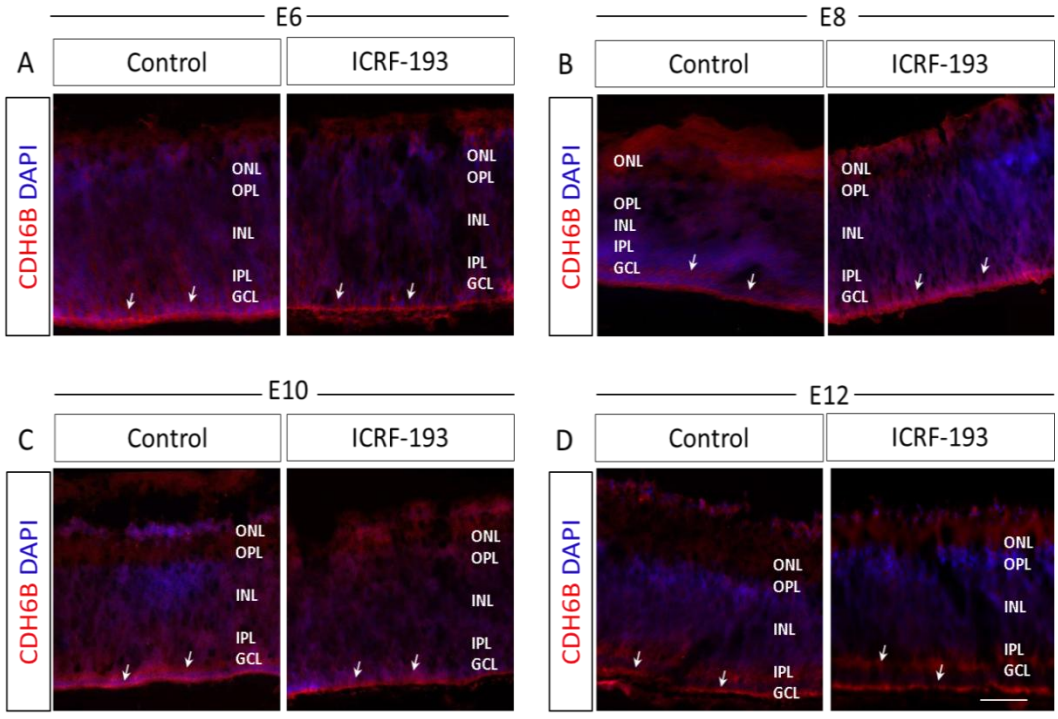

Figure 4.8: Top2b inhibition affects expression of retinal specific marker CCD6B-1 during chick retinal development. Sections of the control and ICRF-193 (500µM) treated retina at E6 (A), E8 (B), E10 (C) and E12 (D) were processed to revealCad6B in RGC layer and IPL (arrows) using CCD6B-1staining and co-stained with DAPI. At E6 and E8 CCD6B-1 labeled Cad6B in control retinas, but its expression was dramatically reduced in ICRF-193 treated (Top2b inhibited) retinas (A, B, arrows). At E10 control section, showed two prominent narrow sublaminae in IPL as compared to just one sublamina in ICRF-193 treated section (C, arrows). At E12 control and E12 ICRF-193 treated sections showed no significant differences in Cad6B expression, revealing two prominent narrow sublaminae in IPL (arrows), suggesting to the reversible effect of ICRF-193 on later developmental stages (D). Scale bars = 50 µm.

The qualitatively analyzed result from immunohistochemistry was confirmed quantitatively using qRT-PCR. Cad6B expression in control and ICRF-193 treated retinas was quantified by qRT-PCR. Primers designed specifically tailored to chick retinal Cad6B were used to amplify the mRNA by qPCR. The results from qPCR showed the dramatic reduction in expression levels of Cad6B mRNA of ICRF-193 treated samples compared to control samples from embryonic day 6 to 10 (E6-E10). A 2.5-fold reduction was seen at E10 in treated samples (**Fig. 4.9, $p<0.02$**), suggesting that Top2b is required for a healthy expression of Cad6B in developing retina.

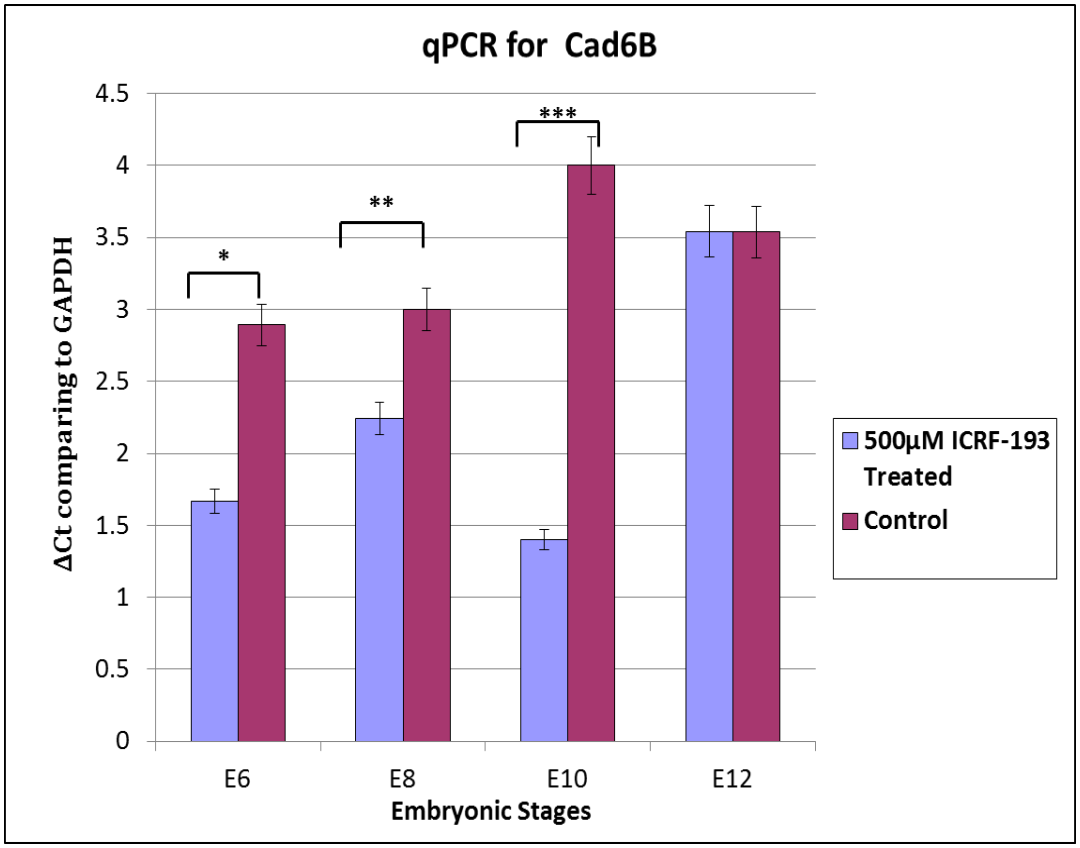

Figure 4.9: Top2b inhibition reduces the expression of Cad6B at E8 and E10. The levels of Cad6B mRNA at various embryonic stages (E6, E8, E10 and E12) normalized against levels of reference GAPDH were determined by qPCR and a sharp reduction in Cad6B mRNA level was seen at E10 of ICRF-193 treated sample compared to control. * p-value < 0.1, ** p-value < 0.05;***, p-value < 0.02, $n \geq 3$.

4.8 Top2b inhibition by ICRF-193 affects expression of Cdh7

Cdh7 was labeled using cell-specific differentiation markers CCD7-1. Cdh7 expression was seen in differentiating ganglion cell layer, their neuritis in developing IPL. Processes of bipolar cells projected to the IPL and OPL, showed expression of Cdh7. These processes show a wide band in the outer one-third of the IPL.

Cad6B and Cdh7 have similar expression pattern of forming the sublaminae at E8. However, the sublaminae were well spaced in Cdh7 expressing control sections compared to narrow sublaminae of Cad6B expressing sections. Chick retinas of embryonic day 4 treated with 500µM ICRF-193 and analyzed at E6-E12 stages. Top2b inhibited retina sections revealed dramatically reduced Cdh7 expression compared to the control retina. By E8 IPL displays two prominent Cdh7+ well-spaced sublaminae (**Fig. 4.10B-D, Control, arrows**), representing projected processes extending into the inner plexiform layer. However, only one sublaminae was seen in the Top2b inhibited retina (**Fig. 4.10B-D, ICRF-193, arrows**), suggesting that ICRF-193 affected the expression of Cdh7 involved in projecting neuritesto the nerve fiber layer during retinogenesis. For E12 retinas the expression of Cdh7 was regained in both control and ICRF-193 treated sections which showed two prominent well-spaced sublaminae in IPL (**Fig. 4.10D, Control, ICRF-193, arrows**), referring to the reversible effect of ICRF-193 on later developmental stages.

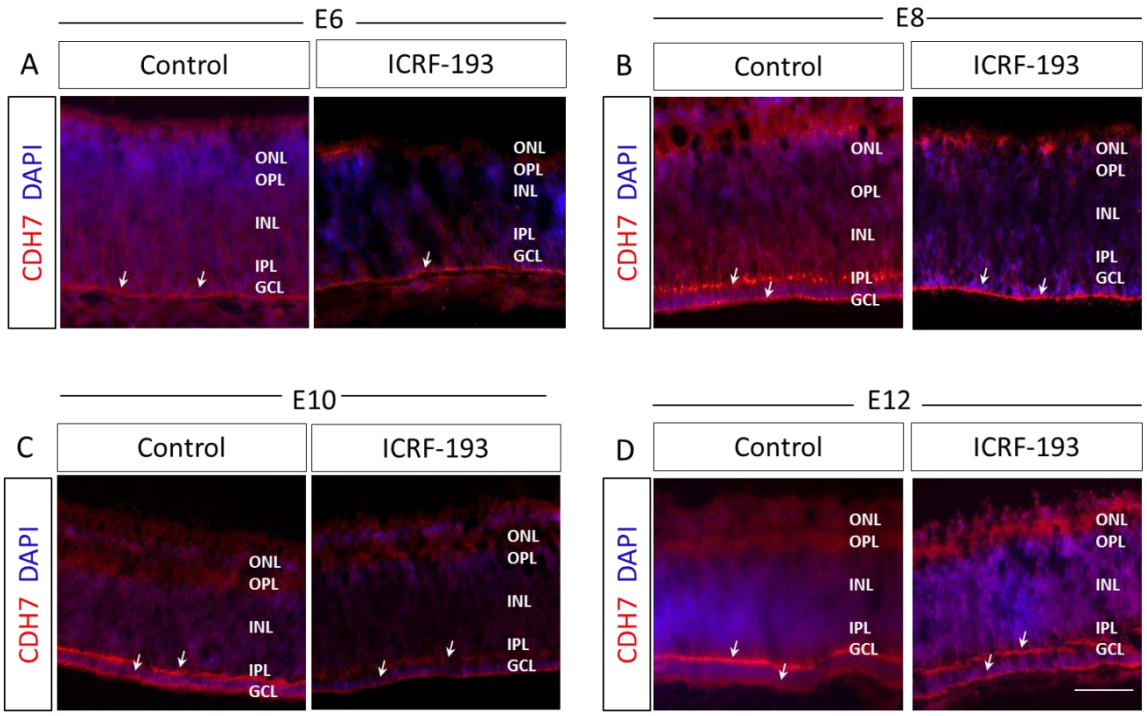

Figure 4.10: Top2b inhibition affects expression of retinal specific marker CCD7-1 during chick retinal development.Sections of the control and ICRF-193 (500µM) treated retina at E6 (A), E8 (B), E10 (C) and E12 (D) were processed to revealCdh7 in RGC layer and IPL (arrows) using CCD7-1 staining and co-stained with DAPI. At E6 CCD7-1 labeled Cdh7 in the control retinas, but its expression was dramatically reduced in ICRF-193 treated retinas (A, arrows). At E8 and E10 control retina sections, showed two prominent narrow sublaminae in IPL as compared to just one sublamina in ICRF-193 treated sections (B, C, arrows). At E12 both control and E12 ICRF-193 treated sections showed two prominent narrow sublaminae in IPL (D, arrows), suggesting reversible effect of ICRF-193 on later developmental stages. Scale bars = 50 µm.

The qualitatively analyzed result from immunohistochemistry was confirmed quantitatively using qRT-PCR. Cdh7 expression in control and ICRF-193 treated retinas was quantified by qRT-PCR. Primers designed specifically tailored to chick retinal Cdh7 were used to amplify the mRNA by qPCR. The results from qPCR showed the dramatic reduction in expression levels of Cdh7 mRNA of ICRF-193 treated samples compared to control samples from embryonic day 6 to 10 (E6-E10). A 2.5 fold reduction was seen at E10 in treated samples (**Fig. 4.11, $p<0.006$**), suggesting that Top2b is required for a healthy expression of Cdh7 in developing retina.

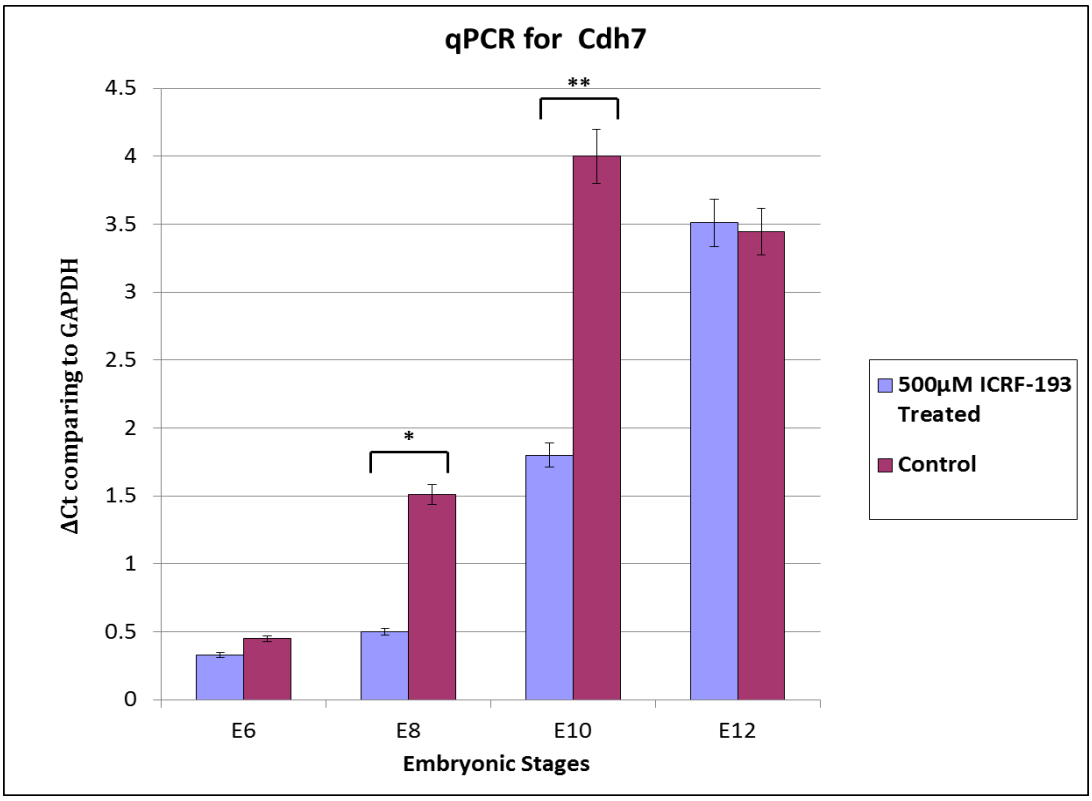

Figure 4.11:Top2b inhibition reduces the expression of Cdh7 at E8 and E10. The levels of Cdh7 mRNA at various embryonic stages (E6, E8, E10, E12) normalized against levels of reference GAPDH were determined by qPCR and a sharp reduction in Cdh7 mRNA level was seen at E10 of ICRF-193 Treated sample compared to Control. * p-value < 0.04, ** p-value < 0.006, $n \geq 3$.

4.9 Top2b inhibition by ICRF-193 affects expression of Cdh2

Retinal neuritis and neural cadherins were labeled using cell-specific differentiation markers such as MNCD2. N-cadherin labeled the neuritis of RGCs and photoreceptors in the RGC layer and photoreceptor layer respectively.Chick retinas of embryonic day 4 treated with 500µM ICRF-193 and analyzed at E6-E12 stages. Both control and Top2b inhibited retina sections revealed uniform expression of Cdh2 in the RGC layer and photoreceptor layer with faint expression overlapping in ONL and IPL, due to the neuritis extension in these regions. Cdh2 expression was continuous along the cell-cell borders. Expression of Cdh2 appeared punctuated and restricted to the microvilli on the cells (**Fig. 4.12A-D arrows**). This qualitatively analyzed result from immunohistochemistry was confirmed quantitatively using qRT-PCR. N-cadherin expression in control and ICRF-193 treated retinas was quantified by qRT-PCR. Primers designed specifically tailored to chick retinal Cdh2 were used to amplify the mRNA by qPCR, which showed Cdh2 mRNA level was not affected by the ICRF-193 treatment at any embryonic stages (E6-E12), (**Fig 4.13 $p<0.05$**), suggesting that Top2b inhibitor ICRF-193 has no effect on Cdh2 expression in developing retina.

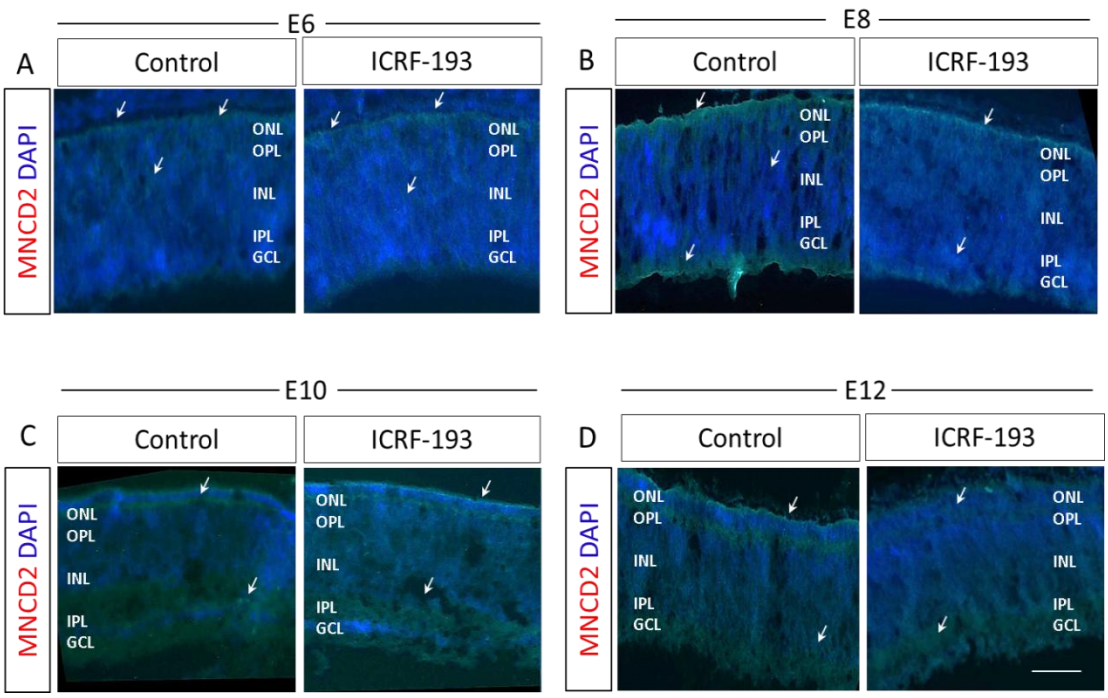

Figure 4.12: Top2b inhibition affects expression of retinal specific marker MNCD2 during chick retinal development. Sections of the control and ICRF-193 (500µM) treated retina at E6 (A), E8 (B), E10 (C) and E12 (D) were processed to reveal retinal neuritis (N-cadherins), using MNCD2 staining and co-stained with DAPI. At E6, E8, E10 and E12MNCD2 labels retinal neuritis of RGCs and photoreceptors (A, B, C, D, arrows). N-cadherin is expression was uniform in these structures and was not affected by Top2b inhibition. Scale bars = 100 µm.

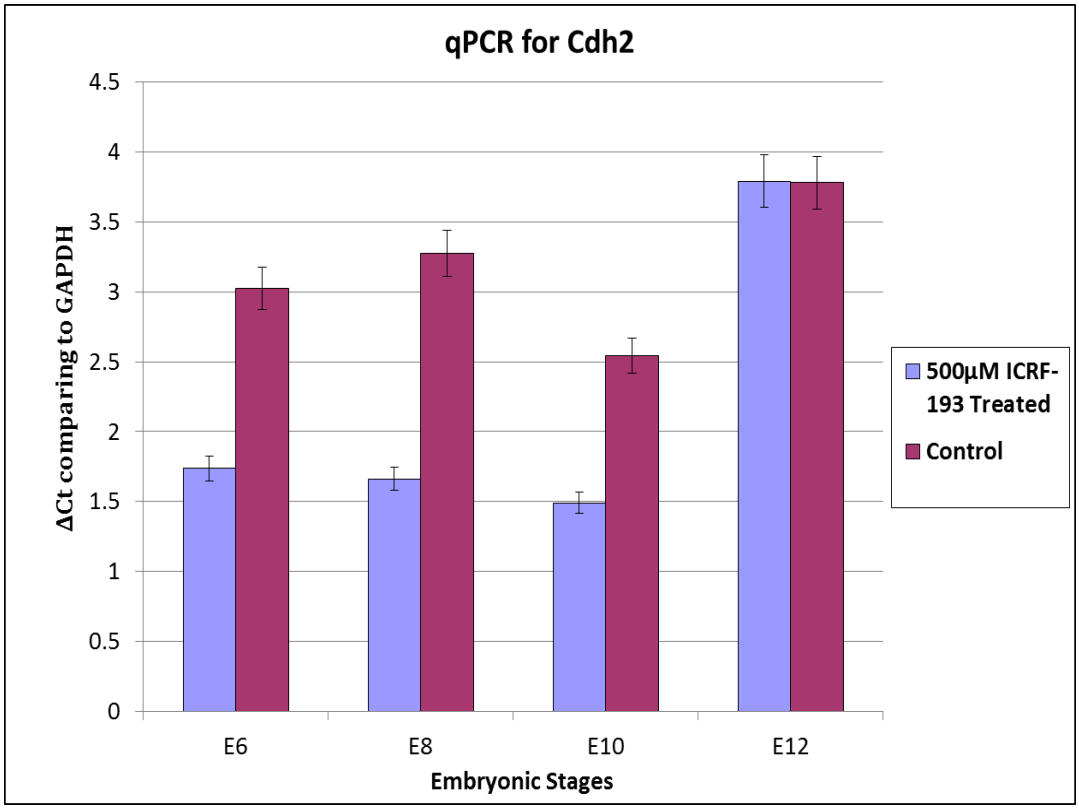

Figure 4.13: Top2b inhibition has no effect on Cdh2expression.

The levels of Cdh2 mRNA at various embryonic stages (E6, E8, E10 and E12) normalized against levels of reference GAPDH were determined by qPCR. No significant change in expression levels were observed for control and ICRF-193 treated samples ($n \geq 3$).

CHAPTER 5

5.1 DISCUSSION

In this study, using developing chick retina model we analyzed the effect of Top2b inhibitor ICRF-193 on retinogenesis and reported the functional role of Top2b. We determined that Top2b is required for migration, process development, proper laminar formation and terminal differentiation of developing retina. Top2b is expressed in all post-mitotic retinal cells; and has role in transcription regulation. Top2b inhibited retinas showed altered phenotypes, as the repercussion of altered gene expression. Results from qRT-PCR confirmed the differential expression of affected genes associated with neurological system development of retina.

Though retinas with Top2b inhibition showed lamination defects and neurodegeneration, but retinal cell types like GCs, HCs, BCs and amacrine cells were produced normally (**Fig. 4.1, 4.2, 4.3A-D, Control**). The time line of cell-specific makers expression was seen in an orderly pattern corresponding to retinal cell types (**Fig. 4.1, 4.2, 4.3A-D, Control**) (McConnell and Kaznowski 1991; McConnell 1991).

We deduced that Top2b inhibition in the retina caused delayed phenotype differentiation, mis-positioning of RGCs, HCs and severe irregularities in the plexiform layers, implying role of Top2b in neurite outgrowth, cell differentiation and laminar formation. Previous studies on RNA-sequencing revealed a down regulation of several developmental genes in the conditional knock out retinas, explaining (Rice and Curran 2001; Basu, Taylor, and Williams 2015; Duan et al. 2014; Hatta and Takeichi 1986; Li et al. 2014) mis-positioned HCs and GCs (**Fig. 4.1, 4.2, 4.3A-B, ICRF-193**). Similar results were seen in current study of Top2b inhibition using ICRF-193.

Studies have showed Top2b deficiency affects target finding of axons (Yang et al. 2000) causes short neurite growth and length (Nur et al. 2007). Also it has been identified that Top2b is the main regulator in zebrafish retinal development of ganglion cell axon path finding (Nevin et al. 2011b). In this study, we showed that Top2b has a role in neurite growth, as its inhibition caused disrupted neurite growth and contributed to the abnormal lamination, declination of neurofilaments and loss of synapses in the IPL, OPL, and caused in ICRF-193 treated retina (**Fig. 4.1, 4.2, 4.3, Treated**).

Mechanism underlying Top2b inhibition leading to differential cadherin expression remains largely unanswered. Several *in vitro* and *in vivo* investigations have shown that Top2b is a prime transcription regulating gene (Ju et al. 2006; King et al. 2013; Lyu et al. 2006; Lyu and Wang 2003b; Tiwari et al. 2012; Tsutsui, Hosoya, et al. 2001). In current study, we observed the mis-organised laminar structure and slow migration of retinal cells owing to the transcriptional role of Top2b in differential expression of cadherin genes.

Studies have shown the expression of Cdh2 uniformly in optic nerve and chiasm structures along the retinal neuritis. Cdh2 expression is uniform along the cell-cell borders. Identical results were observed in current study in spite of inhibition of Top2b, suggesting that Cdh2 expression is independent of Top2b.

Previous studies have showed that deficiency of Cad6B and Cdh7 caused formation of one sublaminae representing dendritic extensions into the inner plexiform layer instead of prominent two narrow sublaminae (Basu, Taylor, and Williams 2015; Duan et al. 2014; Ruan, Wedlich, and Koehler 2006), indistinguishable results were obtained in current study with inhibition of Top2b, suggesting role of Top2b in expression of these

genes (**Fig. 4.8, 4.10 A-C, arrows**). Also deficiency of Cdh8 caused monostratified bipolar cells with displaced arbors, selectively affecting the placement of bipolar cell and their arborization and directing axonal targeting, (Lin, Luo, and Redies 2008), or maintaining lamina-restricted arbors once formed. In current study with Top2b inhibition similar effects were observed, suggesting the role of Top2b in Cdh8 expression.

To summarize, our studies demonstrate that Top2b has a key role in migration, neurite outgrowth and laminar organization of retinal cells. Top2b was found to directly control and regulate cadherins like Cad6B, Cdh7 and Cdh8 involved in retinal development.

REFERENCES

Adachi, N., H. Suzuki, S. Iiizumi, and H. Koyama. 2003. 'Hypersensitivity of nonhomologous DNA end-joining mutants to VP-16 and ICRF-193 - Implications for the repair of topoisomerase II-mediated DNA damage', *Journal of Biological Chemistry*, 278: 35897-902.

Angst, B. D., C. Marcozzi, and A. I. Magee. 2001. 'The cadherin superfamily: diversity in form and function', *Journal of Cell Science*, 114: 629-41.

Arboleda-Estudillo, Y., M. Krieg, J. Stuhmer, N. A. Licata, D. J. Muller, and C. P. Heisenberg. 2010. 'Movement Directionality in Collective Migration of Germ Layer Progenitors', *Current Biology*, 20: 161-69.

Badea, T. C., H. Cahill, J. Ecker, S. Hattar, and J. Nathans. 2009. 'Distinct roles of transcription factors brn3a and brn3b in controlling the development, morphology, and function of retinal ganglion cells', *Neuron*, 61: 852-64.

Basu, R., M. R. Taylor, and M. E. Williams. 2015. 'The classic cadherins in synaptic specificity', *Cell Adhesion & Migration*, 9: 193-201.

Belecky-Adams, T. L., T. Haynes, J. M. Wilson, and K. Del Rio-Tsonis. 2008. 'The Chick as a Model for Retina Development and Regeneration', *Animal Models in Eye Research*: 102-19.

Boije, H., P. H. Edqvist, and F. Hallbook. 2008. 'Temporal and spatial expression of transcription factors FoxN4, Ptf1a, Prox1, Isl1 and Lim1 mRNA in the developing chick retina', *Gene Expr Patterns*, 8: 117-23.

Centanin, L., and J. Wittbrodt. 2014. 'Retinal neurogenesis', *Development*, 141: 241-44.

Chen, L. X., X. W. Zhu, Y. R. Zou, J. Xing, E. Gilson, Y. M. Lu, and J. Ye. 2015. 'The topoisomerase II catalytic inhibitor ICRF-193 preferentially targets telomeres that are capped by TRF2', *American Journal of Physiology-Cell Physiology*, 308: C372-C77.

Chintalapudi, S. R., L. Djenderedjian, A. B. Stiemke, J. J. Steinle, M. M. Jablonski, and V. M. Morales-Tirado. 2016. 'Isolation and Molecular Profiling of Primary Mouse Retinal Ganglion Cells: Comparison of Phenotypes from Healthy and Glaucomatous Retinas', *Front Aging Neurosci*, 8: 93.

Coulombre JL , Coulombre AJ 1965. 'Regeneration of neural retina from the pigmented epithelium in the chick embryo', *Developmental Biology*, 12 (1): 79 – 92

Darnell, D. K., M. R. Stark, and G. C. Schoenwolf. 1999. 'Timing and cell interactions underlying neural induction in the chick embryo', *Development*, 126: 2505-14.

Doh, S. T., H. Hao, S. C. Loh, T. Patel, H. Y. Tawil, D. K. Chen, A. Pashkova, A. Shen, H. Wang, and L. Cai. 2010. 'Analysis of retinal cell development in chick embryo by immunohistochemistry and in ovo electroporation techniques', *BMC Dev Biol*, 10: 8.

Duan, X., A. Krishnaswamy, I. De la Huerta, and J. R. Sanes. 2014. 'Type II Cadherins Guide Assembly of a Direction-Selective Retinal Circuit', *Cell*, 158: 793-807.

Dyer, M. A., and C. L. Cepko. 2001. 'Regulating proliferation during retinal development', *Nature Reviews Neuroscience*, 2: 333-42.

Edqvist, P. H., S. M. Myers, and F. Hallbook. 2006. 'Early identification of retinal subtypes in the developing, pre-laminated chick retina using the transcription

factors Prox1, Lim1, Ap2alpha, Pax6, Isl1, Isl2, Lim3 and Chx10', *Eur J Histochem*, 50: 147-54.

Fannon, A. M., and D. R. Colman. 1996. 'A model for central synaptic junctional complex formation based on the differential adhesive specificities of the cadherins', *Neuron*, 17: 423-34.

Ferreira-Cornwell, M. C., R. W. Veneziale, G. B. Grunwald, and A. S. Menko. 2000. 'N-cadherin function is required for differentiation-dependent cytoskeletal reorganization in lens cells in vitro', *Experimental Cell Research*, 256: 237-47.

Ganzler, S. I. I., and C. Redies. 1995. 'R-Cadherin Expression during Nucleus Formation in Chicken Forebrain Neuromeres', *Journal of Neuroscience*, 15: 4157-72.

Hatta, K., M. Hosoda, and M. Takeichi. 1986. 'Pattern of Expression of N-Cadherin Adhesion Molecules Associated with Morphogenesis of Chicken Embryos', *Development Growth & Differentiation*, 28: 389-89.

Hatta, K., and M. Takeichi. 1986. 'Expression of N-Cadherin Adhesion Molecules Associated with Early Morphogenetic Events in Chick Development', *Nature*, 320: 447-49.

Hayashi, T., and R. W. Carthew. 2004. 'Surface mechanics mediate pattern formation in the developing retina', *Nature*, 431: 647-52.

Huang, E. J., W. Liu, B. Fritzsch, L. M. Bianchi, L. F. Reichardt, and M. Xiang. 2001. 'Brn3a is a transcriptional regulator of soma size, target field innervation and axon pathfinding of inner ear sensory neurons', *Development*, 128: 2421-32.

Isik, S., K. Sano, K. Tsutsui, M. Seki, T. Enomoto, H. Saitoh, and K. Tsutsui. 2003. 'The SUMO pathway is required for selective degradation of DNA topoisomerase II beta induced by a catalytic inhibitor ICRF-193', *Febs Letters*, 546: 374-78.

Islam, M. M., S. T. Doh, and L. Cai. 2012. 'In ovo electroporation in embryonic chick retina', *J Vis Exp*.

Jan G. Hengstler, Carolin K. Heimerdinger, Ilka B. Schiffer , Susanne Gebhard, Jens Sagemüller, Berno Tanner, Hermann M. Bolt, Franz Oesch. 2002. 'Review: Dietary topoisomerase II-poisons: contribution of soy products to infant leukemia?', *EXCLI Journal*: 8-14.

Jeon, C. J., E. Strettoi, and R. H. Masland. 1998. 'The major cell populations of the mouse retina', *Journal of Neuroscience*, 18: 8936-46.

Ju, B. G., V. V. Lunyak, V. Perissi, I. Garcia-Bassets, D. W. Rose, C. K. Glass, and M. G. Rosenfeld. 2006. 'A topoisomerase IIbeta-mediated dsDNA break required for regulated transcription', *Science*, 312: 1798-802.

King, I. F., C. N. Yandava, A. M. Mabb, J. S. Hsiao, H. S. Huang, B. L. Pearson, J. M. Calabrese, J. Starmer, J. S. Parker, T. Magnuson, S. J. Chamberlain, B. D. Philpot, and M. J. Zylka. 2013. 'Topoisomerases facilitate transcription of long genes linked to autism', *Nature*, 501: 58-62.

Korematsu, K., and C. Redies. 1997. 'Expression of cadherin-8 mRNA in the developing mouse central nervous system', *Journal of Comparative Neurology*, 387: 291-306.

Kulesa PM, Fraser SE. 2011. "Live imaging of avian embryos. Imaging in Developmental Biology: A Laboratory Manual." In *Cold Spring Harbor NY, 85-99*, 85-99. Cold Spring Harbor Laboratory Press.

Lagunowich, L. A., and G. B. Grunwald. 1989. 'Expression of Calcium-Dependent Cell-Adhesion during Ocular Development - a Biochemical, Histochemical and Functional-Analysis', *Developmental Biology*, 135: 158-71.

Lee, S., K. Choi, H. Ahn, K. Song, J. Choe, and I. Lee. 2005. 'TuJ1 (class III beta-tubulin) expression suggests dynamic redistribution of follicular dendritic cells in lymphoid tissue', *European Journal of Cell Biology*, 84: 453-9.

Li, X. M., C. Yu, Z. W. Wang, Y. L. Zhang, X. M. Liu, D. W. Zhou, Q. Y. Sun, and H. Y. Fan. 2013. 'DNA Topoisomerase II Is Dispensable for Oocyte Meiotic Resumption but Is Essential for Meiotic Chromosome Condensation and Separation in Mice', *Biology of Reproduction*, 89.

Li, Y., H. Hao, E. Tzatzalos, R. K. Lin, S. Doh, L. F. Liu, Y. L. Lyu, and L. Cai. 2014. 'Topoisomerase IIbeta is required for proper retinal development and survival of postmitotic cells', *Biol Open*, 3: 172-84.

Lin, J., J. Luo, and C. Redies. 2008. 'Molecular cloning and expression analysis of three cadherin-8 isoforms in the embryonic chicken brain', *Brain Research*, 1201: 1-14.

Lin, J. T., C. R. Wang, and C. Redies. 2014. 'Restricted expression of classic cadherins in the spinal cord of the chicken embryo', *Frontiers in Neuroanatomy*, 8.

Liu, W., S. L. Khare, X. Liang, M. A. Peters, X. Liu, C. L. Cepko, and M. Xiang. 2000. 'All Brn3 genes can promote retinal ganglion cell differentiation in the chick', *Development*, 127: 3237-47.

Livak, K. J., and T. D. Schmittgen. 2001. 'Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method', *Methods*, 25: 402-08.

Lorthongpanich, C., T. P. Y. Doris, V. Limviphuvadh, B. B. Knowles, and D. Solter. 2012. 'Developmental fate and lineage commitment of singled mouse blastomeres', *Development*, 139: 3722-31.

Lyu, Y. L., C. P. Lin, A. M. Azarova, L. Cai, J. C. Wang, and L. F. Liu. 2006. 'Role of topoisomerase IIbeta in the expression of developmentally regulated genes', *Mol Cell Biol*, 26: 7929-41.

Lyu, Y. L., and J. C. Wang. 2003a. 'Aberrant lamination in the cerebral cortex of mouse embryos lacking DNA topoisomerase IIbeta', *Proc Natl Acad Sci U S A*, 100: 7123-8.

———. 2003b. 'Aberrant lamination in the cerebral cortex of mouse embryos lacking DNA topoisomerase IIbeta', *Proc Natl Acad Sci U S A*, 100: 7123-8.

Maitre, J. L., and C. P. Heisenberg. 2013. 'Three Functions of Cadherins in Cell Adhesion Review', *Current Biology*, 23: R626-R33.

Margeta, M. A. 2008. 'Transcription factor Lim1 specifies horizontal cell laminar position in the retina', *J Neurosci*, 28: 3835-6.

Masland, R. H. 1986. 'The Functional Architecture of the Retina', *Scientific American*, 255: 102-&.

———. 2001. 'The fundamental plan of the retina', *Nature Neuroscience*, 4: 877-86.

Masland, R. H., and E. Raviola. 2000. 'Confronting complexity: Strategies for understanding the microcircuitry of the retina', *Annual Review of Neuroscience*, 23: 249-84.

Matsunaga, M., K. Hatta, A. Nagafuchi, and M. Takeichi. 1988. 'Guidance of Optic-Nerve Fibers by N-Cadherin Adhesion Molecules', *Nature*, 334: 62-64.

Matsunaga, M., K. Hatta, and M. Takeichi. 1988. 'Role of N-Cadherin Cell-Adhesion Molecules in the Histogenesis of Neural Retina', *Neuron*, 1: 289-95.

McConnell, S. K. 1991. 'The generation of neuronal diversity in the central nervous system', *Annu Rev Neurosci*, 14: 269-300.

McConnell, S. K., and C. E. Kaznowski. 1991. 'Cell cycle dependence of laminar determination in developing neocortex', *Science*, 254: 282-5.

Meyer-Ficca, M. L., J. D. Lonchar, M. Ihara, M. L. Meistrich, C. A. Austin, and R. G. Meyer. 2011. 'Poly(ADP-ribose) polymerases PARP1 and PARP2 modulate topoisomerase II beta (TOP2B) function during chromatin condensation in mouse spermiogenesis', *Biol Reprod*, 84: 900-9.

Miyatani, S., N. G. Copeland, D. J. Gilbert, N. A. Jenkins, and M. Takeichi. 1992. 'Genomic Structure and Chromosomal Mapping of the Mouse N-Cadherin Gene', *Proc Natl Acad Sci U S A*, 89: 8443-47.

Momose, T., A. Tonegawa, J. Takeuchi, H. Ogawa, K. Umesono, and K. Yasuda. 1999. 'Efficient targeting of gene expression in chick embryos by microelectroporation', *Development Growth & Differentiation*, 41: 335-44.

Nadal-Nicolas, F. M., M. Jimenez-Lopez, P. Sobrado-Calvo, L. Nieto-Lopez, I. Canovas-Martinez, M. Salinas-Navarro, M. Vidal-Sanz, and M. Agudo. 2009. 'Brn3a as a marker of retinal ganglion cells: qualitative and quantitative time course studies in naive and optic nerve-injured retinas', *Invest Ophthalmol Vis Sci*, 50: 3860-8.

Nevin, L. M., T. Xiao, W. Staub, and H. Baier. 2011a. 'Topoisomerase II{beta} is required for lamina-specific targeting of retinal ganglion cell axons and dendrites', *Development*, 138: 2457-65.

———. 2011b. 'Topoisomerase IIbeta is required for lamina-specific targeting of retinal ganglion cell axons and dendrites', *Development*, 138: 2457-65.

Niessen, C. M., D. Leckband, and A. S. Yap. 2011. 'Tissue Organization by Cadherin Adhesion Molecules: Dynamic Molecular and Cellular Mechanisms of Morphogenetic Regulation', *Physiological Reviews*, 91: 691-731.

Nitiss, J. L. 2009a. 'DNA topoisomerase II and its growing repertoire of biological functions', *Nature Reviews Cancer*, 9: 327-37.

———. 2009b. 'DNA topoisomerase II and its growing repertoire of biological functions', *Nat Rev Cancer*, 9: 327-37.

Nur, E. Kamal A., S. Meiners, I. Ahmed, A. Azarova, C. P. Lin, Y. L. Lyu, and L. F. Liu. 2007. 'Role of DNA topoisomerase IIbeta in neurite outgrowth', *Brain Res*, 1154: 50-60.

Paulson, A. F., M. S. Prasad, A. H. Thuringer, and P. Manzerra. 2014. 'Regulation of cadherin expression in nervous system development', *Cell Adhesion & Migration*, 8: 19-28.

Poche, R. A., K. M. Kwan, M. A. Raven, Y. Furuta, B. E. Reese, and R. R. Behringer. 2007. 'Lim1 is essential for the correct laminar positioning of retinal horizontal cells', *J Neurosci*, 27: 14099-107.

Prada, C., J. Puga, L. Perezmendez, R. Lopez, and G. Ramirez. 1991. 'Spatial and Temporal Patterns of Neurogenesis in the Chick Retina', *European Journal of Neuroscience*, 3: 559-69.

Redies, C. 1995. 'Cadherin Expression in the Developing Vertebrate Cns - from Neuromeres to Brain Nuclei and Neural Circuits', *Experimental Cell Research*, 220: 243-56.

Rice, D. S., and T. Curran. 2001. 'Role of the reelin signaling pathway in central nervous system development', *Annu Rev Neurosci*, 24: 1005-39.

Robinson, G. A., and R. D. Madison. 2004. 'Axotomized mouse retinal ganglion cells containing melanopsin show enhanced survival, but not enhanced axon regrowth into a peripheral nerve graft', *Vision Research*, 44: 2667-74.

Roca, J., R. Ishida, J. M. Berger, T. Andoh, and J. C. Wang. 1994. 'Antitumor Bisdioxopiperazines Inhibit Yeast DNA Topoisomerase-Ii by Trapping the Enzyme in the Form of a Closed Protein Clamp', *Proc Natl Acad Sci U S A*, 91: 1781-85.

Ruan, G., D. Wedlich, and A. Koehler. 2006. 'Xenopus cadherin-6 regulates growth and epithelial development of the retina', *Mechanisms of Development*, 123: 881-92.

Schlegelmilch, K., Mohseni, M., Kirak, O., Pruszak, J., Rodriguez, J.R., Zhou, D., Kreger, B.T., Vasioukhin, V., Avruch, J., Brummelkamp, T.R., . 2011. 'Yap1 acts downstream of a-catenin to control epidermal proliferation', *Cell*, 144: 782-95.

Schuman, E. M., and S. Murase. 2003. 'Cadherins and synaptic plasticity: activity-dependent cyclin-dependent kinase 5 regulation of synaptic beta-catenin-cadherin interactions', *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, 358: 749-56.

Shibuya, Y., A. Mizoguchi, M. Takeichi, K. Shimada, and C. Ide. 1995. 'Localization of N-Cadherin in the Normal and Regenerating Nerve-Fibers of the Chicken Peripheral Nervous-System', *Neuroscience*, 67: 253-61.

Strauss, O. 2005. 'The retinal pigment epithelium in visual function', *Physiological Reviews*, 85: 845-81.

Suga, A., M. Taira, and S. Nakagawa. 2009. 'LIM family transcription factors regulate the subtype-specific morphogenesis of retinal horizontal cells at post-migratory stages', *Dev Biol*, 330: 318-28.

Tiwari, V. K., L. Burger, V. Nikoletopoulou, R. Deogracias, S. Thakurela, C. Wirbelauer, J. Kaut, R. Terranova, L. Hoerner, C. Mielke, F. Boege, R. Murr, A. H. Peters, Y. A. Barde, and D. Schubeler. 2012. 'Target genes of Topoisomerase IIbeta regulate neuronal survival and are defined by their chromatin state', *Proceedings of the National Academy of Sciences of the United States of America*, 109: E934-43.

Treubert-Zimmermann, U., D. Heyers, and C. Redies. 2002. 'Targeting axons to specific fiber tracts in vivo by altering cadherin expression', *Journal of Neuroscience*, 22: 7617-26.

Tsutsui, K, S Okada, M Watanabe, T Shohmori, S Seki, and Y Inoue. 1993. 'Molecular cloning of partial cDNAs for rat DNA topoisomerase II isoforms and their differential expression in brain development', *Journal of Biological Chemistry*, 268: 19076-83.

Tsutsui, K., O. Hosoya, K. Sano, and A. Tokunaga. 2001. 'Immunohistochemical analyses of DNA topoisomerase II isoforms in developing rat cerebellum', *J. Comp. Neurol.*, 431: 228-39.

Tsutsui, K., K. Sano, A. Kikuchi, and A. Tokunaga. 2001. 'Involvement of DNA topoisomerase IIbeta in neuronal differentiation', *J Biol Chem*, 276: 5769-78.

Vergara, M. N., and M. V. Canto-Soler. 2012. 'Rediscovering the chick embryo as a model to study retinal development', *Neural Development*, 7.

Wang, J. C. 2002. 'Cellular roles of DNA topoisomerases: a molecular perspective', *Nat Rev Mol Cell Biol*, 3: 430-40.

Wohrn, J. C. P., S. Nakagawa, M. Ast, M. Takeichi, and C. Redies. 1999. 'Combinatorial expression of cadherins in the tectum and the sorting of neurites in the tectofugal pathways of the chicken embryo', *Neuroscience*, 90: 985-1000.

Wohrn, J. C. P., L. Puelles, S. Nakagama, M. Takeichi, and C. Redies. 1998. 'Cadherin expression in the retina and retinofugal pathways of the chicken embryo', *Journal of Comparative Neurology*, 396: 20-38.

Xiao, H., Y. Mao, S. D. Desai, N. Zhou, C. Y. Ting, J. L. Hwang, and L. F. Liu. 2003. 'The topoisomerase II beta circular clamp arrests transcription and signals a 26S proteasome pathway', *Proc Natl Acad Sci U S A*, 100: 3239-44.

Xu, L., P. A. Overbeek, and L. W. Reneker. 2002. 'Systematic analysis of E-, N- and P-cadherin expression in mouse eye development', *Experimental Eye Research*, 74: 753-60.

Yang, X., W. Li, E. D. Prescott, S. J. Burden, and J. C. Wang. 2000. 'DNA topoisomerase IIbeta and neural development', *Science*, 287: 131-4.

Young, R. W. 1985a. 'Cell-Differentiation in the Retina of the Mouse', *Anatomical Record*, 212: 199-205.

———. 1985b. 'Cell-Proliferation during Postnatal-Development of the Retina in the Mouse', *Developmental Brain Research*, 21: 229-39.