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GRADE / DEGREE

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FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Requirement for E2F4 in Development of Ventral Telencephalon and Visual System

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Requirement for E2F4 in Development of Ventral Telencephalon and Visual System

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Thesis submitted to the Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements for the PhD degree in
Cellular and Molecular Medicine

August, 2007

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Your file Votre référence
ISBN: 978-0-494-50754-4
Our file Notre référence
ISBN: 978-0-494-50754-4

ABSTRACT

New functions of the Rb/E2F pathway in developmental processes are emerging. The goal of this research was to identify the role of the E2F4 transcription factor during early nervous system development. First, I addressed whether E2F4 was required for the development of the telencephalon. Analysis of E2F4 deficient mouse embryos revealed that loss of E2F4 led to severe morphological defects in early ventral telencephalic development. Specifically, ganglionic eminences were absent and expression of the regional homeobox transcription factors was significantly reduced, suggesting that E2F4 is involved in the regulation of genes important for brain patterning. E2F4 deficiency also caused a reduction in neural stem cell number, indicating the requirement for E2F4 in regulation of neural stem cell self-renewal. To account for the aberrant telencephalic development, we showed that E2F4 deficiency lead to aberrant Shh signaling and impaired activity of forebrain-specific *Shh* enhancers. Activation of the Shh pathway with a Hh agonist restored cells expressing *Nkx2.1* in explant cultures, as well as neural stem cell self-renewal. Finally, I identified a novel genetic interaction between E2F4 and Shh pathway in early telencephalic development. These studies demonstrated that E2F4 was required for ventral telencephalic development. Next, I determined whether E2F4 was required for eye patterning, which is closely linked to ventral telencephalic development and is regulated by Shh. I found that early eye morphogenesis and patterning was perturbed, such that optic stalk specific genes extended into the ventral optic cup with simultaneous reduction of the neural retinal markers. Also, Shh signaling was disrupted in the absence of E2F4. The *Shh* expression domain in the ventral hypothalamus was split and Shh activity was expanded into the ventral optic cup. These studies revealed a key

role for E2F4 in early eye patterning. Overall, my findings reveal a novel requirement for E2F4 during early telencephalic and eye development, as well as in neural stem cells regulation.

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LIST OF ABBREVIATIONS

A – anterior

ash - acheate-scute

AP – anterior-posterior

ANR – anterior neural ridge

BF1 – Brain Factor 1

bHLH – basic helix-loop-helix

Bmi - B lymphoma Mo-MLV insertion region

BMP – bone morphogenic protein

BmpR – BMP receptor

BrdU – 5-bromo-2-deoxyuridine

Cdc – cell division control

Cdk – cyclin-dependent kinase

cDNA – complementary DNA

CIHR – Canadian Institutes of Health Research

CKI – cyclin-dependent kinase inhibitor

CNS – central nervous system

CP – commissural plate

cyc – cyclops

D – dorsal

DBD – DNA binding domain

DE – diencephalon

de2f – Drosophila E2F

DIG – digoxigenin

Dlx – distalless homeobox

DMEM - Dulbecco's Modified Eagle's Medium

DNA – deoxyribonucleic acid

dOS – dorsal optic stalk

DP – dimerizing protein

DPBS - Dulbecco's phosphate buffered saline

DV – dorsal-ventral

E – eye

E2F – E2 promoter binding factor

E# - embryonic day # (ex. E9)

Emx – empty spiracle homeobox

Evf – embryonic ventral forebrain

Fgf – fibroblast growth factor

FGFR – Fgf receptor

FP – floor plate

Frz – frizzeled

G – gap

GABA - gamma-aminobutyric acid

GAD – glutamic acid decarboxylase

Gal – galactosidase

GFAP – glial fibrillary acidic protein

Gli – glioma-associated

Gsh2 – genomic screened homeobox 2

H – hypothalamus

Hes – hairy and enhancer-of-split

Hesx – homeobox gene expressed in embryonic stem cells

HH – Hamburger-Hamilton (stage of chick embryo development)

Hh - hedgehog

Hox – homeobox

HPE – holoprosencephaly

INK – inhibitors of CDK4

ISH – in situ hybridization

Isl – islet

Kip – kinase inhibitor protein

KO – knock-out

L – lateral

LGE – lateral ganglionic eminence

Lhx – Lim homeobox

Lim - Lin11, Isl-1 and Mec-3 domain

LP – lens placode

LT – lamina terminalis

LV – lateral ventricle

LZ – leucine zipper

Mash – mouse acheate-scute homologue

Math – mouse athonal

MB – marked box

ME – mesencephalon

MGE – medial ganglionic eminence

mRNA – matrix ribonucleic acid

Msx – musashi-like homeobox

MZ – marginal zone

NBL – neuroblastic layer

NC – notochord

NES – nuclear export signal

Ngn - neurogenin

Nkx – Niremberg-Kim homeobox

NLS – nuclear localization signal

NR – neural retina

NSC – neural stem cells

OCT - Optimal Cutting Temperature

OGS – Ontario Graduate Scholarship

Olig – oligodendrocyte transcription factor

Orc – origin complex

Otx - orthodenticle homeobox

P – posterior

P# – postnatal day

Pax – paired homeobox

PBS – phosphate buffered saline

Pc-G – polycomb group

PCP – prechordal plate

PCR - polymerase chain reaction

PFA – paraformaldehyde

pRb – retinoblastoma protein

PE – prosencephalon

PH3 – phosphor-histone H3

Ptc - Patched

Ptx – paired-like homeodomain transcription factor

Rb – retinoblastoma

RGC – retinal ganglion cell

RE – rhombencephalon

RPE – retinal pigmented epithelium

RT-PCR - reverse transcription polymerase chain reaction

Rx – retinal homeobox

SBE – Shh brain enhancer

SCN – Stem Cell Network

SCv – spinal cord ventral

SE – surface ectoderm

s.e.m. – standard error mean

Shh – Sonic hedgehog

Six – sine oculis homeobox

Smad – mothers against decapentaplegic homolog

Smo – smoothened

st – striatum

SVZ – subventricular zone

Tbr - T-box brain

Tcf/LEF – T cell factor/lymphoid enhancer factor

TE – telencephalon

TGF – transforming growth factor

V – ventral

Vax – ventral anterior homeobox

VM – ventral midline

vOS – ventral optic stalk

VZ – ventricular zone

Wnt – wingless/int

WT – wild type

Zash – zebrafish acheate-scute homologue

ZLI – zona limitans interthalamica

ACKNOWLEDMENTS

I am enormously grateful for the generous support and guidance of my research supervisor Dr. Ruth Slack, who gave me the opportunity to be a member of her lab. She has created stimulating working and learning environment, but at the same time, she has always encouraged balancing scientific work and life outside the lab. Under her tutorship I have sharpened my critical thinking and scientific writing skills. She provided numerous opportunities to attend scientific conferences, where I was able to share my work and stay up-to-date with the most recent advances in neuroscience. Importantly, Dr. Slack has always taught me to keep the 'big picture' in mind while staying focused on a particular problem.

I am thankful to Dr. Jackie Vanderluit who taught me a lot of fundamental lab procedures and introduced me to the exciting science and techniques of 'neurospherology'. I am sincerely grateful to Kelly McClellan for helpful discussions, encouragement and help on matters far beyond research, as well as for tremendous assistance in writing and editing my written 'creations'. My special thanks to Jason MacLaurin and Carl McIntosh for sharing their expertise and tremendous technical support. Many thanks go to Lisa Julian for immense editorial assistance. I would like to thank Dr. Noel Ghanem for valuable critical reviews and constructive feedback. Thanks to all 'life side' members for scientific discussions during lab meetings and 'life side' seminars. I am grateful to all Slack lab members for making every day of my graduate work enjoyable.

My gratitude also extends to my advisory committee members, Dr. Valerie Wallace, Dr. David Park and Dr. Luc Sabourin for their productive critique, advice, and guidance. I am particularly thankful to Dr. Valerie A. Wallace, as well as to a former postdoc in her lab

Dr. Marosh Furimsky, whose expert advice has been crucial in expanding my scientific horizons during the study of visual system development.

Thank you to my family and friends for moral support.

I would like to acknowledge financial support from Ontario Graduate Scholarship and Stem Cell Network.

Chapter 1. Introduction

Our ability to interact with the environment and generate adequate responses relies on the function of the nervous system which has peripheral and central components. The central nervous system (CNS) plays an essential role as an ultimate integrating and coordinating centre of sensory, motor, behavioral and cognitive functions. Numerous exogenous or endogenous factors may alter the normal CNS development and lead to neurological and psychiatric disorders (Pombero et al., 2007). Knowledge of the mechanisms which direct CNS development is essential for understanding the pathophysiology of neurodevelopmental disorders. The first part of this introduction provides an overview of the brain and eye development, focusing on the early stages of morphogenesis. This is followed by a presentation of the Rb/E2F pathway and its roles in cell cycle regulation and developmental processes. Finally, I will put forward the objectives and hypothesis to be tested in this thesis.

1.1. Brain development

1.1.1. CNS morphological structure and development overview

The CNS is composed of two major subdivisions: the spinal cord posteriorly and brainstem, diencephalon and telencephalon anteriorly (Kandel et al., 2000). The brainstem, which includes medulla oblongata, cerebellum and midbrain, connects the spinal cord to the diencephalon and telencephalon. The telencephalon is the most complex and evolutionary advanced part of the brain. Morphologically it is represented by two cerebral hemispheres connected by the nerve fibers of corpus callosum. The telencephalon is composed of the outer cortex, containing the layered composition of the neuronal bodies, and inner white matter, which is made of neuronal axons. The

collections of the neuronal bodies within the white matter of the telencephalon form nuclei of the basal ganglia. At the rostral end of the telencephalon the olfactory bulbs are structurally linked to the rostral telencephalic hemispheres.

The complex CNS morphology evolves from a single uniform sheet of cells. The dorso-medial part of the embryonic ectoderm acquires neural character and forms the neural plate (reviewed in (Colas and Schoenwolf, 2001)). During neurulation the lateral neural plate edges fold up and close at the dorsal midline along the anterior-posterior axis, which forms the neural tube (Fig. 1.1) (Colas and Schoenwolf, 2001). After neural tube formation the lateral regions of the neural plate become dorsal, whereas the medial part assumes a ventral position. While the posterior part of the neural tube develops into the spinal cord, the anterior end of the neural tube transiently transforms into three consecutive enlargements (vesicles): prosencephalon, mesencephalon, and rhombencephalon. Two of those vesicles, prosencephalon and rhombencephalon, are further subdivided into two vesicles each: diencephalon, secondary prosencephalon and metencephalon, myelencephalon, respectively (Fig. 1.2) (Puelles and Rubenstein, 1993, 2003). Each vesicle represents a primordium of the corresponding brain regions. Thus, the neural plate generates complex brain morphology through a series of complex morphological transformations.

1.1.2. Neural tissue

The CNS originates from the embryonic ectoderm as a single layer of neuroepithelial precursor cells, which form the neural plate (Lumsden and Krumlauf, 1996; Stern, 2001; Stern, 2005). These cells represent the earliest neural stem cells (NSC)

Figure 1.1. Subdivisions of the anterior neural tube at the end of neurulation. Antero-lateral view of the neural tube. During neurulation lateral (L) edges of the neural plate fold up and close at the dorsal (D) midline (light blue arrows). Anterior part of the neural tube forms three consecutive vesicles: prosencephalon (PE), mesencephalon (ME), and rhombencephalon (RE) (approximate borders are indicated by thin black lines). Fgf8 signaling centre (blue) is located in the lamina terminalis (LT) and forming commissural plate (CP). The *Shh* expression domain (red) occupies the ventral part of the neural tube. The telencephalon develops from the secondary prosencephalon. Abbreviations: A – anterior, E – eye, P – posterior, LP – lens placode, TE – telencephalon, V – ventral.

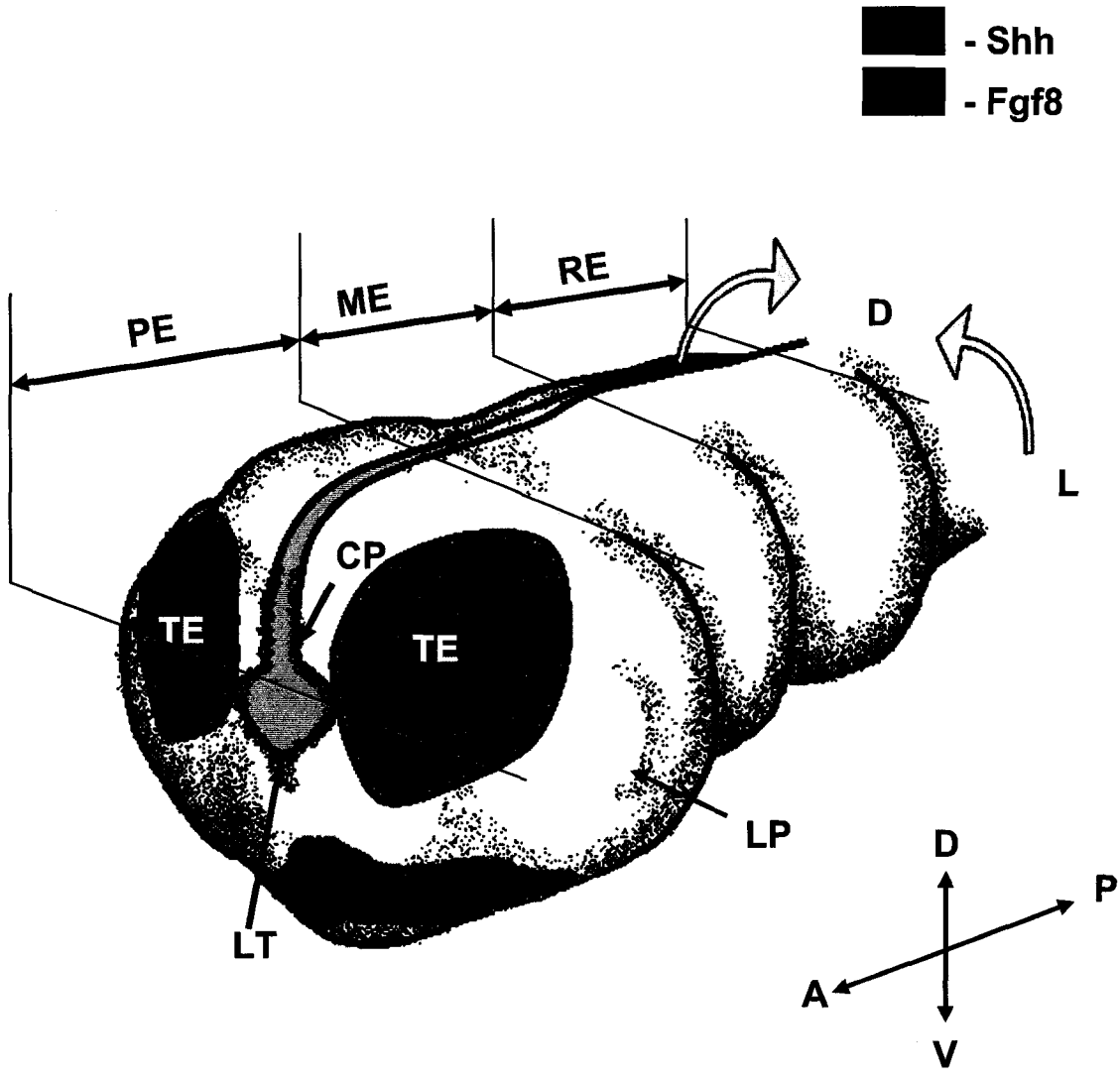
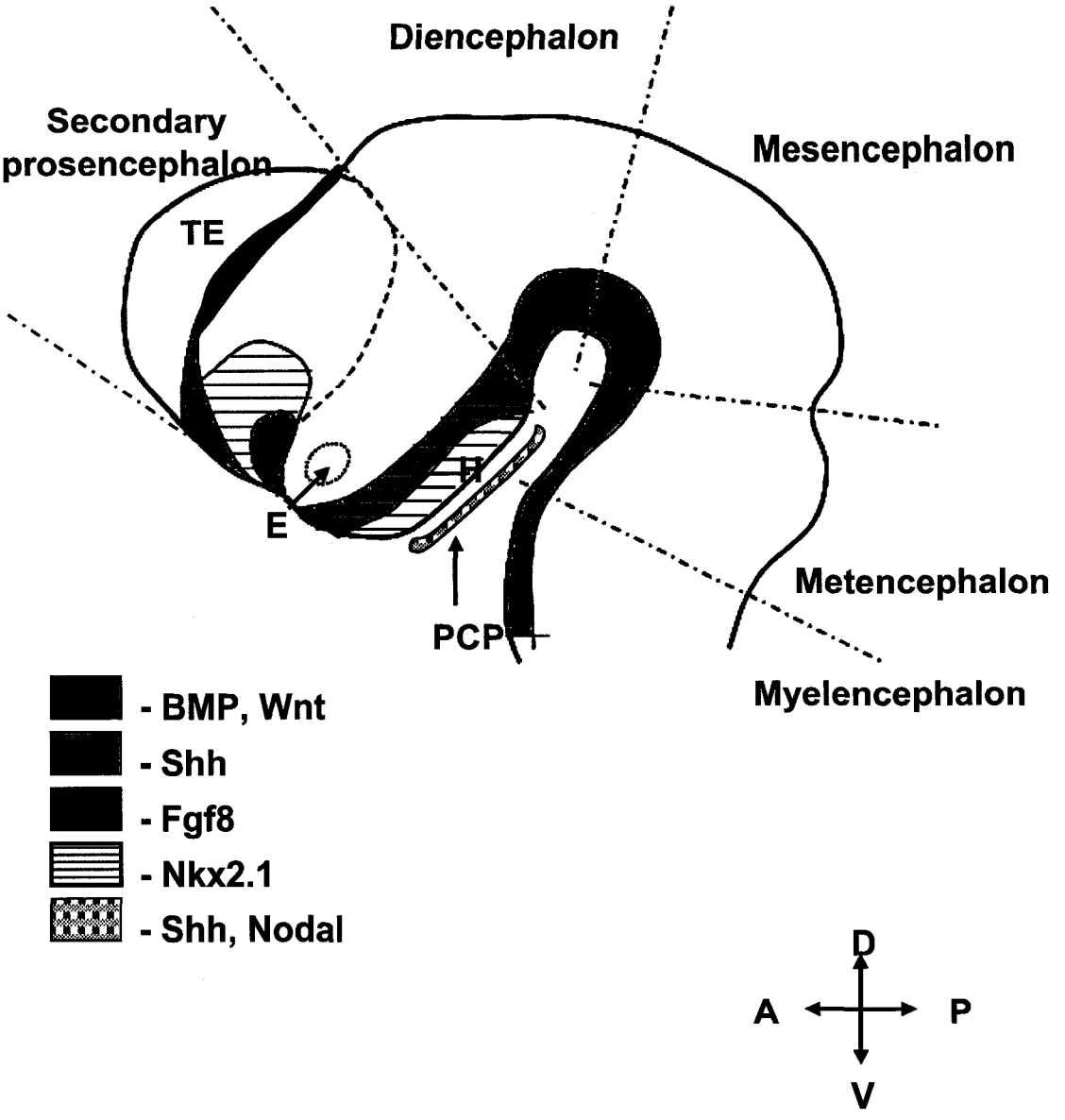

- Shh
- Fgf8
PE
ME
RE
D
L
CP
TE
TE
LP
LT
D
P
A
V

Figure 1.2. Signaling centres regulating patterning of the developing telencephalon. Lateral view of the developing mouse brain, which consists of five major subdivisions (separated by dash-dot lines). Members of BMP and Wnt families regulate patterning of the pallial telencephalic structures from the dorsal midline. Fgf8, Shh, and Nodal signaling from the rostral and ventral midline, as well as from the ventral hypothalamus and prechordal plate (PCP) regulate the expression of the regional transcription factors, such as *Nkx2.1*. Projection of the ventral-posterior border of the telencephalic vesicle is indicated by black dashed line.

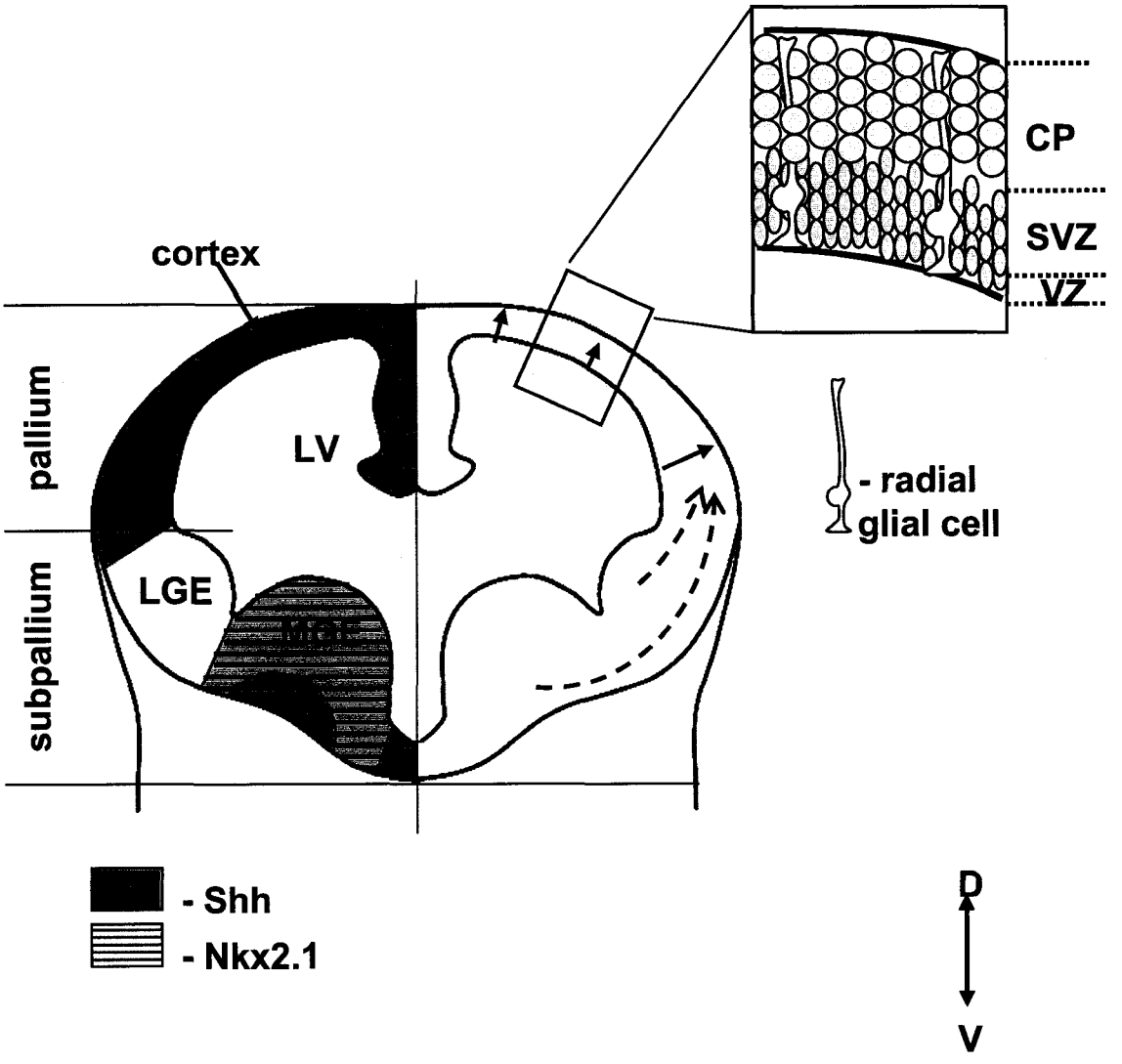

Diencephalon
Secondary prosencephalon
Mesencephalon
TE
H
E
PCP
Metencephalon
Myelencephalon
- BMP, Wnt
- Shh
- Fgf8
- Nkx2.1
- Shh, Nodal
D
A
P
V

(Merkle and Alvarez-Buylla, 2006). At this stage neuroepithelial precursors are multipotent and can generate all cells of the neural tissue: neurons, astrocytes and oligodendrocytes. Neuroepithelial precursors mature into radial glial cells, which serve as a scaffold for radially migrating newly born neurons (Fig. 1.3). Moreover, radial glia perform the function of NSCs (Anthony et al., 2004). NSCs undergo rounds of self-renewing and differentiating cell divisions. Differentiating cell divisions generate a pool of limited neural progenitors, which have limited differentiation potential as they can only generate either neurons or glia. Neurogenic activity of the NSCs and dramatic amplification of the neural progenitors coupled with terminal differentiation and migration generate the structures of the mature CNS. Following differentiation, neural tissue is composed of neurons and glia. Remarkably, increasing evidence indicates that a subpopulation of the astrocytic glial cells in adults represent neural stem cells (reviewed in (Doetsch, 2003)). For example, inducible ablation of the dividing cells expressing astroglial marker glial fibrillary acidic protein (GFAP) abrogates the generation of new neurons in the adult olfactory bulb and dentate gyrus of the hippocampus (Garcia et al., 2004). Thus, differentiated neurons and glia of the brain are generated by neural stem cells, which themselves undergo the process of maturation from early neuroepithelial cells to radial glia and, finally, become astrocytic-like glia in the adult brain.

1.1.3. Origin and general structure of telencephalon

The results of fate mapping studies in mouse, chick, xenopus and other vertebrate species indicate that telencephalic primordia are located at the rostrolateral edge of the

Figure 1.3. Organization of the developing mouse telencephalon at the start of neurogenesis. Diagram of the telencephalic coronal section shows dorsally located pallium and medial and lateral ganglionic eminences of the subpallium. *Shh* expression is restricted to the separate domains of the ventral midline and mantle region of the MGE, whereas *Nkx2.1* is also expressed in the MGE proliferative region. Insert on the right shows the early layered organization of the cortical plate. New cortical projection neurons are generated within the proliferative zone (VZ and SVZ) and radially migrate (blue arrows) to the final destination. The subpallium generates cortical interneurons, which migrate tangentially from MGE and LGE (black dashed arrows) to the cortex. Abbreviations: D – dorsal, LGE – lateral ganglionic eminence, LV – lateral ventricle, MGE – medial ganglionic eminence, SVZ – subventricula zone, V – ventral, VZ – ventricula zone.

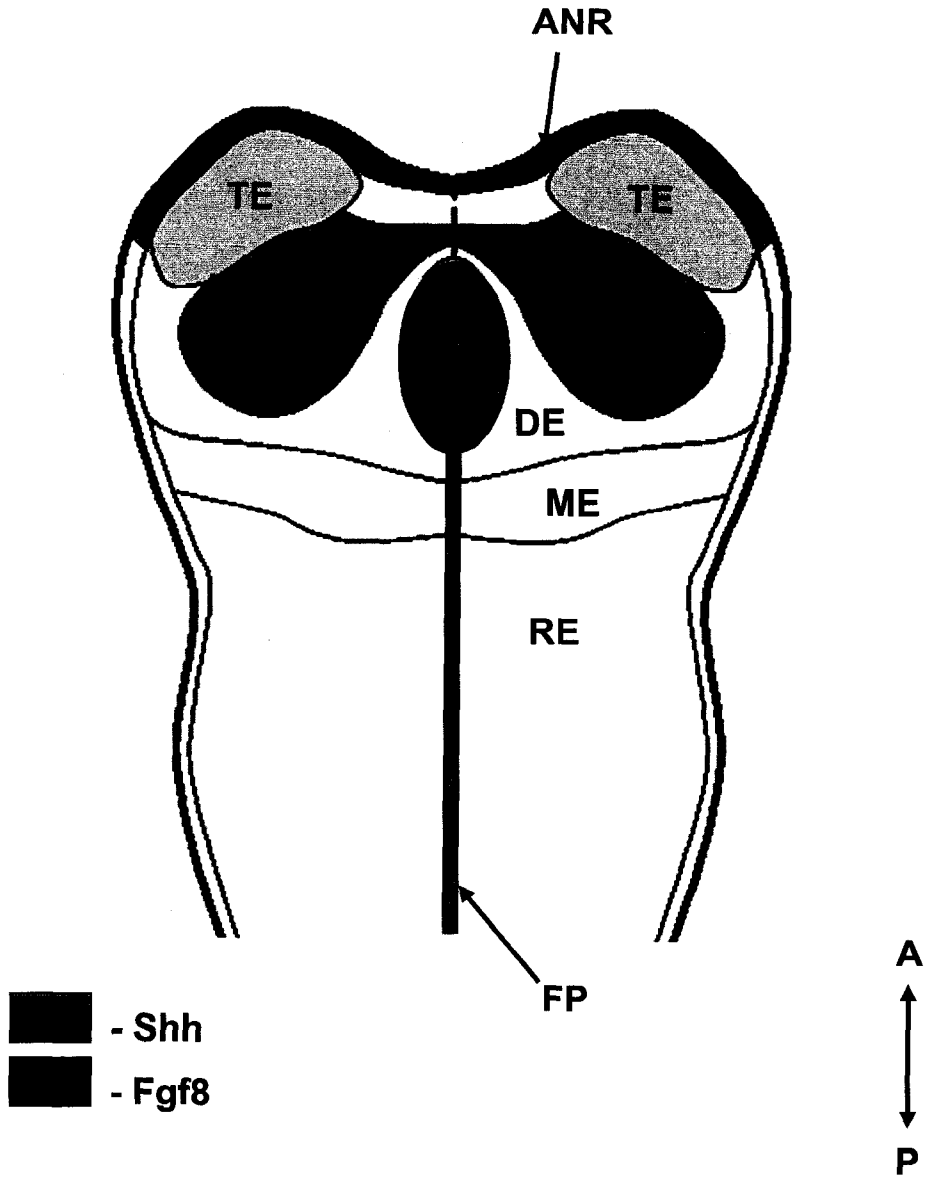

cortex
pallium
subpallium
LV
LGE
CP
SVZ
VZ
- radial
glial cell
- Shh
- Nkx2.1
D
V

neural plate (Fig. 1.4) (Eagleson et al., 1995; Rubenstein et al., 1998; Inoue et al., 2000; Cobos et al., 2001). Telencephalic vesicles develop as evaginations from the dorsal part of the secondary prosencephalon (Fig. 1.1) (Rubenstein et al., 1994; Puelles and Rubenstein, 2003). Dorsal-ventral patterning of each telencephalic vesicle establishes pallial (dorsal) and subpallial (ventral) domains (Fig. 1.3). The pallium is comprised of the developing cerebral cortex, cortical hem and hippocampus, whereas the subpallium is represented by medial (MGE) and lateral (LGE) ganglionic eminences. Pallial progenitors produce excitatory projection neurons of the cerebral cortex, which are generated within the proliferative region of the dorsal telencephalon. These progenitors migrate radially to their final destination within the cortical plate. MGE and LGE are the source of local projection neurons and inhibitory interneurons, which tangentially migrate to the cortex and olfactory bulb. In mature cerebral hemispheres the MGE is transformed into the globus pallidus and the LGE forms striatum. Both of these structures constitute the basal ganglia, which play a fundamental role in the initiation of voluntary movements.

1.1.4. Induction of the anterior neural plate

Development of the morphologically complex and cellularly diverse CNS is the result of the coordinated activity of several signaling pathways. These pathways are reactivated during successive stages of neural morphogenesis, and at the same time they have very distinct temporal functions. Initially, the coordinated activity of several inductive signals emanating from the "primary organizing centres" establish the anterior-

Figure 1.4. Anterior end of the neural plate at the end of gastrulation. The approximate topology of the prospective brain regions is indicated. Fgf8 signaling from the anterior neural ridge (ANR) and Shh from the midline region of the hypothalamus guide telencephalic development. Abbreviations: A – anterior, ANR – anterior neural ridge, DE – diencephalon, E – eye, FP – floor plate, H – hypothalamus, ME – mesencephalon, P – posterior, RE – rhombencephalon, TE – telencephalon.

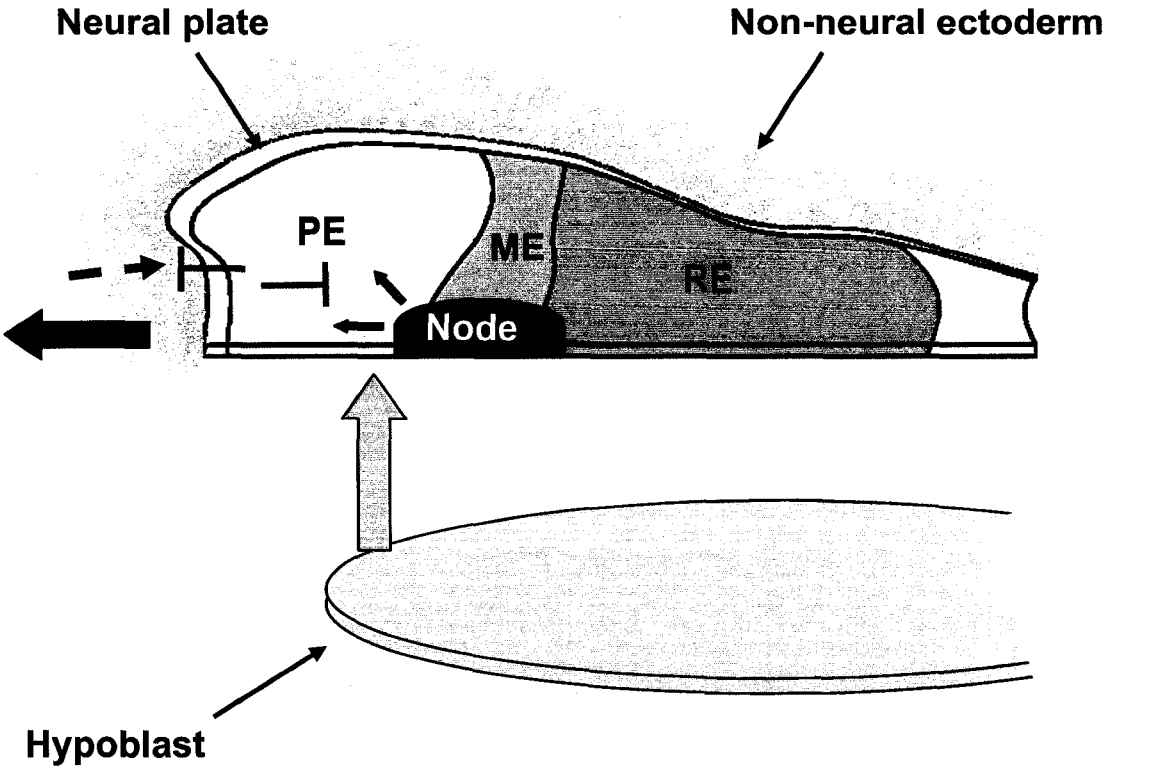

ANR
TE
TE
DE
ME
RE
FP
- Shh
- Fgf8
A
P

posterior and medial-lateral patterning of the neural plate during pre- and early gastrula stage of embryonic development (Fig. 1.4) (Echevarria, et al., 2003; Wilson and Rubenstein,2004). These organizing centres include node (Spemann organizer in amphibians), perineural ectoderm, embryonic mesendoderm and extraembryonic tissues (Beddington and Robertson, 1998; Spemann and Mangold, 2001; Stern, 2001; Echevarria et al., 2003). It has been suggested that Fibroblast growth factor (Fgf) signaling from the extraembryonic anterior visceral endoderm or hypoblast induces the pre-neural state of the anterior epiblast at the time preceding gastrulation (Fig. 1.5) (Stern, 2001; Londin et al., 2005; Stern, 2005). Furthermore, disruption of other anterior visceral endoderm specific genes, such as *Lim1* (Shawlot and Behringer, 1995), *Otx2* (Acampora et al., 1995; Acampora et al., 1997; Rhinn et al., 1998), or *Hesx1* (Dattani et al., 1998; Martinez-Barbera et al., 2000) causes either severe truncation or complete absence of the forebrain structures. Similarly, surgical excision of the AVE severely affects the early patterning of the anterior neuroectoderm (Thomas and Beddington, 1996). These results indicate that inductive signaling from the anterior visceral endoderm is essential during early stages of forebrain development and its disruption results in the absence or severe morphological defects of the telencephalon.

1.1.5. Anterior neural plate is protected from caudalizing factors

During early gastrulation the neural character of prospective forebrain cells is stabilized and protected from the caudalizing activity of the Bone Morphogenic Protein (BMP), retinoid acid and Wnt signaling (Spemann and Mangold, 2001; Wilson and Houart, 2004). On one hand, this is achieved by inducing expression of a number of BMP

Figure 1.5. Induction of the neural plate and establishment of anterior neural character. Signaling from the hypoblast (Fgf) (yellow arrow) induces neural character of the overlying ectoderm. BMP and Wnt signalings from the node (dark blue arrows) and non-neural ectoderm (black dashed arrow) impose caudalizing character of the neural plate. Anterior neural plate is protected from the caudalizing activity by generating BMP and Wnt antagonists (red blunt-ended arrow) and undergoing anterior morphogenetic movement (thick black arrow).


Neural plate
Non-neural ectoderm
PE
ME
RE
Node
Hypoblast
A
P

and Wnt antagonists such as *noggin, chordin, follistatin, dickkopf,* and Frizzled(Frz)-related protein, *Frzb*, in the anterior embryonic tissues (De Robertis et al., 1997; Glinka et al., 1998; Schier and Shen, 2000; Anderson et al., 2002; Rallu et al., 2002a). On the other hand, rostral movement of the anterior neural plate distances the developing forebrain anlage from caudalizing signaling molecules (Fig. 1.5) (Varga et al., 1999; Foley and Stern, 2001). The activity of anti-caudalizing factors and morphogenetic movement allows further refinement of the anterior neural character and subsequent establishment of the coarse pattern of regional transcription factor expression (Lumsden and Krumlauf, 1996). Disruption of these processes leads to severe defects of brain development. For example, mutant mice deficient for BMP antagonists *chordin* and *noggin* develop variable phenotypes characterized by rostral truncation of the brain, holoprosencephaly, and craniofacial defects (Anderson et al., 2002). This and other findings show that inhibition of BMP and Wnt activity during gastrulation is required for forebrain morphogenesis. Thus, expression of BMP and Wnt antagonists and rostral movement of the neural plate protects the anterior end of the neural plate from caudalizing activities.

1.1.6. Early regional specification of the forebrain

Primary organizing centres provide vital cues for the establishment of "secondary" patterning centers in the developing nervous system (Wilson and Rubenstein, 2000; Echevarria et al., 2003). The major secondary organizer of the developing telencephalon, the anterior neural ridge, is located at the junction of the rostral neural plate and surrounding non-neural ectoderm (Fig. 1.4) (Shimamura and

Rubenstein, 1997; Houart et al., 1998). Moreover, signaling emanating from the prechordal plate (Fig. 1.2) and non-neural ectoderm (Fig. 1.5) which are located underneath and along the sides of the developing neural plate, respectively, set up medial-lateral pattering (Wilson and Houart, 2004). Therefore, the secondary signaling centres refine the coarse initial anterior-posterior patterning and induce the expression of region-specific transcription factors.

1.1.7. Anterior neural ridge

The activity of the anterior neural ridge (Fig. 1.4) is crucial for the regional patterning of the developing telencephalon (Shimamura and Rubenstein, 1997; Echevarria et al., 2003). Ablation of the population of cells within the neural and non-neural ectodermal border at mid-gastrulation causes the loss of mRNA expression of the telencephalon-specific homeobox genes *Emx1* and *Dlx2*, as well as the loss of zebrafish acheate-scute (ash) homologue *Zash-1a* (Houart et al., 1998). Similarly, excision of the anterior neural ridge in mouse anterior neural plate explants at somite stage 4 embryos (~E8.25) abolishes the expression of the winged-helix transcription factor brain factor 1 (*BF1*) (Xuan et al., 1995) in the telencephalic anlage (Shimamura and Rubenstein, 1997). In contrast, excised anterior neural ridge restores brain factor 1 expression in the anterior neural plate (Shimamura and Rubenstein, 1997). Moreover, ectopic transplantation of anterior neural ridge cells induces expression of *Emx1* and *Dlx2* in adjacent tissue (Shimamura and Rubenstein, 1997). These studies highlight the requirement for and inductive role of the anterior neural ridge for telencephalic development.

Among the signaling molecules expressed in the anterior neural ridge are several Fgf family members (Shimamura and Rubenstein, 1997; Gimeno et al., 2003; Walshe and Mason, 2003). One of them, *Fgf8*, is expressed in the anterior neural ridge from E8 and has been implicated in anterior neural plate patterning (Crossley and Martin, 1995; Shimamura and Rubenstein, 1997). Similar to the application of the excised anterior neural ridge, beads soaked with recombinant Fgf8 protein rescue the expression of *BF1* in E8.25 mouse anterior neural plate explants (Shimamura and Rubenstein, 1997). Inhibition of Wnt signaling is also important for specification of the developing telencephalon. The secreted Frz-related Wnt antagonist, *Tlc*, is expressed in the anterior neural ridge (Houart et al., 2002). Inhibition of Tlc activity in zebrafish embryos by antisense morpholinos dramatically decreases *Emx1*, *BF1* and *Fgf8* expression in the developing telencephalon (Houart et al., 2002). These studies point to a key role for the anterior neural ridge as a major regional specification and patterning centre of the developing telencephalon.

1.1.8. Prechordal plate

Another important signaling centre controlling the specification of the neural plate is located in the prechordal plate, which is located under the anterior end of the neural ectoderm (Fig. 1.2) (Dale et al., 1997; Pera and Kessel, 1997; Shimamura and Rubenstein, 1997; Rubenstein et al., 1998). Prechordal plate removal at E7.5 in mouse and at Hamburger-Hamilton stage 5 (HH5) chick embryos leads to the loss of expression of the ventral-specific marker, *Nkx2.1*, in the medial neural plate (Pera and Kessel, 1997; Shimamura and Rubenstein, 1997). One of the secreted signaling molecules emanating

from the prechordal plate is Sonic hedgehog (Shh). Shh is a crucial regulator of ventral neural tube patterning at all levels of the neural axis (Chiang et al., 1996; Rallu et al., 2002a; Lupo et al., 2006). Loss of Shh signaling from the prechordal plate and notochord leads to loss of the ventral midline along the entire neural axis (Chiang et al., 1996). The severe defect in telencephalic morphogenesis in Shh deficient mice is characterized by the development of a single telencephalic vesicle and the absence of ventral markers, such as *Otx2* (Chiang et al., 1996). The resultant phenotype of holoprosencephaly is one of the most frequently observed brain defects in humans (Wallis and Muenke, 1999). A similar phenotype is displayed by the zebrafish mutant *Cyclops* (Sampath et al., 1998). The gene *cyc*, which is affected by this mutation, encodes a transforming growth factor-β-related molecule similar to mouse *Nodal*. Moreover, Shh is a mediator of Nodal activity since it can rescue the expression of *Nk2.1b* (homologue of *Nkx2.1*) in the ventral telencephalon of Nodal signaling mutants (Rohr et al., 2001). These results show that Nodal and Shh signaling from the prechordal plate are essential for specification of the developing telencephalon.

In summary, early forebrain development is controlled by signaling from the structures surrounding the rostral end of the neural plate, which induces its anterior character. This initial patterning is further refined and protected from caudalizing activities. Simultaneously, signaling centres that are established within the neural plate specify the development of discrete brain regions. Defective signaling during this stage of development leads to the absence of or a dramatically reduced and grossly mispatterned telencephalon.

1.1.9. Regional transcription code

Activities of the patterning centres during the initial stage of CNS development specify the anterior-posterior and medial-lateral grid of the combinatorial expression of regional transcription factors (Campbell, 2003). Before the start of neurogenesis (~E11) distinct dorsal (pallial) and ventral (subpallial) structures are established within the developing telencephalon (Fig. 1.3). These territories express specific sets of transcription factors (transcription code) (Schuurmans and Guillemot, 2002). *Neurogenin 1 (Ngn1)* and *Neurogenin 2 (Ngn2)* are expressed in the pallial ventricular zone, whereas *Mash1* expression is restricted to the neural progenitors of the subpallium (Guillemot and Joyner, 1993; Sommer et al., 1996). Other dorsal markers, such as Pax6 and Emx2 homeodomain transcription factors, are required for morphogenesis of the cerebral cortex where they establish a complementary anterior-posterior gradient of expression (Muzio et al., 2002b; Grove and Fukuchi-Shimogori, 2003). Simultaneous deletion of *Emx2* and *Pax6* in mouse dorsal telencephalon abrogates the expression of the pallial markers *Tbr2, Ngn1* and *Ngn2* and result in the dorsal expansion of the striatal markers *Mash1, Islet1*, and *GAD65/67* (Muzio et al., 2002a). Transcription factors Nkx2.1, Dlx2, and Gsh2 mark the region of the ventral telencephalon where they establish the identity of the ventral neural progenitors (Sussel et al., 1999; Toresson et al., 2000; Corbin et al., 2003). For example, Nkx2.1 is required for patterning and specification of the developing ventral forebrain. Nkx2.1 deficiency leads to the loss of *Shh* expression in the basal forebrain, respecification of the MGE into LGE and dorsalization of the ventral telencephalon (Sussel et al., 1999). Nkx2.1 is also required for specification of MGE derived

cholinergic, parvalbumin-, somatostatin- and neuropeptide Y-expressing interneurons at later time points of development (Sussel et al., 1999; Xu et al., 2004). These results indicate that Nkx2.1 is indispensable for ventral telencephalic patterning. Thus, homeodomain and basic helix-loop-helix (bHLH) transcription factors demarcate distinct dorsal and ventral domains within the developing telencephalon where they are required for specification and maintenance of neuronal and glial progenitors of the corresponding regions.

1.1.10. Telencephalic signaling centres

Each region of the developing telencephalon displays distinctive regional and temporal responsiveness to inductive patterning signals (Kobayashi et al., 2002). These signals are generated by midline signaling centres within the telencephalon. Closure of the neural tube transforms the anterior neural ridge into the rostral midline, whereas fusion of the lateral edges of the neural plate creates the dorsal midline. The ventral midline develops from the middle area of the neural plate.

1.1.11. Dorsal midline

The dorsal midline expresses several members of the BMP family (Fig. 5) (Furuta et al., 1997). The high degree of compensation among different BMPs precludes conclusive establishment of their role in loss-of-function mutant models. Telencephalon-specific deletion of the *BmpR1a* in mice disrupts specification of the most dorsomedial derivative of the dorsal midline: the choroid plexus (Hebert et al., 2002). At the same time, application of BMP2 and BMP4 in explant cultures induces expression of the dorsal

midline marker *Msx1* (Furuta et al., 1997). Moreover, activity of the *Emx2* enhancer in mouse dorsal telencephalon is regulated by the tranducers of BMP and Wnt signaling, Smad and Tcf, respectively (Theil et al., 2002). Zebrafish *Swirl*/BMP2b deficient mutants display loss of dorsal and expansion of ventral neurons at all levels of the neural axis including developing forebrain (Barth et al., 1999).

Besides BMPs, several genes of Wnt signaling are expressed in the dorsal midline (Parr et al., 1993; Lee et al., 2000; Backman et al., 2005). Loss of either *Wnt3a* or *Tcf,* transducers of Wnt signaling (Huelsken and Behrens, 2002), abrogates hippocampal development (Galceran et al., 2000; Lee et al., 2000). Canonical Wnt signaling is mediated by β-catenin (Huelsken and Behrens, 2002). Telencephalon-specific *β-catenin* inactivation before E9 in mice results in the downregulation of the dorsal markers *Emx1*, *Emx2* and *Ngn2* as well as dorsal expansion of ventral markers (Backman et al., 2005). In contrast, ectopic activation of *β-catenin* in the ventral telencephalon reduces expression of the subpallial markers *Nkx2.1, Dlx2, Gsh2, Olig2* and *Mash1* and causes a ventral shift of the pallial markers *Pax6* and *Ngn2* (Backman et al., 2005). These results indicate that the activity of the BMP and Wnt signaling pathways is required for the establishment of the dorsal telencephalic midline and dorsal-ventral patterning of the developing telencephalon.

1.1.12. Rostral midline

The rostral midline within the region of the commissural plate and lamina terminalis expresses several members of the Fgf family, which regulate telencephalic dorsal-ventral patterning and specify positional identity of cortical neurons (Fig. 1.1,

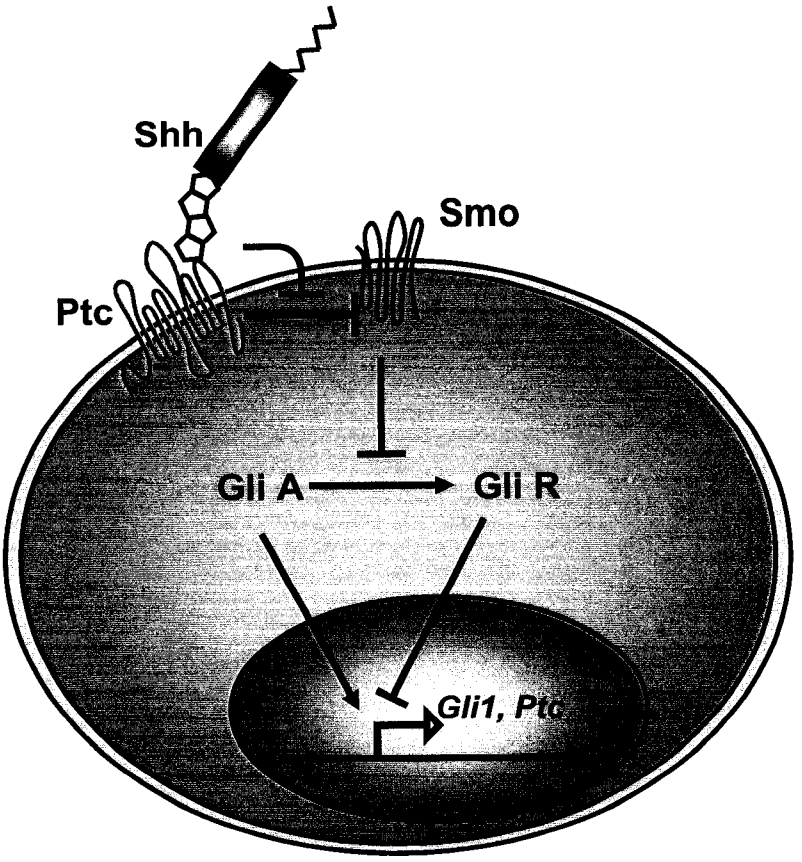
Fig.1.2) (Fukuchi-Shimogori and Grove, 2001). Conditional mutation analysis revealed that reduction of Fgf8 signaling leads to a reduced general size of the developing telencephalon, as well as a specific defect in the ventral telencephalon (Meyers et al., 1998; Storm et al., 2006). Furthermore, combined conditional inactivation of Fgf Receptors (FgfR) leads to a reduced size of ventral structures, decreased expression of the ventral markers *Nkx2.1* and *Dlx2*, and expansion of the dorsal markers *Pax6* and *Emx* into the ventral regions of the ventral telencephalon (Gutin et al., 2006). Fgf8 signaling also initiates regionalization of the ventral telencephalon by inducing the expression of the homebox transcription factor *Nkx2.1* (Fig. 1.2) (Crossley et al., 2001; Shinya et al., 2001; Storm et al., 2006), which then induces the expression of *Shh* within the MGE (Sussel et al., 1999; Jeong et al., 2006; Storm et al., 2006). These results show that Fgf8 activity from the rostral midline regulates specification and patterning of the developing telencephalon.

1.1.13. Ventral midline

Specification and patterning of the subpallial telencephalic region is also governed by signaling from the telencephalic ventral midline (Lupo et al., 2006), which is established by Shh and BMP7 signaling from the prechordal mesendoderm (Dale et al., 1997). Ventral telencephalic midline tissue can induce the expression of *Evf-1*, which labels neural progenitor in the ventral subventricular zone (Kohtz et al., 1998). As a result of prechordal mesendoderm inductive activity, *Shh* expression appears initially in the ventral midline of the hypothalamus and later (around E9.5) within the telencephalon (Shimamura et al., 1995). Ectopic activation of Shh signaling *in vivo* and in the explant

cultures also results in specification of ventral neuronal progenitors and up-regulates the expression of *Nkx2.1*, *Dlx2* and *Gsh2* (Muller et al., 2001; Ren et al., 2002; Dimova et al., 2003; Korenjak and Brehm, 2005; Iwanaga et al., 2006). Moreover, specification of distinct MGE and LGE identities within the subpallium shows temporal changes in the responsiveness to Shh signaling (Kohtz et al., 1998; Gunhaga et al., 2000; Fuccillo et al., 2004). Telencephalon-specific deletion of *Smoothened (Smo)*, which is an obligate transmembrane receptor of Shh signaling (Fig. 1.6), results in the loss of early ventral telencephalic patterning and complete dorsalization (Fuccillo et al., 2004). This and other studies have also shown the requirement of Shh signaling for differentiation of GABAergic interneurons and oligodendrocytes during neurogenesis (Machold et al., 2003; Fuccillo et al., 2004). Transcriptional mediators of Shh signaling are zinc-finger Gli transcription factors (Matise and Joyner, 1999). Deletions of *Gli1* or *Gli2*, which are primary transcriptional activators in response to Shh pathway stimulation, display relatively normal ventral telencephalic patterning (Park et al., 2000). In contrast, deficiency of Gli3, which acts as a transcriptional repressor in the absence of Shh activity, causes severe dorsal-ventral patterning defects (Theil et al., 1999; Tole et al., 2000). Expression of the dorsal telencephalic markers is lost in these animals, whereas ventral markers are expanded into the dorsal regions. This phenotype is opposite to the patterning defect of Shh deficient embryos, which suggests that mutual repression between Shh and Gli3 may establish dorsal-ventral patterning (Tole et al., 2000; Rallu et al., 2002a). Indeed, combined deletion of *Shh* or *Smo* and *Gli3* practically restores normal dorsal-ventral development in the telencephalon (Rallu et al., 2002b). This observation indicates that reciprocal interaction between Shh and Gli3 represents a crucial mechanism

Figure 1.6. Diagram of the Shh signaling pathway. Shh binding to the Ptc receptor derepresses Smo, which inhibits formation of the repressive form of Gli (GliR) and promotes accumulation of the activating form of Gli (GliA). GliA translocates to the nucleus and activates transcription of the target genes.


Shh
Smo
Ptc
Gli A
Gli R
Gli1, Ptc

of dorsal-ventral telencephalic patterning. Moreover, these results suggest that other signaling mechanisms are employed to pattern ventral telencephalon.

In summary, early telencephalic development is induced before gastrula stage by signaling from the structures surrounding the prospective neural plate. Primary patterning centres establish initial anterior-posterior and medial-lateral domains within neural plate and protect anterior neural identities from caudilizing signaling. Moreover, these centres initiate the development of the secondary signaling centres within the developing telencephalon, which control regional specification. Ultimately, before the start of neurogenesis, the telencephalon has distinct dorsal and ventral structures, which express a unique set of patterning genes. These genes create a combinatorial transcription code of neuronal and glial progenitors, which will shape the cerebral hemispheres.

1.2. Embryonic eye development

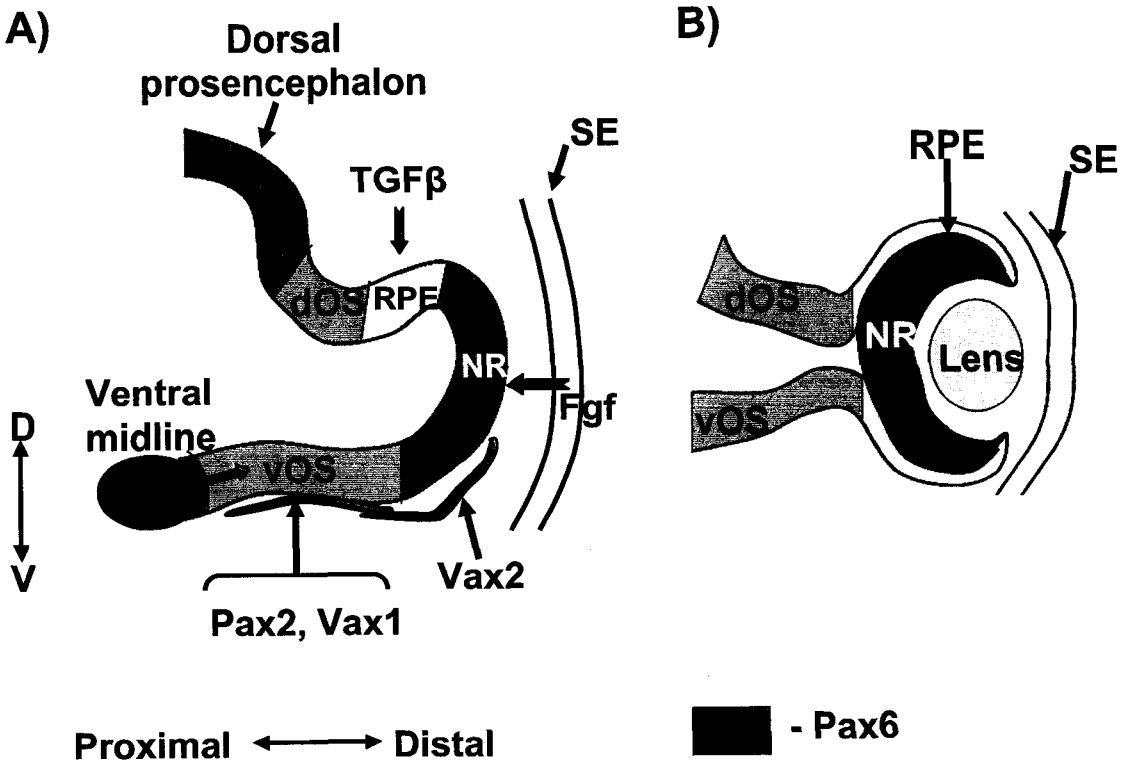
1.2.1. Morphogenetic transformations during early eye patterning

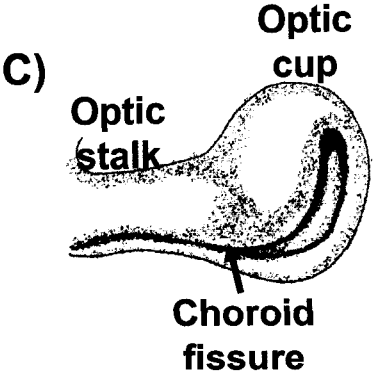
Early eye development is intimately linked to the morphogenesis of the anterior end of the neural tube. Signaling from extraembryonic tissue surrounding the anterior neural plate regulates the initial inductive events within the anterior neural plate, as described earlier. The primordium of the developing eyes is initially represented by a single eye field, which occupies the position between the telencephalic anlage rostrally and diencephalic cells caudally (Fig. 1.4) (Woo and Fraser, 1995; Varga et al., 1999; England et al., 2006). This tissue already expresses a unique combination of transcription factors, such as Rx, Pax6, Six3, Otx2, and Hesx1, which guide the subsequent steps of

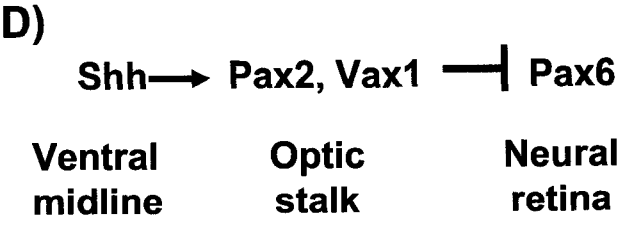
eye specification and morphogenesis (Gruss and Walther, 1992; Simeone et al., 1993; Hermesz et al., 1996; Mathers et al., 1997; Bovolenta et al., 1998). Complex morphogenetic movements of the diencephalic and telencephalic primordia lead to the eyefield separation (England et al., 2006). Shh and Nodal-related genes *Cyclops*, *one-eyed pinhead* play crucial roles in this process (Chiang et al., 1996; Hammerschmidt et al., 1996; Strahle et al., 1997; Rebagliati et al., 1998; Sampath et al., 1998). Loss of either one of these genes not only severely affects telencephalic development but also leads to the development of single-eye mutants, known as cyclops, which is found in humans and animals (Wallis and Muenke, 1999). Next, optic vesicles evaginate in the ventrolateral hypothalamic region. This leads to the formation of the optic vesicle and optic stalk and establishment of the proximal-distal patterning of the developing eyes (Fig. 1.7) (Lupo et al., 2006). Homeodomain transcription factors are essential mediators of this process. *Pax2* and the ventral anterior homeobox genes *Vax1* and *Vax2* are expressed in the ventroproximal part of the optic vesicle, whereas *Pax6* marks the dorsodistal optic vesicle (Nornes et al., 1990; Gruss and Walther, 1992; Hallonet et al., 1999). *Pax2* deletion leads to the development of coloboma, which is the failure of the choroid fissure closure (Fig. 1.7C) (Torres et al., 1996). *Pax6* mutant mice have no eyes and failure of lens development (Hill et al., 1992). Thus, similar to telencephalic development, coordinated activity of the signaling pathways, including Shh, leads to the initial specification of eye primordia, which express distinct homeodomain transcription factors.

Contact of the distal end of the optic vesicle with the lens placode region of the surface ectoderm induces invagination of both structures and formation of the double-layered optic cup and lens vesicle (Fig. 1.7) (Hyer et al., 2003). Invagination at the distal

Figure 1.7. Early eye patterning. A) The optic vesicle evaginates from the neural epithelium of the lateral-ventral prosencephalon and reaches the surface ectoderm at E9. TGFβ and Fgf signaling cooperate with Shh in eye morphogenesis. Fate maps of the different eye regions are indicated in colors. B) The optic cup forms after invagination of the optic vesicle at the distal end by E11. C) Proximal (optic stalk) and distal (optic cup) subdivisions of the developing eye. D) Molecular signaling cascade from the ventral midline regulating early eye morphogenesis. Abbreviations: D – dorsal, dOS – dosral optic stalk, NR – neural retina, RPE – retinal pigmented epithelium, SE – surface ectoderm, V – ventral, vOS – ventral optic stalk.


A)
Dorsal prosencephalon
SE
TGFβ
dOS
RPE
NR
Fgf
Ventral midline
D
V
vOS
Vax2
Pax2, Vax1
B)
RPE
SE
dOS
NR
Lens
vOS
Proximal ⟷ Distal
- Pax6


C)
Optic cup
Optic stalk
Choroid fissure


D)
Shh → Pax2, Vax1 ⊣ Pax6
Ventral midline
Optic stalk
Neural retina

end of the optic vesicle extends along the ventral side of the optic stalk to form the choroid fissure, which subsequently closes along the length of the optic stalk and at the ventral optic cup, surrounding the optic vessels (Fig. 1.7C). Failure of the optic fissure closure leads to the development of coloboma in humans and in animal models (Gregory-Evans et al., 2004). The outer layer of the optic cup generates the retinal pigmented epithelium, the inner layer forms the neural retina, and the optic stalk is transformed into the optic nerve. At this stage *Pax2* and *Vax1* are expressed in the optic nerve, *Vax2* is limited to the ventral optic cup, and *Pax6* labels the neural retina. This morphogenetic process is complete by ~E11 in mice.

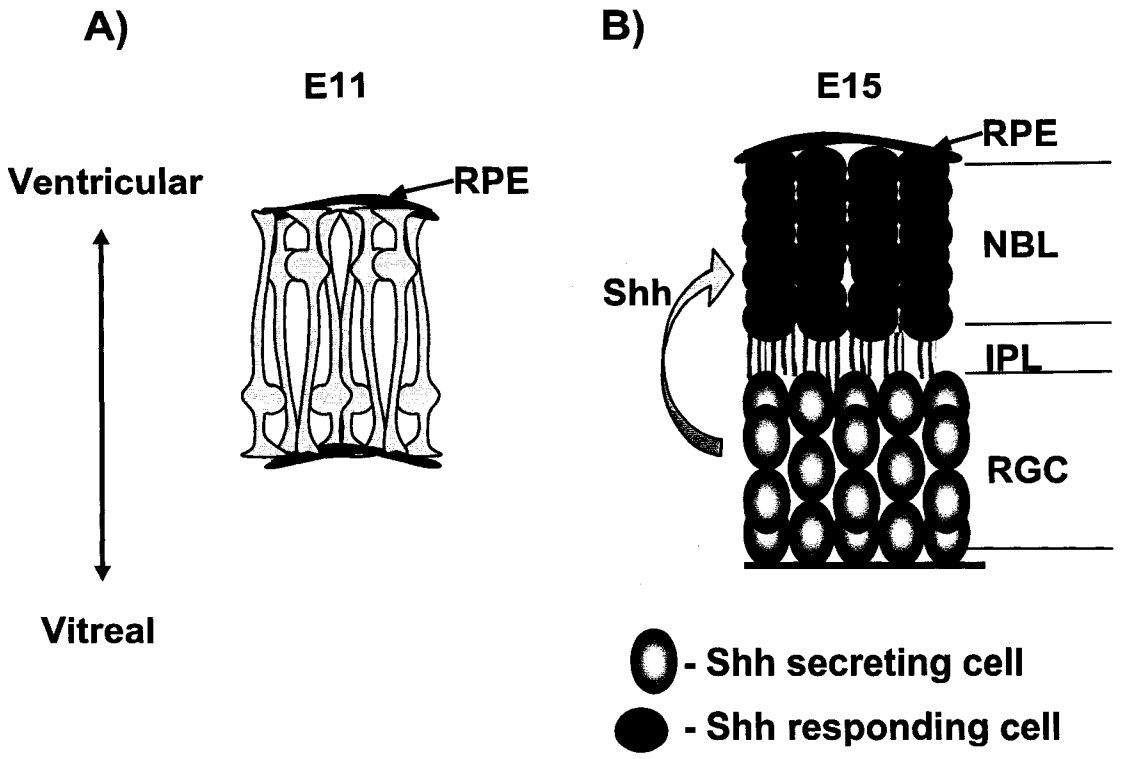
Shh signaling from the ventral midline of the hypothalamus controls the process of early proximal-distal patterning, as well as dorsal-ventral regionalization within the neural retina. Shh induces the expression of *Vax1, Vax2*, and *Pax2*, which in turn repress *Pax6* (Fig. 1.7) (Chow and Lang, 2001; Take-uchi et al., 2003; Gregory-Evans et al., 2004). Ectopic expression of *Shh* induces *Pax2* in the distal structures and leads to the repression of *Pax6* (Ekker et al., 1995; Zhang and Yang, 2001b). Later, Shh signaling from the telencephalon is also required for the development of the ventral optic structures. Ablation of the telencephalon-specific transcription factor, *BF1*, results in the loss of *Shh* in the ventral telencephalon and absence of the optic stalk and ventral optic cup (Huh et al., 1999). Clearly, ventral proximal structures of the developing eye are very sensitive to any changes in Shh signaling from the forebrain midline. Furthermore, there are complex interactions between Shh and retinoic acid, BMP and Fgf signaling during early retinal patterning (Chow and Lang, 2001; Lupo et al., 2006). Fgf signaling from the surface ectoderm induces neural retinal character of the distal optic vesicle (Fig. 1.7A)

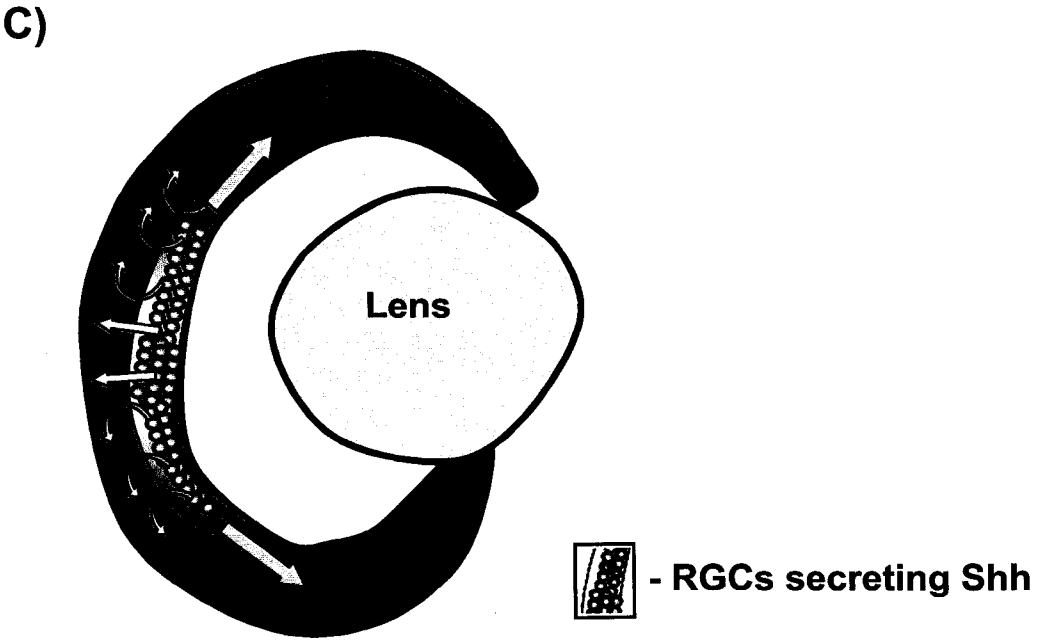
(Pittack et al., 1997). At the same time, TGFβ signaling from periocular mesenchyme specifies retinal pigmented epithelium by inducing expression of microphthalmia-associated transcription factor (Fig. 1.7A) (Fuhrmann et al., 2000). Later BMP activity is required for specification of the dorsal retinal domains. Disruption of BMP signaling leads to the loss of dorsal retinal markers, such as *Tbx5*, dorsal expansion of the ventral-specific *Vax2*, and aberrant eye morphology (Murali et al., 2005). Though, the exact epistatic interaction between Shh and BMP signaling is not clear presently and appears to be species specific. On one hand, activation or inhibition of Shh can induce and decrease BMP expression, respectively, in chick embryos (Zhang and Yang, 2001b). On the other hand, *Pax2* induction by BMP is mediated by Shh in mouse retinae (Weston et al., 2003). Taken together, these results point to the crucial role of Shh during multiple stages of early eye development.

1.2.2. Early retinogenesis

Early eye patterning events are followed by the complex process of retinogenesis in the neural retina (reviewed in (Esteve and Bovolenta, 2006)). Initial differentiation of the pseudostratified neuroepithelial retinal cells is believed to be induced by Fgf signaling from the optic stalk organizer (Martinez-Morales et al., 2005). The first postmitotic retinal ganglion progenitor cells appear in the centre of the retina (Fig. 1.8C). The wave of differentiation then spreads from the centre to the periphery of the retina. Committed progenitors transiently express the bHLH transcription factor *Math5* (Wang et al., 2001). Terminal differentiation of the retinal ganglion cells is marked by the appearance of *Islet1* expression (Yamada et al., 1993). An important step in retinogenesis is the

Figure 1.8. Early retinogenesis. A) E11 prospective retinal neuroepethelium consists of one layer of dividing progenitor cells. At E15, neural retina consists of a layer of postmitotic retinal ganglion cells (RGC) which secret Shh, and a neuroblastic layer (NBL) which consists of proliferating progenitors and committed precursors. These two layers are separated by the inner plexiform layer. RPE – retinal pigmented epithelium. Differentiation of the Shh-secreting RGCs starts at the centre of the retina and spreads to the periphery (arrows).


A)
E11
Ventricular
RPE
Vitreal
B)
E15
RPE
NBL
Shh
IPL
RGC
- Shh secreting cell
- Shh responding cell


C)
Lens
- RGCs secreting Shh

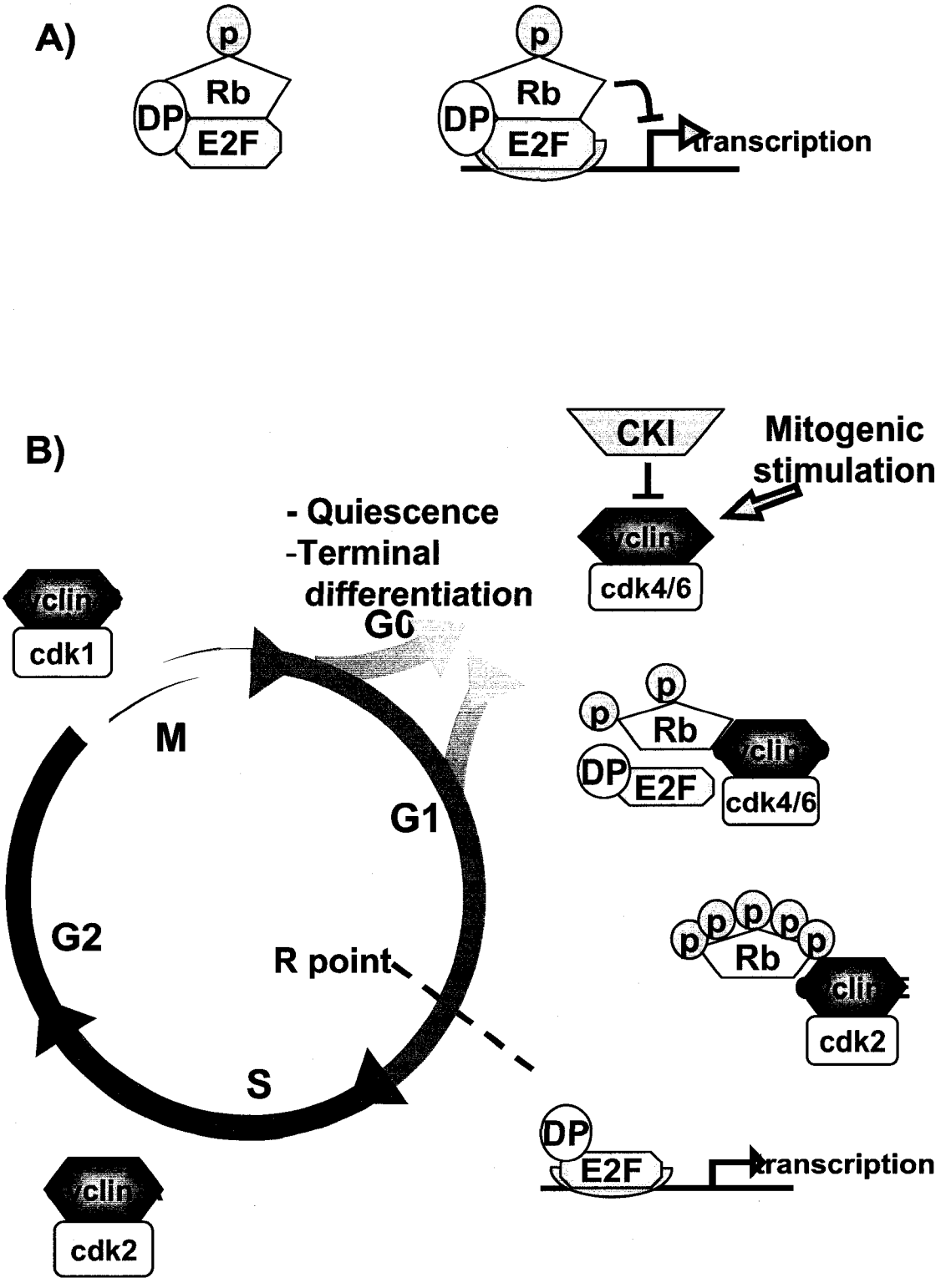
induction of *Shh* expression in retinal ganglion cells. Shh then induces proliferation of retinal progenitors in the outer nuclear layer and at the same time provides negative-feedback signaling to limit the development of retinal ganglion cells (Zhang and Yang, 2001a; Wang et al., 2005). Shh also regulates differentiation of later born retinal neurons and cell survival (Wallace, 2007). During retinogenesis, committed precursors express cell type specific bHLH and homeodomain transcription factors. Ultimately, seven major retinal cell subtypes are generated during sequential competence stages, and form three nuclear layers of the mature retina (Livesey and Cepko, 2001; Wallace, 2007). In murine retina, the process of retinogenesis continues after birth and is completed by postnatal day (P) 11 (Young, 1985).

Taken together, early eye development is intimately coupled with forebrain morphogenesis. The same signaling pathways regulate initial inductive events and subsequent specification and patterning. Shh signaling is required for specification and early eye pattering, as well as for control of proliferation, cell survival and fate determination during retinogenesis.

1.3. Retinoblastoma (Rb)/E2F pathway in cell cycle regulation and development

Complex morphological transformations of the CNS during early stages of development are tightly coordinated with extensive proliferation and initiation of differentiation within the neural tissue. During proliferation, each cell proceeds through distinct cell cycle phases (Fig. 1.9). The cell enters the cell cycle at G1 (gap phase 1)

Figure 1.9. Rb/E2F pathway in cell cycle regulation. A) Rb represses transcription by sequestering activating E2Fs (left) or as part of the repressive complex, which binds to the target promoters (right). B) Diagram represents phases of the cell cycle and its regulation by CKI-cyclin-cdk-Rb-E2F machinery. DP – dimerization partner.


A)
p
Rb
DP
E2F
p
Rb
DP
E2F
transcription
B)
CKI
Mitogenic stimulation
clin
cdk4/6
- Quiescence
-Terminal differentiation
G0
clin
cdk1
M
G1
G2
R point
S
p
p
Rb
clin
DP
E2F
cdk4/6
p
p
p
p
p
p
Rb
clin
cdk2
DP
E2F
transcription
clin
cdk2

when it grows and prepares for DNA synthesis. Next, duplication of the genetic material takes place during S phase (DNA synthesis). Then, during the time of G2 (gap phase 2) the cell intensely grows and synthesizes proteins necessary for an upcoming cell division. Finally, mitotic separation of chromosomes and ensuing cell division occurs in M phase, after which the cells re-enter G1 phase, thus, completing the cycle. During this phase cells can become quiescent in G0 phase and undergo terminal differentiation.

Proteins that regulate proliferation affect several aspects of neurodevelopment. First, the overall number and length of cell cycles influence the size of the developing structures. For example, deletion of the cell cycle inhibitor $p27^{Kip1}$ in mice results in a 30% increase in telencephalic neuronal cell density (Fero et al., 1996). Second, precise regional control of neural precursor proliferation generates morphologically distinct structures within the developing nervous system (ex. cortex dorsally vs ganglionic eminences ventrally). In this case proliferation is thought to be controlled by local patterning molecules (reviewed in (Fuccillo et al., 2006)). The proto-oncogene, *N-myc*, stimulates proliferation of cerebellar granule neuronal precursors as direct transcriptional target of Shh/Gli signaling (Kenney et al., 2003), whereas β-catenin promotes cell cycle re-entry of cortical neural progenitors at the expense of differentiation (Chenn and Walsh, 2002). Third, proliferating neural stem cells and neural progenitors change their differentiation potential depending on stage of neurogenesis or cell cycle. For example, different neuronal subtypes are generated at the beginning and at the end of neurogenesis (McConnell and Kaznowski, 1991; Shen et al., 2006). Additionally, it has been shown that the generation of neurons precedes glial differentiation *in vivo* and *in vitro* (Qian et al., 2000; Marshall et al., 2003; Vaccarino et al., 2007). Finally, the fate of neural

progenitors is determined at the time of terminal mitosis (Takahashi et al., 1999; Cremisi et al., 2003), therefore, the genes that regulate proliferation play crucial roles in development of the CNS. One of the key components of the cell cycle regulatory machinery is Rb/E2F pathway.

1.3.1. Rb family of proteins

Retinoblastoma is a heritable malignant tumor of the retina, which occurs during early childhood and accounts for 3% of all childhood cancers (CanadianCancerSociety, 2007). The genetic locus responsible for the development of this cancer was localized to Chromosome 13 (Cavenee et al., 1985). Cloning of the gene and identification of the *Rb* loss-of-function mutations established *Rb* as the first discovered human tumor suppressor gene (Friend et al., 1986; Huang et al., 1988; Yandell et al., 1989; Goodrich and Lee, 1990). A tumor suppressive function of Rb was supported by the fact that *Rb* mutations or deletions are associated with at least one third of human tumors, including osteosarcoma, bladder carcinoma, and small-cell lung carcinoma (Horowitz et al., 1990; Liu et al., 2004). Subsequent extensive studies and generation of the first targeted *Rb* deletion mutant mice established the key requirement for Rb as a negative regulator of cell cycle progression and for the development of a number of body systems (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992; Lipinski and Jacks, 1999; Galderisi et al., 2006; Khidr and Chen, 2006).

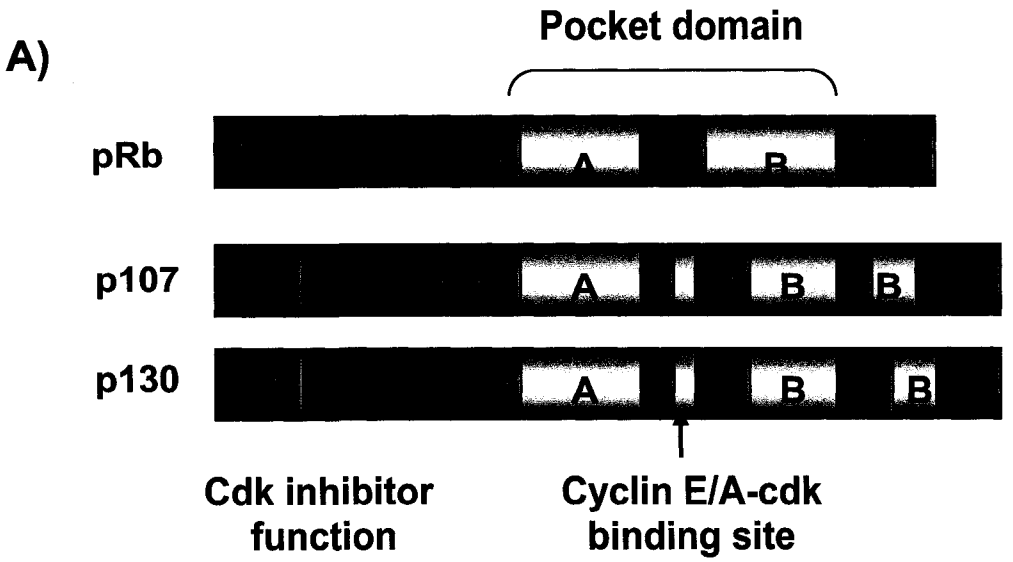
Rb is the founding member of the Rb family of proteins, which also includes p107 and p130 (Du and Pogoriler, 2006; Vidal et al., 2007). All of these proteins share genomic sequence and protein structure similarity, which is higher between p107 and

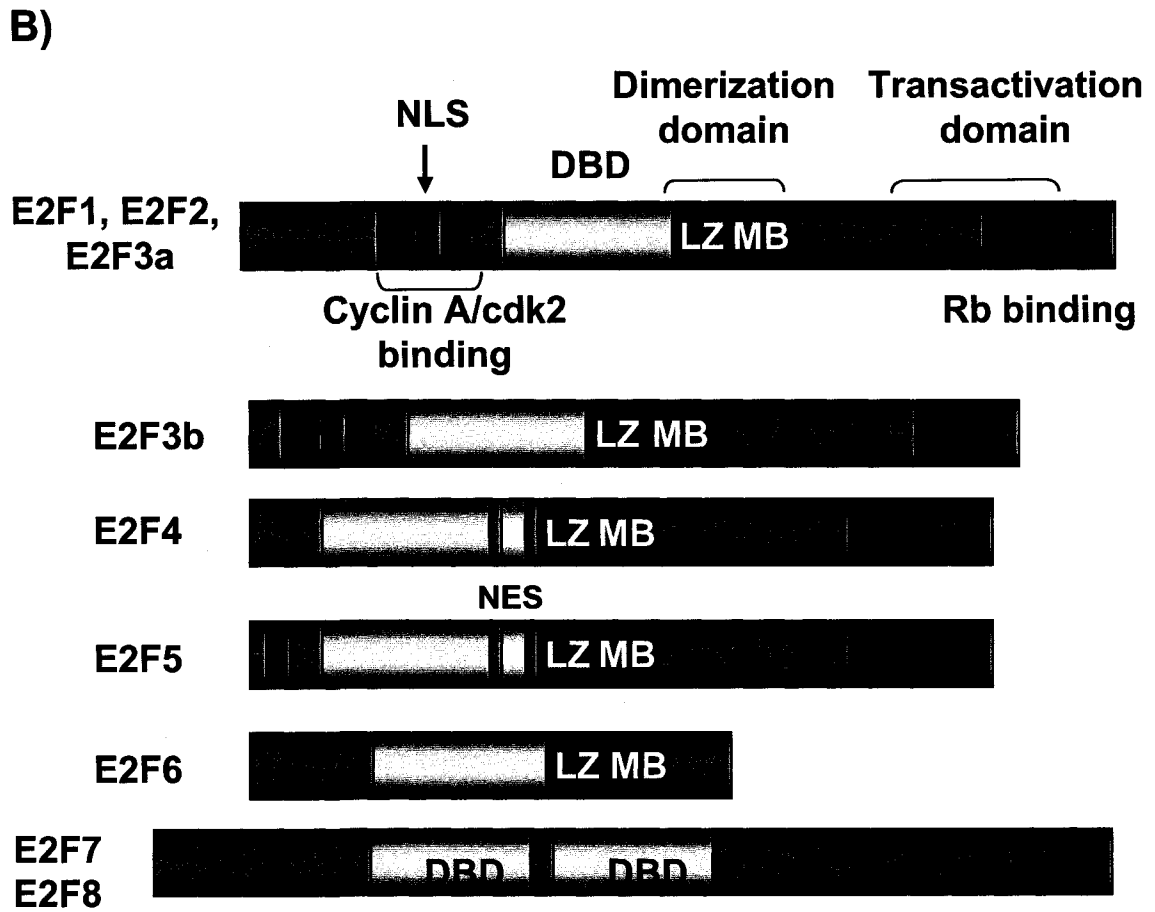
p130 (Fig. 1.10) (Classon and Dyson, 2001). The presence of the conserved pocket region, which consists of A and B domains separated by a spacer region, is a common feature of these proteins (Claudio et al., 2002). The Rb pocket region is required for the protein-protein interaction of Rb with E2F transcription factors (Hiebert et al., 1992; Nevins, 1992, 1998; Lee et al., 2002a). In addition to the E2F binding site, Rb pocket proteins contains an LXCXE motif, which is involved in interactions with viral oncoproteins, D-type cyclins, and chromatin modifying proteins (Whyte et al., 1988; Dyson et al., 1989; Chellappan et al., 1992; Dowdy et al., 1993; Lee et al., 1998; Stiegler et al., 1998; Wang et al., 1998; Dahiya et al., 2000; Dick et al., 2000; Singh et al., 2005). Such a broad spectrum of Rb interacting partners places it in the centre of multiple regulatory pathways controlling proliferation and other cellular processes.

1.3.2. Regulation of pocket protein activity

The level of Rb protein (pRb) does not fluctuate during the cell cycle, whereas p130 and p107 expression exhibit cell cycle phase-dependent changes, such that during G0 phase p130 is highly expressed and the level of p107 is low, but their expression pattern is reversed when cells progress to S phase (Classon and Dyson, 2001). The regulation of pocket protein activity is achieved by post-translational modifications. The activity of pRb is mainly regulated by phosphorylation, though other mechanism, such as acetylation has been suggested (Chan et al., 2001; Giacinti and Giordano, 2006). pRb has multiple phosphorylation sites, which regulate its activity (Ezhevsky et al., 2001). In quiescent cells pRb is hypophosphorylated, while during G1 phase of the cell cycle pRb becomes progressively phosphorylated (Mittnacht et al., 1994).

Figure 1.10. Protein structure of Rb and E2F family members. A) Rb proteins interact with E2Fs through the pocket region, which consists of A and B domains. p107 and p130 have a spacer within the B domain of the pocket region, and contain a Cyclin E/A-cdk binding site and a cyclin inhibitor motif (Classon and Dyson, 2001). B) E2F family members have a high degree of homology within DNA binding domain (DBD). Leucine-zipper (LZ) and marked-box (MB) motifs form the dimerization domain for interaction with DP proteins. Rb binds to E2F1-5 within the region of the transactivation domain. Note the absence of Rb binding motifs in E2F6-8. E2F4 and E2F5 have nuclear export signal (NES), and E2F4 does not have a nuclear localization signal (NLS).


A)
Pocket domain
pRb
A
B
p107
A
B
B
p130
A
B
B
Cdk inhibitor function
Cyclin E/A-cdk binding site


B)
NLS
Dimerization domain
Transactivation domain
DBD
E2F1, E2F2, E2F3a
LZ MB
Cyclin A/cdk2 binding
Rb binding
E2F3b
LZ MB
E2F4
LZ MB
NES
E2F5
LZ MB
E2F6
LZ MB
E2F7
E2F8
DBD
DBD

Cyclin-dependent kinases (cdk) and their interacting partners, the cyclins, are well-established regulators of pRb phosphorylation (Mittnacht et al., 1994; Lundberg and Weinberg, 1998). Growth signaling leads to the sequential assembly and activation of specific cyclin-cdk complexes (Fig. 1.9) (Lundberg and Weinberg, 1998), which causes progressive phosphorylation of Rb proteins leading to its inactivation and consequent cell cycle progression (Giacinti and Giordano, 2006). Inactivation of Cyclin D-cdk4/6 with TAT-p16 (cdk inhibitor) in primary peripheral blood lymphocytes dephosphorylates Rb protein (Ezhevsky et al., 2001). Moreover, increased pRb protein phosporylation is observed in a number of tumors associated with *cdk* and *cyclin* gain-of-function mutations (Loden et al., 1999; Tashiro et al., 2007). Cells from Cyclin E deficient mice are also defective in their ability to re-enter the cell cycle, and similar to *Cyclin D* mutants, are resistant to oncogenic transformation (Geng et al., 2003). Therefore, cyclins are essential regulators of pRb phosphorylation during development and tumorigenesis.

Cyclin-cdk activity is controlled by a family of cyclin-dependent kinase inhibitors (CKI). The CKI family consists of two classes depending on the structure and their preferential interacting partners (reviewed in (Sherr and Roberts, 1999)). The INK4 class of CKIs includes p15, p16, p18, and p19 proteins, which specifically interact and inhibit the activity of cdk4 and cdk6. The Cip/Kip CKIs includes p21, p27, and p57, which can bind to both cyclins and cdk's. Binding of p16 to Cyclin D-cdk4/6 results in their inactivation, decreases pRb phosphorylation, and leads to cell cycle arrest (Fig. 1.9) (Sherr and Roberts, 1999; Massague, 2004). A crucial role for CKIs as regulators of the cell cycle has been established in a number of studies reviewed in (Sherr and Roberts, 1999; Massague, 2004). For example, *p16* overexpression causes pRb-dependent cell

cycle arrest (Medema et al., 1995), and deletion of *p16* is found in a number of primary tumors (Yonghao et al., 1999).

Thus, Rb proteins are key regulators of cell cycle progression. Mitogenic stimuli through a number of signaling pathways converge on and inhibit the activity of CKIs. This, in turn, activates Cyclin-Cdk complexes, which sequentially increase pRb phophorylation and allow for cell cycle progression.

1.3.3. E2F transcription factors are key targets of Rb activity

Rb pocket proteins control the expression of their target genes by directly regulating the activity of E2F transcription factors (Stevaux and Dyson, 2002), by interacting with chromatin structure remodeling proteins (Frolov and Dyson, 2004; Macaluso et al., 2006), and also by E2F-independent mechanisms (Giacinti and Giordano, 2006). The ultimate decision to advance through the cell cycle is made at R (Restriction) point of G1 phase (Fig. 1.9) (Pardee, 1989; Giacinti and Giordano, 2006). A change in pRb phosphorylation status represents an important biochemical mechanism of transcriptional control. Hypophosphorylated pRb is complexed with E2F transcription factors, while increasing pRb phosphorylation during G1 phase of the cell cycle leads to the dissociation of the Rb-E2F complexes. Free E2Fs can then bind to DNA regulatory elements and induce transcription of genes required for cell cycle progression. Thus, control of transcription and cell cycle progression relies on the function of E2F transcription factors.

1.3.4. E2F family members

E2F was originally identified as a downstream target for adenoviral oncoprotein E1A, which drives cells in the cell cycle (Kovesdi et al., 1986). In the past two decades an extensive family of E2F transcription factors has been identified comprising 8 E2F and 3 DP members (Trimarchi and Lees, 2002; Rowland and Bernards, 2006). E2F-DP heterodimerization generates a functional E2F unit (Wu et al., 1995; Trimarchi and Lees, 2002). Structurally all E2Fs share a high degree of homology within the winged-helix DNA binding domain (Fig. 1.10) (Zheng et al., 1999; DeGregori and Johnson, 2006). E2Fs 1-6 also have a dimerization domain for interaction with DP proteins, which consists of leucine zipper and marked box motifs (Tao et al., 1997; Cartwright et al., 1998; Milton et al., 2006). Out of eight E2Fs only E2F1- E2F5 contain a pocket protein binding domain, located in the carboxyl-terminal region (Jost et al., 1996; Du and Pogoriler, 2006), while other E2Fs function independently of pocket protein interaction. The transactivation region is located between the DNA binding and dimerization domains. E2F1-3 and E2F5 possess a nuclear localization signal, whereas only E2F4 and E2F5 have a nuclear export signal (de la Luna et al., 1996; Gaubatz et al., 2001; Apostolova et al., 2002; Trimarchi and Lees, 2002). These motifs provide another level of regulation of E2F-dependent transcription (Gill and Hamel, 2000). Moreover, E2F1 can be negatively regulated directly by Cyclin A-cdk2 through the region within N terminus, which is also present in E2F2 and E2F3 (Krek et al., 1995; DeGregori and Johnson, 2006). Additional diversity to the E2F family is brought by the discovery of two E2F3 isoforms – E2F3a and E2F3b (Leone et al., 2000). The last two recently discovered members of the E2F family E2F7 and E2F8 possess only two consecutive DNA binding

domains and are devoid of any other characteristic functional motifs (de Bruin et al., 2003a; Maiti et al., 2005). The role of these new E2Fs remains to be discovered.

1.3.5. Mechanism of E2F-mediated activation and repression

Based on the functional ability of different E2Fs to activate or repress transcription, all E2Fs have been subdivided into two categories. E2F1, E2F2, and E2F3a are considered to be "activating" E2Fs, which can induce cell proliferation and transcriptional activation of target genes (Lees et al., 1993; Araki et al., 2003; DeGregori and Johnson, 2006; Kong et al., 2007). The levels of E2F1-3a activity and mRNA increase when cell proliferation is induced (Shirodkar et al., 1992; Leone et al., 2000). E2F3b through E2F8 are generally viewed as "repressive" E2Fs. E2F3b interacts with pRb, whereas E2F4 and E2F5 preferentially bind to p107 or p130 and repress transcriptional activity of target promoters, such as *E2F1, c-myc, cyclin E, cyclin A, Cdc2, Cdc6, Orc1, pRB, dihydrofolate reductase*, and others (Leone et al., 2000; Chen et al., 2002; Araki et al., 2003; Attwooll et al., 2004). Several properties distinguish the E2F4 transcription factor from other E2F family members. First, the level of *E2F4* expression does not fluctuate through different phases of the cell cycle and it constitutes a major part of the total E2F activity in quiescent cells and almost all of the free E2F activity during G1 phase of the cell cycle (Moberg et al., 1996). Finally, unlike other E2Fs, E2F4 can form complexes with all Rb family members (Trimarchi and Lees, 2002; Vidal et al., 2007). E2F6-8 have exhibited pRb-independent repressive activity (Trimarchi et al., 2001; de Bruin et al., 2003a; Maiti et al., 2005). E2F6 represses transcription in association with Polycomb transcriptional repressor complexes, whereas

the mechanism of E2F7 and E2F8 mediated repression is presently under investigation (Trimarchi et al., 2001; DeGregori and Johnson, 2006). A recent report has pointed to a novel, non-transcriptional function of E2F8 as a guanine nucleotide exchange factor and an activator of G proteins (Hagemann et al., 2007).

E2F transcriptional activity depends on their association with Rb proteins. In quiescent cells and during early G1 phase of the cell cycle E2Fs are bound by pocket proteins, which result in repression of transcription. This repression could be achieved by two mechanisms: prevention of activation or active repression (Trimarchi and Lees, 2002). Repressive E2F4 and E2F5 predominantly interact with p107 or p130 (Dyson et al., 1993; Beijersbergen et al., 1994; Vairo et al., 1995). These complexes translocate to the nucleus, involve chromatin remodeling proteins and actively repress target gene promoters (Ferreira et al., 1998; Iavarone and Massague, 1999; Gaubatz et al., 2000; Vandel et al., 2001; Chen et al., 2002; Rayman et al., 2002). At the same time E2F1, E2F2, and E2F3 are sequestered by pRb. This prevents the activating function of E2F1-3 (Lees et al., 1993; Trimarchi and Lees, 2002). Growth stimulation and pRb hyperphosphorylation leads to the release of E2Fs and stimulation of E2F dependent transcription. The prevailing view has been that E2Fs actively stimulate cell cycle progression through their transactivation domain (Trimarchi and Lees, 2002). The wealth of new data in the past several years have led to the refinement of this model and proposal of more complex mechanism of transcriptional regulation by E2Fs (Rowland and Bernards, 2006). For example, the loss of repressive *de2f2* rescues the phenotype of *de2f1* deficient *Drosophila*, which is characterized by slow growth, downregulation of E2F-regulated genes, and reduced proliferation (Frolov et al., 2001). Thus, the results of

this study suggest that the presence of activating E2Fs on the promoters of the proliferating cells simply displaces repressive E2Fs, which prevents repression and cell cycle exit. At the same time, the function of the E2Fs transactivating domain is required for cell cycle re-entry (Rayman et al., 2002; Frolov and Dyson, 2004). Overall, this suggests that E2F transcription factors are not inducers of transcriptional activity, but rather they perform an essential permissive and regulatory function for cell cycle progression (Rowland and Bernards, 2006).

1.3.6. Physiological functions of Rb

The role of pRb as a negative regulator of cell cycle progression is clearly evident from the fact that pRb mutations are the underlying cause of numerous types of tumors (Liu et al., 2004). Moreover, a number of Cyclin, cdk and CKI mutations, which lead to hyperphosphorylation of pRb and subsequent release of E2F transcriptional activity, also lead to uncontrolled proliferation and tumor development (Sherr and Roberts, 2004; Berthet et al., 2006; Uziel et al., 2006). Generation of germline and tissue-specifie Rb deficient mice suggested a much broader physiological role of Rb for regulation of proliferation, apoptosis, and differentiation of the nervous (see next subchapter) and hematopoietic systems, as well as the eye, muscles, adipose tissue, and placenta (de Bruin et al., 2003b; MacPherson et al., 2003; Clark et al., 2004; Huh et al., 2004; Sage et al., 2005; Scime et al., 2005). The developmental defects observed in germline *Rb* mutant mice are complicated by disruption of its function in extraembryonic tissues (de Bruin et al., 2003b; Wu et al., 2003; Wenzel et al., 2007). Conditional inactivation of *Rb* in trophoblast stem cells disrupts placental morphogenesis, increases the number of

immature erythrocytes and causes embryonic lethality by E15.5. Unlike the massive cell death found in whole-embryo *Rb* knockouts, these conditional mutants exhibit apoptosis confined to a small region in the brainstem (Wenzel et al., 2007). Tissue-specific inactivation of *Rb* points to the essential cell-autonomous role in the embryo. Conditional deletion of *Rb* in mouse myoblasts caused perinatal lethality, increased apoptosis and failure of myotube differentiation (Huh et al., 2004). These findings indicate that Rb function in extraembryonic and fetal tissues is required for normal delelopment. Other Rb family members, p107 and p130, are required for repression of the target genes in quiescent cells and cellular senescence (Balciunaite et al., 2005; Helmbold et al., 2006; Litovchick et al., 2007). At the same time, it has been shown that pRb, p107 and p130 are recruited and regulate distinct sets of target gene promoters (Hurford et al., 1997; Balciunaite et al., 2005). Loss of *Rb* and simultaneous loss of *p107* and *p130* causes aberrant expression of different groups of E2F target genes (Hurford et al., 1997). Germline deletions of *p107* or *p130* in mice on a mixed background are indistinguishable from wild type controls, suggesting compensation between Rb family members (LeCouter et al., 1998b; LeCouter et al., 1998a). Simultaneous deletion of *p107* and *p130* leads to neonatal lethality and abnormal bone development, a phenotype which is different from *Rb* mutant mice (Cobrinik et al., 1996). Moreover, p107 deficient mice show a requirement for this protein in neural precursor self-renewal and commitment to neuronal fate ((Vanderluit et al., 2004; Vanderluit et al., 2007); see next subchapter). Therefore, Rb proteins have overlapping and unique roles in developing and mature systems and are important for control of proliferation, apoptosis and target gene repression.

1.3.7. Physiological function of E2F proteins

In agreement with the model of Rb/E2F transcriptional regulation, Rb deficiency causes deregulated E2F activity, which can be rescued by inactivation of *E2F1* or *E2F3* (Tsai et al., 1998; Callaghan et al., 1999; Ziebold et al., 2001). Cells deficient for E2F1 or E2F3 show impaired ability to re-enter the cell cycle, while deletion of all three activating E2Fs stops proliferation of mouse embryonic fibroblasts (Wu et al., 2001; Kong et al., 2007). This defect is primarily attributed to the loss of E2F3, since its reintroduction almost completely rescues the proliferation defect (Wu et al., 2001). *E2F3* deletion also significantly alters the expression of the genes required for cell cycle regulation, DNA replication, and mitotic control, which are not affected by *E2F1* deletion (Kong et al., 2007). E2F1, on the other hand, is considered a major factor in inducing p53-dependent and -independent apoptosis (Lazzerini Denchi and Helin, 2005).

E2F4 and E2F5 are the major contributors of Rb/E2F-mediated repression (DeGregori and Johnson, 2006). According to a widely accepted model, both E2F4 and E2F5 heterodimerize with DP proteins and together with pocket proteins translocate to the nucleus where they repress target promoters. In quiescent cells repressive E2Fs in association with p107 and p130 occupy the majority of E2F site containing promoters (Takahashi et al., 2000; Wells et al., 2000; Litovchick et al., 2007). E2F4 and E2F5 are dispensable for proliferation, but they are required for CKI-induced cell cycle arrest (Gaubatz et al., 2000). Though it has been suggested that E2F4 is a "repressive" E2F, several reports showed that it can function as a transcriptional activator (reviewed in (DeGregori and Johnson, 2006)). E2F4 displays nuclear localization in cycling

keratinocytes, fibroblasts, myocytes, lens fiber cells, intestinal crypt cells and erythroid compartment cells, where it can induce proliferation and cell cycle progression (Beijersbergen et al., 1994; Gill and Hamel, 2000; Wang et al., 2000; Chen et al., 2004b; Deschenes et al., 2004; Ebelt et al., 2005; Kinross et al., 2006). Moreover, a genomewide location analysis showed that E2F4 regulates a subset of target genes independently of interaction with Rb proteins in proliferating cells (Balciunaite et al., 2005). These results strongly suggest that E2F4 may function as a transcriptional activator.

Whereas the majority of the initially described genes regulated by the E2Fs are involved in the regulation of the cell cycle (Blake and Azizkhan, 1989; Hiebert et al., 1989; Nevins, 1998; Chen et al., 2002) a number of recent reports have identified non-cell cycle related E2F target genes, including homeobox genes, such as a subset of the *HoxA* genes, *Ptx1* (Muller et al., 2001; Ren et al., 2002; Dimova et al., 2003; Korenjak and Brehm, 2005; Iwanaga et al., 2006). This is consistent with the complex *in vivo* phenotypes generated by single and combined deletions of different E2Fs. E2F1 deficient mice are viable, though they exhibit testicular atrophy, exocrine gland dysplasia, aberrant apoptosis and predisposition for tumor development (Field et al., 1996; Yamasaki et al., 1996). Combined loss of *E2F1* and *E2F2* results in impaired hematopoietic cell proliferation and differentiation, pancreatic degeneration, and a high incidence of tumors (Zhu et al., 2001; Li et al., 2003a; Li et al., 2003b). E2F3 deficiency leads to downregulation of cell cycle genes and embryonic lethality (Humbert et al., 2000a; Cloud et al., 2002). Moreover, mice carrying compound E2F1 and E2F3 deficiency show exacerbation of the phenotypes characteristic to the single mutants (Cloud et al., 2002). E2F4 deficient mouse embryos exhibit growth retardation, a high rate of early postnatal

lethality, and gastrointestinal and craniofacial defects (Humbert et al., 2000b; Rempel et al., 2000).

Though, it has been shown that E2F4 is required for erythrocyte proliferation and cell cycle progression (Kinross et al., 2006), analysis of different hematopoietic compartments and peripheral blood samples indicates that the absence of E2F4 leads to defects in erythroid, myeloid and B-cell lineages with an increased fraction of immature cells, suggesting a requirement for E2F4 in cellular differentiation (Humbert et al., 2000b; Rempel et al., 2000). Consistent with a developmental role, *E2F4* deletion disrupts the development of the ciliated epithelium in the entire respiratory tract (Danielian et al., 2007). Furthermore, *E2F4* deletion can suppress development of thyroid and pituitary tumors in Rb heterozygous mice (Lee et al., 2002b). Remarkably, loss of *E2F4* in this case leads to an aberrant association of p107 and p130 with activating E2Fs (Lee et al., 2002b). These results further demonstrate the complexity of Rb/E2F interaction in the living organism.

E2F6 deficient mice are viable and healthy, but they develop posterior homeotic skeletal transformation in the lumbar and sacral vertebral regions (Storre et al., 2002). This finding is consistent with previously described pocket protein-independent function of E2F6 as a component of repressive mammalian multiprotein Polycomb Group (Pc-G) complex (Trimarchi et al., 2001). Transcriptional repressesion of *Hox* genes by Pc-G establishes the anterior-posterior Hox code, and the Pc-G mutant, such as *Bmi1*, exhibits similar homeotic transformation phenotype (van der Lugt et al., 1994).

Taken together, the presence of the distinct phenotypic features of the individual and combined E2F mouse mutants indicates that each E2F transcription factor performs

unique biological functions *in vivo*, such as control of proliferation, differentiation, and apoptosis. At the same time the full scope of the functions of each E2F is obscured by the complex compensatory interactions between E2F family members.

1.3.8. Rb/E2F pathway in nervous system development

A number of studies have described the specific functions of Rb/E2F members in the nervous system and eye development (reviewed in (Ferguson and Slack, 2001; Ohnuma and Harris, 2003; McClellan and Slack, 2006)). A crucial role for the Rb protein in the development of the nervous system has been shown by analysis of germline Rb deficient mice. These animals die before E15 and display ectopic proliferation and increased apoptosis throughout the central and peripheral nervous system (Jacks et al., 1992; Lee et al., 1992). Rb is required for terminal cell cycle exit of committed neural progenitors (Slack et al., 1998; Callaghan et al., 1999). Detailed analysis of conditional telencephalon-specific Rb deficient mice reveals that widespread apoptosis in the nervous system is primarily due to a placental defect (Ferguson et al., 2002; de Bruin et al., 2003b). At the same time, analysis of these mice shows ectopic proliferation and increased neurogenesis leading to aberrant cortical morphology and lamination (Ferguson et al., 2002). Moreover, telencephalon-specific *Rb* deletion leads to abnormal migration of the ventrally derived subpopulation of the GABAergic inhibitory interneurons, thus demonstrating a novel cell-autonomous requirement for Rb in interneuronal migration (Ferguson et al., 2005).

Besides Rb, other members of the Rb family also perform unique functions during nervous system development. p107 is a negative regulator of neural stem cell self-

renewal in embryonic and adult brain (Vanderluit et al., 2004). Furthermore, in the absence of p107 the expression of *Hes1* is upregulated, which inhibits proneural genes and disrupts commitment of neural progenitors to a neuronal fate (Vanderluit et al., 2007). *p130* deletion causes abnormal proliferation and apoptosis in developing nervous system, but these defects are strain-dependent (LeCouter et al., 1998a). Overexpression of Rb and p130 in rat neural stem cells indicate that they cooperate with extrinsic factors to regulate lineage specification (Jori et al., 2007). Conditional inactivation of all three pocket proteins in neural progenitors at the beginning of neurogenesis causes increasing ectopic proliferation and apoptosis, which is evident from E13.5, and also induces premature differentiation, aberrant cortical morphology and cerebellar development, and behavioral abnormalities (McLear et al., 2006).

E2F transcription factors have also been shown to perform distinct and overlapping functions during neurogenesis (McClellan and Slack, 2006). *E2F1* deletion in mouse embryos show no abnormalities in the developing brain, but display a decreased number of neural stem cells and reduced progenitor proliferation, as well as cerebellar atrophy in the adult brain (Cooper-Kuhn et al., 2002). Consistent with role of E2F3 as an activator of cell cycle progression, it has been shown that E2F3 is required for positive regulation of neural progenitor proliferation in the telencephalon of embryonic and adult brain (McClellan et al., 2007). Moreover, pRb mediated regulation of neuronal migration is specifically mediated by the E2F3 transcription factor, whereas both E2F1 and E2F3 can interact with pRb to regulate neural precursor proliferation, cell cycle exit and cortical lamination (McClellan et al., 2007). Also, in the context of Rb deficiency, deletions of either *E2F1* or *E2F3* almost completely and *E2F2* partially rescue ectopic

proliferation and increased apoptosis (Saavedra et al., 2002). Development of the nervous system has not been specifically evaluated in *E2F4* mouse mutant. Overall, the analysis of Rb/E2F mutant mice indicates that each member of the pathway performs multifaceted and often distinct roles in the developing nervous system.

1.3.9. Rb/E2F in retinal development

Rb is essential for normal eye development, since its mutation in humans leads to the development of early childhood tumor. In contrast to the human retina, Rb deficiency in mice does not cause retinoblastoma or defects in eye development (Clarke et al., 1992; Jacks et al., 1992; Maandag et al., 1994). *Rb* is expressed in proliferating postnatal retinal progenitors (Donovan et al., 2006), and it is critically required for cell cycle exit and terminal differentiation during late retinogenesis. Committed post-mitotic retinal precursors ectopically proliferate within the differentiating ganglion cell layer and undergo p53-independent apoptosis in the absence of Rb. There is also specific loss of photoreceptors ganglion, and bipolar cells (Bremner et al., 2004; MacPherson et al., 2004). Importantly, simultaneous inactivation of *Rb* and *p130* causes retinoblastoma development, indicating that p130 can compensate for Rb tumor suppressor function (MacPherson et al., 2004). Recently the involvement of the Rb/E2F pathway in early nervous system and eye development has been identified in *humdy* mouse mutants (Kim et al., 2007). These embryos exhibit severe early developmental defects, such as increased proliferation, exencephaly, and coloboma. The mutant gene encodes protein phospatase family member Phactr4, which is required for Rb dephosphorylation and control of E2F activity. Disruption of *Phactr4* causes accumulation of

hyperphosphorylated pRb and derepression of E2F target genes. *Humdy* mutant phenotype can be rescued by E2F1 deletion (Kim et al., 2007). This study emphasizes the critical intermediary role Rb/E2F pathway in cell cycle regulation, nervous system and eye development.

Mutant eye phenotypes of individual E2Fs so far have not been reported, but they perform distinct and overlapping functions as pRb targets in retinal development. Loss of *E2F1, E2F2*, or *E2F3* can rescue defective proliferation in *Rb* mutant retina and lens (Saavedra et al., 2002), but E2F1 has a unique role in mediating anti-apoptotic function of Rb (Saavedra et al., 2002). Surprisingly, it has been shown that Rb-mediated differentiation of starburst amacrine cells is mediated by E2F3a, which is considered to be an activator of cell cycle progression (Chen et al., 2007).

In summary, E2F transcription factors perform a wide range of vital physiological functions during development and in the growth of the organism. E2F proteins are an integral part of the cell cycle machinery. They can either stimulate cell cycle progression or participate in repression of the target genes in association with Rb and chromatin remodeling proteins. Moreover, the analysis of *in vivo* E2F mutant models combined with genome wide location analysis and microarray data greatly broadens the spectrum of E2F functions beyond cell cycle control. New evidence highlights the profound roles for E2F transcription factors in the development of the nervous system. The following studies reveal a novel role for E2F4 in nervous system development and function, never before described for cell cycle family members.

1.4. Statement of objectives and hypothesis

Recent studies point to important spatial and temporal roles of the cell cycle regulatory Rb/E2F pathway in neurodevelopmental processes beyond cell cycle control, such as neural precursor self-renewal, differentiation, neuronal migration and patterning (Ferguson et al., 2002; Vanderluit et al., 2004; Ferguson et al., 2005; McClellan and Slack, 2006; McClellan et al., 2007; Vanderluit et al., 2007). Though a number of reports have shown that E2F transcription factors regulate an array of non-cell cycle related genes, examples of physiological relevance of these functions to nervous system development are limited.

E2F4 deficient mouse embryos display the widest range of reported developmental defects compared to the other E2F mutants (Field et al., 1996; Lindeman et al., 1998; Humbert et al., 2000b; Humbert et al., 2000a; Rempel et al., 2000; Cloud et al., 2002). This suggests that E2F4 performs a number of unique functions, which could not be compensated by other E2Fs. A key role for E2F4 in CNS development has not yet been reported.

Therefore, the **primary objective** of this study was to establish the requirement for E2F4 in telencephalic development and neural stem cell regulation (Chapter 2).

I hypothesized that E2F4 would be required for early telencephalic morphogenesis and neural stem cell self-renewal.

To address this hypothesis I analyzed morphology and regional patterning of the developing telencephalon in wild type and E2F4 deficient embryos (Rempel et al., 2000).

This model allows for the study of E2F4 function as an integral part of the complex physiological regulatory network. I employed an *in vitro* neurosphere assay to assess NSC self-renewal (Reynolds and Weiss, 1996). Interbreeding of *E2F4* knockouts with Shh or Gli3 deficient mice was used to establish genetic interactions between E2F4 and the Shh morphogenetic pathway.

The results of the experiments in the first objective (Chapter 2) showed that E2F4 deficiency caused deregulation of Shh signaling in the ventral forebrain. Since Shh signaling from the ventral forebrain midline is essential for the early patterning of the visual system (Lupo et al., 2006) my **second objective** was to evaluate the role of E2F4 in early eye morphogenesis (Chapter 3).

I hypothesized that E2F4 is required for early eye patterning.

Since Shh signaling performs distinct functions at different stages of eye development I analyzed eye morphology and patterning in wild type and E2F deficient mouse embryos at two different developmental timepoints (E11.5 and E15.5). I also performed analysis of proliferation and cell survival on eye coronal sections.

The proposed studies significantly advance our knowledge about the role of the Rb/E2F pathway in patterning and specification of developing forebrain structures. Moreover, these novel functions of the Rb/E2F pathway in telencephalic and eye morphogenesis will help us to understand the etiology and pathophysiology of neural

developmental disorders so that we may subsequently design appropriate therapeutic strategies for their treatment.

Chapter 2.

Vladimir A. Ruzhynsky, Kelly A. McClellan, Jacqueline L. Vanderluit, Yongsu Jeong, Marosh Furimsky, David S. Park, Douglas J. Epstein, Valerie A. Wallace, and Ruth S. Slack (2007). Cell cycle regulator E2F4 is essential for the development of the ventral telencephalon. *J. Neurosci.* **27**: 5926 - 5935.

Title: Cell cycle regulator E2F4 is essential for the development of the ventral telencephalon.

Abbreviated title: E2F4 requirement in early telencephalic development

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Number of figures: 9
Number of supplementary figures: 3
Number of pages: 32
Keywords: E2F4; cell cycle; Sonic hedgehog; neural precursors; neural patterning; telencephalon

Contributions of the author and collaborators

I performed the experiments outlined in this study and analyzed the data. Kelly A. McClellan provided expert suggestions and assisted with explant culture experiment, as well as assisted with writing of the manuscript. Dr. Jacqueline L. Vanderluit performed the initial analysis of neural stem cell population, assisted with histological analysis and editing of the manuscript. Dr. Yongsu Jeong and Dr. Douglas J. Epstein generated and provided Shh transgenic reporter mice. Dr. Douglas J. Epstein critically reviewed the manuscript. Marosh Furimsky provided teaching assistance with in situ hybridization, as well as helped with the initial analysis of Shh pathway. Dr. David S. Park provided mice and critically reviewed the manuscript. Dr. Valerie A. Wallace provided essential intellectual input in analysis of Shh pathway in E2F4 deficient mutants, provided reagents for testing Shh pathway activity, helped with data analysis, and reviewed the manuscript. The study was conducted under the guidance and help of Dr. Ruth R. Slack. This manuscript was written by me, and reviewed and edited by Dr. Ruth S. Slack and co-authors.

ACKNOWLEDGEMENTS

We would like to thank Dr. C.-C. Hui for helpful discussion. We thank A. P. McMahon, A. Joyner, J. Rubenstein, D.N. Pringle for for providing cDNA constructs. We are grateful to Dr. Lee Rubin from Curis Inc. (Cambridge, MA, USA) for providing us with the Hh signaling pathway agonist. The authors would like to thank J.G. MacLaurin, W.C. McIntosh, and Chantal Mazerolle for excellent technical assistance and expertise. This work was funded by a CIHR grant to RSS. VAR is a recipient of OGS and SCN studentships, KAM of CIHR Canada Graduate Doctoral Research Award, and JLV is a recipient of a HSFC fellowship. MF is a recipient of a CIHR Postdoctoral Fellowship.

ABSTRACT

Early forebrain development is characterized by extensive proliferation of neural precursors coupled with complex structural transformations, however little is known regarding the mechanisms by which these processes are integrated. Here, we show that deficiency of the cell cycle regulatory protein, E2F4, results in the loss of ventral telencephalic structures and impaired self-renewal of neural precursor cells. The mechanism underlying aberrant ventral patterning lies in a dramatic loss of Shh expression specifically in this region. The E2F4 deficient phenotype can be recapitulated by interbreeding mice heterozygous for E2F4 with those lacking one allele of Shh, suggesting a genetic interaction between these pathways. Treatment of E2F4 deficient cells with a Hh agonist rescues stem cell self-renewal and cells expressing the homeodomain proteins that specify the ventral telencephalic structures. Finally, we show that E2F4 deficiency results in impaired activity of Shh forebrain specific enhancers. In conclusion, these studies establish a novel requirement for the cell cycle regulatory protein, E2F4, in the development of the ventral telencephalon.

INTRODUCTION

Forebrain development is characterized by extensive proliferation of neural precursors combined with complex structural transformations regulated by multiple morphogenic signaling pathways (reviewed in (Fuccillo et al., 2006)). This morphogenetic signaling must be tightly coordinated with cell cycle control to ultimately shape the developing brain. Presently little is known regarding the mechanisms by which the cell cycle machinery is integrated with these key developmental events.

Studies are emerging demonstrating the importance of cell cycle regulatory proteins in nervous system development. In particular members of the retinoblastoma (Rb) family of cell cycle genes, and genes which in turn regulate Rb activity, have been shown to have important roles both in cell cycle dependent and developmental processes that go beyond the mechanics of cell division (reviewed in (McClellan and Slack, 2006)). For example, Rb has been shown to have a critical function in regulating terminal mitosis of neuroblasts in the central and peripheral nervous systems and retina (Chen et al., 2002; Ferguson et al., 2002; MacPherson et al., 2003; Marino et al., 2003). Recently, we and others have shown that Rb exhibits non cell cycle dependent functions. For example, Rb has been shown to have a cell-autonomous function in ventral telencephalon development as telencephalon specific Rb deficient mice exhibit a migration defect in ventrally derived interneurons (Ferguson et al., 2005). The closely related Rb family member, p107, has also been shown to be an important regulator of precursor self-renewal (Vanderluit et al., 2004). This regulation comes about through modulation of the activity of the fate-determining Notch signaling pathway. Clearly, cell cycle regulators, and in particular

members of the Rb family, have functions beyond the regulation of the cell cycle machinery and play a pivotal role in shaping the developing brain.

Rb family proteins execute the function of cell cycle regulation by regulating the activity of E2F transcription factors (reviewed in (Trimarchi and Lees, 2002)). In addition to regulating genes involved in cell cycle progression, E2Fs are ubiquitously expressed and control an array of genes involved in development, differentiation, and apoptosis (Muller et al., 2001). E2F4, a binding partner for Rb and p107, is believed to be a repressor of transcription (Trimarchi and Lees, 2002; Attwooll et al., 2004) and may be involved in the regulation of differentiation. E2F4 deficient mice exhibit growth retardation and developmental defects including hematopoietic cell maturation and gut epithelial development (Humbert et al., 2000b; Rempel et al., 2000). While E2F4 deficient mice exhibit defects in craniofacial development, the role E2F4 of in nervous system development has never been examined. As E2F4 is an interacting partner for Rb and p107, and given the key role of Rb proteins in telencephalic development, we questioned whether E2F4 itself may be required for the regulation of brain development.

Here we show that deficiency of the cell cycle regulatory protein E2F4 results in the loss of ventral telencephalic structures and impaired self-renewal of neural precursor cells. The mechanism underlying aberrant ventral patterning lies in a dramatic loss in Shh expression in this region. Treatment with Hh agonist reveals a rescue of stem cell self-renewal and cells expressing homeodomain proteins that specify the ventral telencephalic structures. Finally, we found that E2F4 deficiency resulted in reduced activity of Shh forebrain specific enhancers demonstrating that E2F4 is required for Shh gene regulation.

In conclusion, these studies establish a novel role for the cell cycle regulatory protein, E2F4, in the development of the ventral telencephalon.

MATERIALS AND METHODS

E2F4, Shh and 447L17ßlacZ mice

$E2F4^{+/-}$ mice were generously provided by Dr. Jacqueline Lees (Massachusetts Institute of Technology) (Humbert et al., 2000b). 447L17ßlacZ transgenic reporter mice were previously described (Jeong et al., 2006). All were maintained on a C57Bl/6 background (Charles River Laboratories International, Inc., Wilmington, MA, USA) except $Gli3^{Xt/+}$ and 447L17ßlacZ mice, which were mixed C57Bl/6xC3HeB/FeJ and BL6xSJL, respectively. To generate Shh/E2F4, Gli3/E2F4 and 447L17ßlacZ/E2F4 embryos, heterozygous $Shh^{+/-}$C57Bl/6 (Lewis et al., 2001), $Gli3^{Xt/+}$ (Hui and Joyner, 1993) and transgenic 447L17ßlacZ mice were bred with $E2F4^{+/-}$ mice. Animals were genotyped as published previously for E2F4 (Humbert et al., 2000b), Shh (Lewis et al., 2001), Gli3 (Hui and Joyner, 1993) and 447L17ßlacZ (Jeong et al., 2006). The day of vaginal plug detection was counted as embryonic day (E) 0.5. All experiments were approved by the University of Ottawa's Animal Care ethics committee adhering to the Guidelines of the Canadian Council on Animal Care.

Immunohistochemistry and phenotypic analysis

Pregnant mice were sacrificed with a lethal injection of 65 mg/ml sodium pentobarbital solution (Somnotol) at indicated time points. To analyze neural progenitor proliferation in the embryonic brains, pregnant mouse dams received intraperitonial injection of 50 mg/kg^{-1} BrdU (Sigma; cat.#B5002) in 0.9% NaCl 30 minutes prior to sacrifice. Embryo heads were dissected and fixed in 4% PFA overnight in phosphate buffer, pH 7.4. Cryoprotection was performed by overnight incubation in 30% sucrose/1XDulbecco's

Phosphate Buffered Saline (DPBS) (Gibco) solution. Embryos were embedded in 1:1 mix of 30% sucrose in 1XDPBS/OCT (TissueTek 4583, Sakura Finetek, USA), and frozen on liquid nitrogen. 14 μm coronal sections were cut on a cryostat and mounted on Superfrost slides (Fisher Scientific, Cat. #12-550-15). Immunohistochemistry was performed using anti-phosphohistone H3 (1:1000, Upstate; cat.#06570), anti-BrdU (1:50; BD Biosciences; cat.#347580) and anti-ßIII-tubulin (mouse monoclonal hybridoma supernatant, 1:50 (Caccamo et al., 1989)) primary antibodies. PH3+ cell nuclei were counted separately along the dorsal and ventral ventricular zones. BrdU-postive cells were counted in the dorsal and ventral (MGE) 5500 μm^2 areas. The lengths and the areas of the dorsal and ventral regions were measured using Northern Eclipse 6.0 software (Empix Imaging Inc., Mississauga, ON, Canada). Cells were counted in every 10^{th} coronal section from the most rostral regions of the telencephalon to the preoptic area. PH3+ cells are expressed as a number of cells per 500 μm of the ventricular surface. Cresyl violet staining was performed as per standard protocols. Sections were analyzed using a Zeiss Axioskop 2 (Oberkochen, Germany) upright microscope and images were captured using Sony Power HAD 3CCD color video camera or QiCam digital video camera (QImaging Corporation, Burnaby, BC, Canada) and Northern Eclipse 6.0 software (Empix Imaging Inc., Mississauga, ON, Canada).

Western blotting

Total protein extracts were prepared from proliferating neurosphere cultures according to the protocols previously described (Ferguson et al., 2000). Immunoblotting was performed with antibodies directed against E2F4 (Chemicon Interantional Inc.,

MAB8894) and actin (sc-1616; Santa Cruz Biotechnology Corp.). Immunoblots were developed using chemiluminescence reagent according to manufacturer's instructions (ECL; Amersham Biosciences).

In situ hybridization, whole mount β-galactosidase analysis, and RT-PCR

Non-radioactive in situ hybridization (ISH) was performed on tissue sections and whole mount embryos and neural tube explants as described previously (Bruhn and Cepko, 1996; Wallace and Raff, 1999). Antisense riboprobes for Shh (Echelard et al., 1993), Gli1 (Hui and Joyner, 1993), Nkx2.1 (Shimamura et al., 1995), Dlx2 (Porteus et al., 1991), and Pax6 were prepared from plasmids, generous gifts from A. P. McMahon, A. Joyner, J. Rubenstein and N. Pringle. E2F4 DIG-labeled riboprobe was generated from pBS-IIKS-E2F4 template, containing 0.7 kb cDNA insert, which was amplified by PCR with primers E2F4-1 (ATCGAAGCTTGATTACATCTACAACC) and E2F4-2 (ATATAGGAATTCATTCGTTACTG). β-galactosidase staining of transgenic 447L17ßlacZ reporter embryos was performed as described (Zerucha et al., 2000). Embryos were cleared in 1:1 benzyl alcohol:benzyl benzoate, analyzed using Zeiss Stemi 2000-C stereo microscope (Oberkochen, Germany) and images were captured with Sony Power HAD 3CCD color video camera. RT-PCR was performed as previously described (Cregan et al., 2004). The primers for Shh detection (forward 5'-GGAAAGAGGCGGCACCCCAAAAAG-3', reverse 5'-CTCATCCCAGCCCTCGGTC ACTCG-3') were designed using DNAStar PrimerSelect software (DNAStar Inc, USA). cDNA synthesis was performed at 46°C for 45 min, followed by a 2 min initial

denaturation step at 94°C. This was followed by 37 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. S12 was used as loading control.

In vitro neurosphere assay

Primary and secondary neurosphere cultures were prepared from the telencephalic neuroepithelia of E13.5 mouse embryos as described previously (Vanderluit et al., 2004). Briefly, neuroepithelium of the ganglionic eminences was dissected, mechanically single-celled, counted and plated at equal cell density (10 cells/μl) for both E2F4-/- and control animals. Multipotentiality was examined by plating proliferating for 4 days neurospheres on poly-L-ornithine-coated dishes in differentiation medium containing 1% fetal bovine serum. After 7 days in vitro cells were fixed with 4% PFA and processed for βIII-tubulin, Glial Fibrillary Acidic Protein (GFAP) and O4 immunocytochemistry using anti-ßIII-tubulin (mouse monoclonal hybridoma supernatant; 1:50 (Caccamo et al., 1989)), anti-GFAP (rabbit polyclonal anti–bovine glial fibrillary acidic protein; 1:400; AB986; DakoCytomation), and anti-O4 (mouse monoclonal; 1:50; MAB345; Chemicon) primary antibodies. To label proliferating cells in monolayer progenitor cultures, 10 μM BrdU (Sigma; cat.#B5002) was added to the culture medium 24 hours prior to fixation. Cells were fixed on day 3 with 1:1 Methanol:Acetone (v/v) for 15 min, washed 3 times with 1×PBS and incubated with primary anti-BrdU primary antibodies (1:50; BD Biosciences; cat.#347580) overnight. Secondary antibodies goat anti-mouse CY3 (1:1000; Jackson Immono.; cat.#115-166-003) were applied for one hour at room temperature. Cell nuclei were stained with Hoechst (1:250; Sigma). The proportion of neuronal (ßIII-tubulin), glial (GFAP), and BrdU-positive cells were calculated as a percentage of the total number

of cells (Hoechst-positive nuclei). A minimum of 5 random fields per genotype were analyzed.

Neurosphere self-renewal rescue experiment was performed using Hh agonist 1.4 (Frank-Kamenetsky et al., 2002) (a kind gift from Curis Inc., Cambridge, MA, USA), which was added to the neurosphere culture media at the time of plating at a final concentration of 20 nM. Data were analyzed for significance by Student's *t*-test.

Neural tube explant cultures

Neural tube explant cultures were performed as previously described (Echevarria et al., 2001) with some modifications. E9.5 explants were cultured on 0.4 μm Polycarbonate Membrane Nunc 10 mm Tissue Culture Inserts (Nalge Nunc International, USA, cat.#137052) in 24-well Nunclon plates (Nunc A/C, Denmark, cat.#142475) containing explant culture medium (DMEM, 10% FBS, 2 mM glutamine, 100 u/ml penicillin-streptomycin) at 37°C in 5% CO_2, 95% humidity for 48 hours. Hh agonist 1.4 was added to the explant culture medium at the time of plating at a final concentration of 20 nM. Explants were fixed in 4% PFA in 1XPBS, pH 7.4 for 2 hours at 4°C and processed for whole mount in situ hybridization. Images of the explants were taken with a Zeiss Axioskop 2 (Oberkochen, Germany) upright microscope.

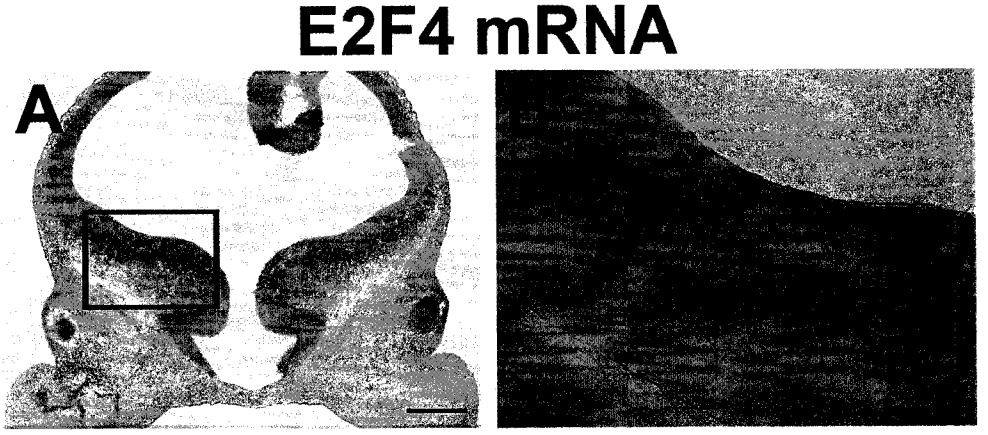
RESULTS

Aberrant development of ventral telencephalon in the absence of E2F4

E2F4 is important for normal embryonic development and is broadly expressed in most tissues (Dagnino et al., 1997; Callaghan et al., 1999). To ask whether E2F4 is differentially expressed in the developing brain, we performed in situ analysis on E11.5 embryos. While E2F4 is ubiquitously expressed, the highest levels of E2F4 expression were detected in the ventricular and subventricular zones (Supp. Fig. 2.1). To determine whether E2F4 was required for forebrain development, we examined E2F4 deficient mice. Recovery of E2F4 deficient embryos at embryonic days (E) E9.5 to E11.5 was at the expected Mendelian ratio. 89% of E2F4 deficient embryos (40 of 45) were smaller and displayed a reduced size of the developing telencephalon (Fig. 2.1A,C). Histological characterization revealed that E2F4 deficient embryos exhibited a complete absence or significant reduction of the medial and lateral ganglionic eminences (MGE and LGE) in the ventral telencephalon. In contrast, pallial regions appeared to be unaffected (Fig. 2.1B,D). These data suggest that E2F4 plays an essential role in the development of ventral telencephalic structures.

To further define the requirement of E2F4 in telencephalic development, we asked if there was any perturbation in the expression of homeodomain-containing transcription factors Nkx2.1, Dlx2, and Pax6. Nkx2.1 expression, which is normally restricted to the ventral midline and MGE (Corbin et al., 2003), was markedly reduced in the ventral telencephalon of the E2F4 deficient mice (Fig. 2.2A,D). Consistent with a reduction in ventral structures, we found that Dlx2, which is expressed in ventricular and subventricular zones of the ventral telencephalon, was also decreased in the E2F4 null

Supplementary figure 2.1. Expression of E2F4 in the developing mouse telencephalon. (A,B) Coronal sections through the telencephalon of E11.5 wild type mouse embryos were processed for in situ hybridization with E2F4 digoxigenin-labeled riboprobe. B – magnified image of indicated area in A (black box). Scale bars: A – 250 μm; B – 50 μm. (C) Analysis of E2F4 protein (~56 kDa) level in the proliferating neurospheres from wild type (WT) and E2F4-/- (KO) embryos by western blot. Non-specific band is indicated by asterisk. Actin was used as a loading control.


E2F4 mRNA
A

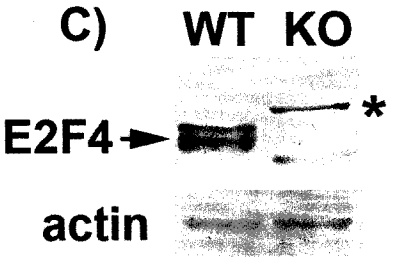

C)
WT
KO
E2F4
*
actin

Figure 2.1. Reduced size of the developing ventral telencephalon in E2F4 deficient mouse embryos. (A,C) Antero-lateral view of the wild type (WT) (A) and E2F4 deficient (E2F4-/-) (C) E11.5 mouse embryos. Note the size difference between WT (n=13) and E2F4-/- (n=17/20) developing telencephalic hemispheres (TE, black doublended arrow) relatively to the total antero-posterior length of the developing head (white line). Scale bar 0.5 mm (A,C). (B,D) Coronal sections through the brains of WT and E2F4-/- E11.5 mouse embryos stained with cresyl violet. Scale bar 250 μm.

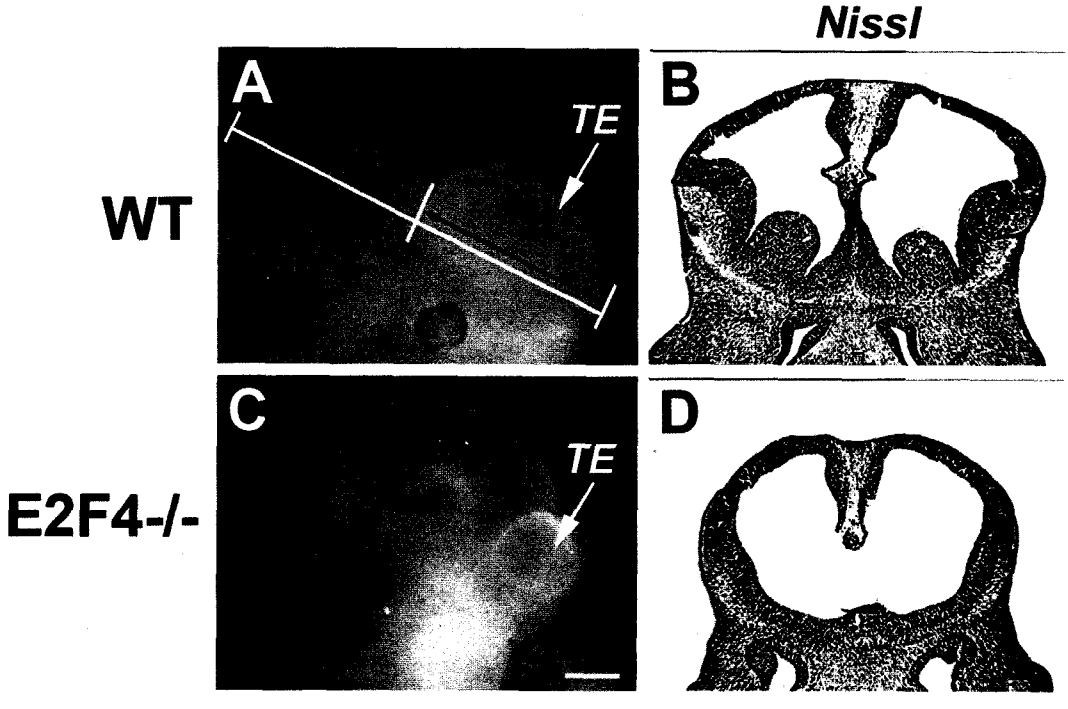
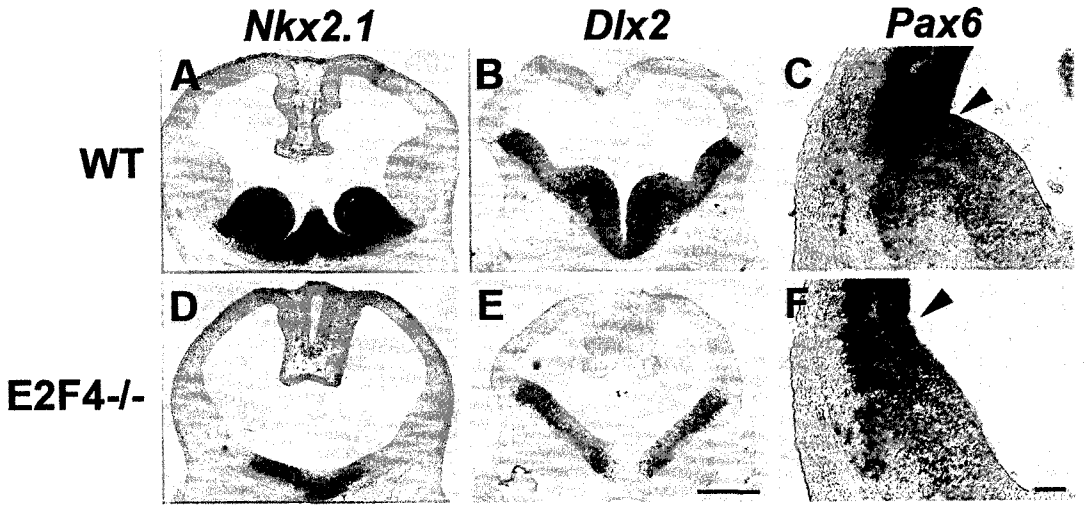

Nissl
A
TE
B
WT
C
TE
D
E2F4-/-

Figure 2.2. Aberrant early ventral telencephalic patterning in E2F4 deficient mouse embryos. (A-F) Region specific mRNA expression of homeobox patterning genes Nkx2.1 (A,D), Dlx2 (B,E) and Pax6 (C,F) in the ventral telencephalon of E11.5 mouse embryos analyzed by in situ hybridization. Note the ventral shift of Pax6 expression at the dorso-ventral boundary (F, black asterisk). At least five animals of each genotype were examined. Scale bars: 500 μm (A,B,D,E), 125 μm (C,F).


Nkx2.1
Dlx2
Pax6
WT
E2F4-/-
A
B
C
D
E
F

telencephalon (Fig. 2.2B,E). Pax6 expression is normally localized to the cortical proliferative zones of the developing brain (Walther and Gruss, 1991). In the absence of E2F4 the expression pattern of Pax6 was similar to the wild type embryos in the rostral telencephalon, whereas in the caudal regions the ventral boundary of Pax6 was extended into the ventral telencephalon (Fig. 2.2F; data not shown), suggesting a moderate dorsalization of the caudal region of the developing telencephalon. The reduced expression of homeodomain proteins that specify ventral telencephalic structures (Nkx2.1, Dlx2) is consistent with the loss of the MGE and LGE. At midneurogenesis (E15.5) the ventral defect continues to persist although some growth of ventral telencephalic structures has occurred. E2F4 deficient embryos exhibit enlarged lateral ventricles due to the impaired development of the MGE and LGE. (Supp Fig. 2.2C). Furthermore, we observed reduced expression of LIM homeodomain transcription factor Lhx6, which labels postmitotic neurons in the ventral telencephalon, as well as MGE-derived migrating interneurons in the dorsal telencephalon (Supp. Fig. 2.2D; (Grigoriou et al., 1998; Lavdas et al., 1999)). These results demonstrate that E2F4 deficiency results in a significant delay in the development of the ventral telencephalon.

Progenitor proliferation and early neuronal differentiation are not affected by the loss of E2F4

As the Rb/E2F pathway is primarily known for its role in cell cycle regulation and E2F4 deficiency results in a dramatic reduction in ventral telencephalic structures we asked whether cell proliferation was impaired in the ventricular zones of E2F4 null mice. Cell proliferation was measured using an M phase marker, PH3, and by BrdU

Supplementary figure 2.2. E2F4 deficient embryos exhibit reduction of the developing striatal neuroepithilium and decreased expression of Lhx6 in the cortical marginal zone. In situ hybridization analysis of Lhx6 mRNA expression in E15.5 wild type (A,B) and E2F4 null (C,D) embryos. Note the reduced Lhx6 expression in the cortical marginal zone (MZ), as well as the smaller size of the striatum (st) and enlarged lateral ventricle (black asterisk). Scale bars: A, C – 250 μm; B, D – 75 μm.

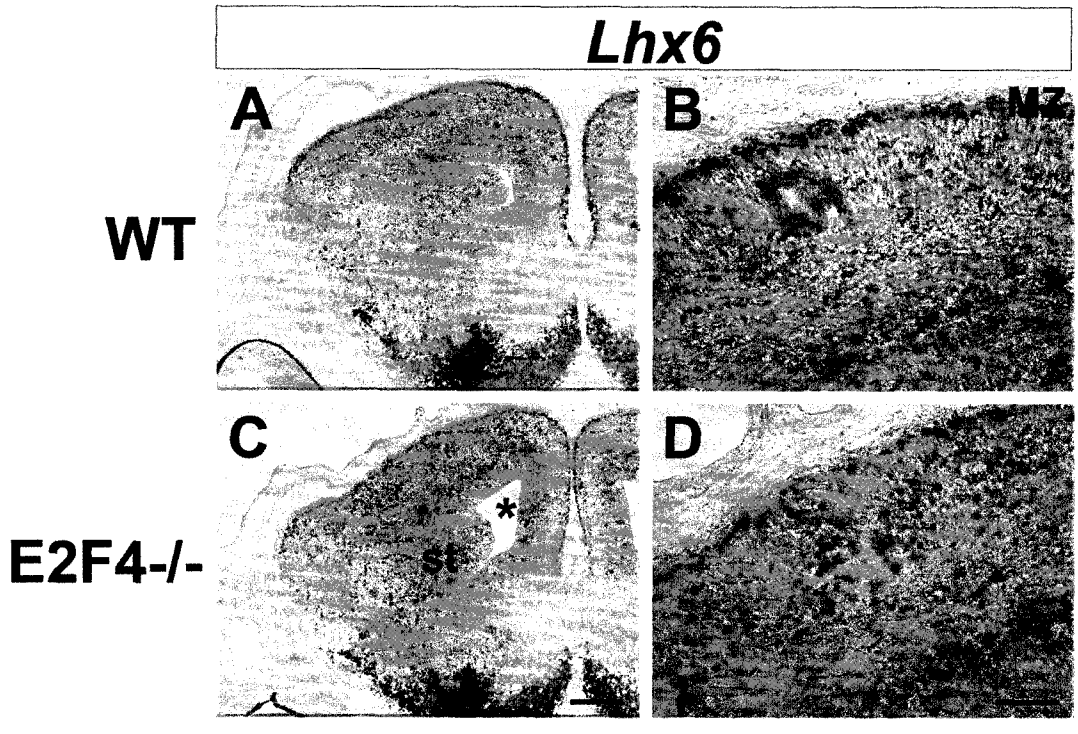
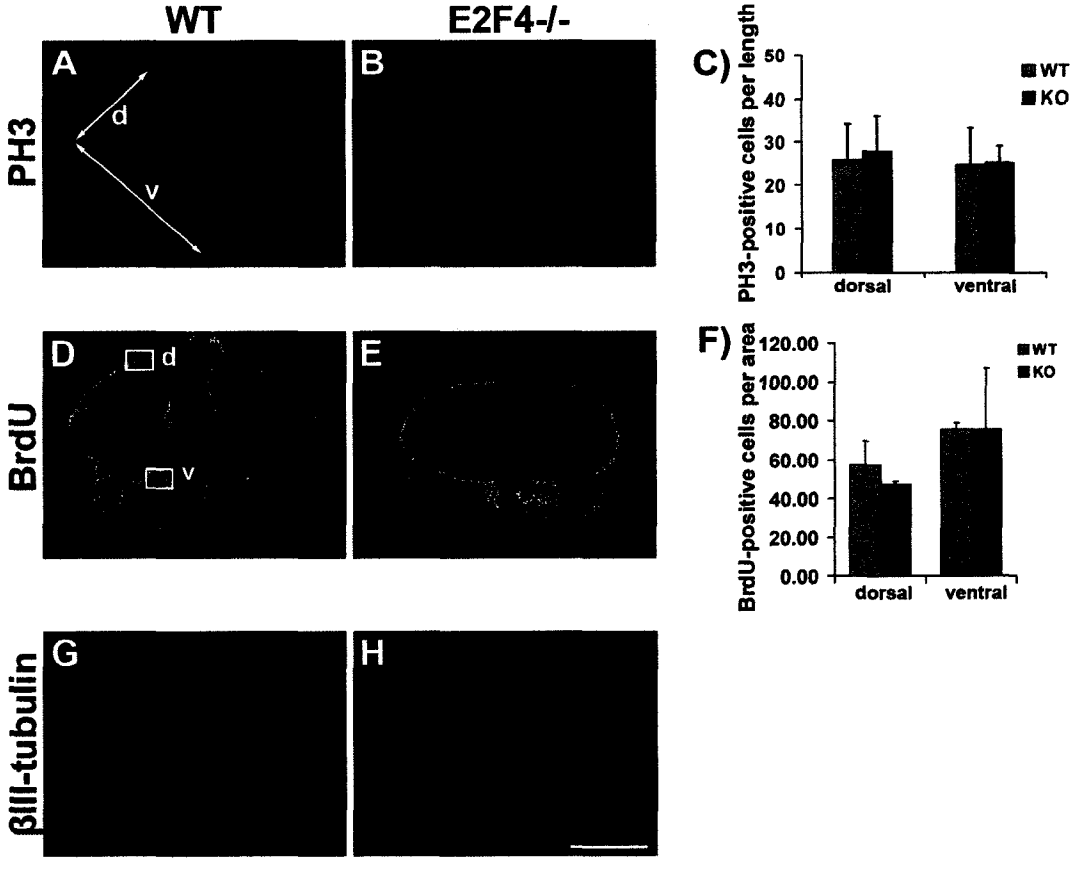

Lhx6
A
B
MZ
WT
C
D
E2F4-/-
*
st

Figure 2.3. Proliferation and early neuronal differentiation appears normal in the developing telencephalon. (A,B,D,E) 14 μm coronal sections through the telencephalon of E11.5 embryos were processed for Phosphohistone-H3 (PH3) and BrdU immunohistochemistry. (C) The mean count of PH3-positive cell per 500 μm of the dorsal (d) and ventral (v) ventricular surface of WT (n=5) and E2F4-/- (n=3) embryos. Error bars indicate S.D. (F) Relative number of BrdU-positive cells per 5500 μm^2 area in the dorsal (d, white box) and ventral (v, white box) telencephalic regions of WT (n=3) and E2F4-/- (n=3) embryos following 30 minute BrdU pulse. Error bars indicate S.D. (G-H) Early differentiating neurons are present in the mantle region of the embryonic telencephalon of E2F4 deficient mice (E) as shown by the βIII-tubulin immunoreactivity (green). Scale bar 500 μm.


WT
E2F4-/-
PH3
BrdU
βIII-tubulin
A
B
C)
D
E
F)
G
H
d
v
PH3-positive cells per length
50
40
30
20
10
0
dorsal
ventral
WT
KO
BrdU-positive cells per area
120.00
100.00
80.00
60.00
40.00
20.00
0.00

incorporation to count cells that have entered S phase. Cells expressing PH3 were counted along the entire dorsal or ventral regions of the ventricular zones while BrdU expressing cells where counted in the dorsal neuroepithelium and MGE (boxed areas Fig. 2.3D). E2F4 mutant embryos did not exhibit any difference in the number of proliferating cells in the dorsal or ventral ventricular zone regions of the telencephalon (Fig. 2.3A-F). Similarly, the differentiation of early born neurons *in vivo* was not affected by the loss of E2F4 as determined by the expression of βIII-tubulin in the mantle region of the developing telencelpalon (Fig. 2.3H). The number of βIII-tubulin positive cells, however, is clearly reduced in the region of the medial ganglionic eminence, consistent with the impaired development of ventrally derived structures. These findings suggest that E2F4 deficiency does not affect the rate of cell division in neural progenitor cells or the timing of the onset of neuronal differentiation in the telencephalon.

Reduced number of neurosphere forming cells in the telencephalon of E2F4 deficient mouse embryos

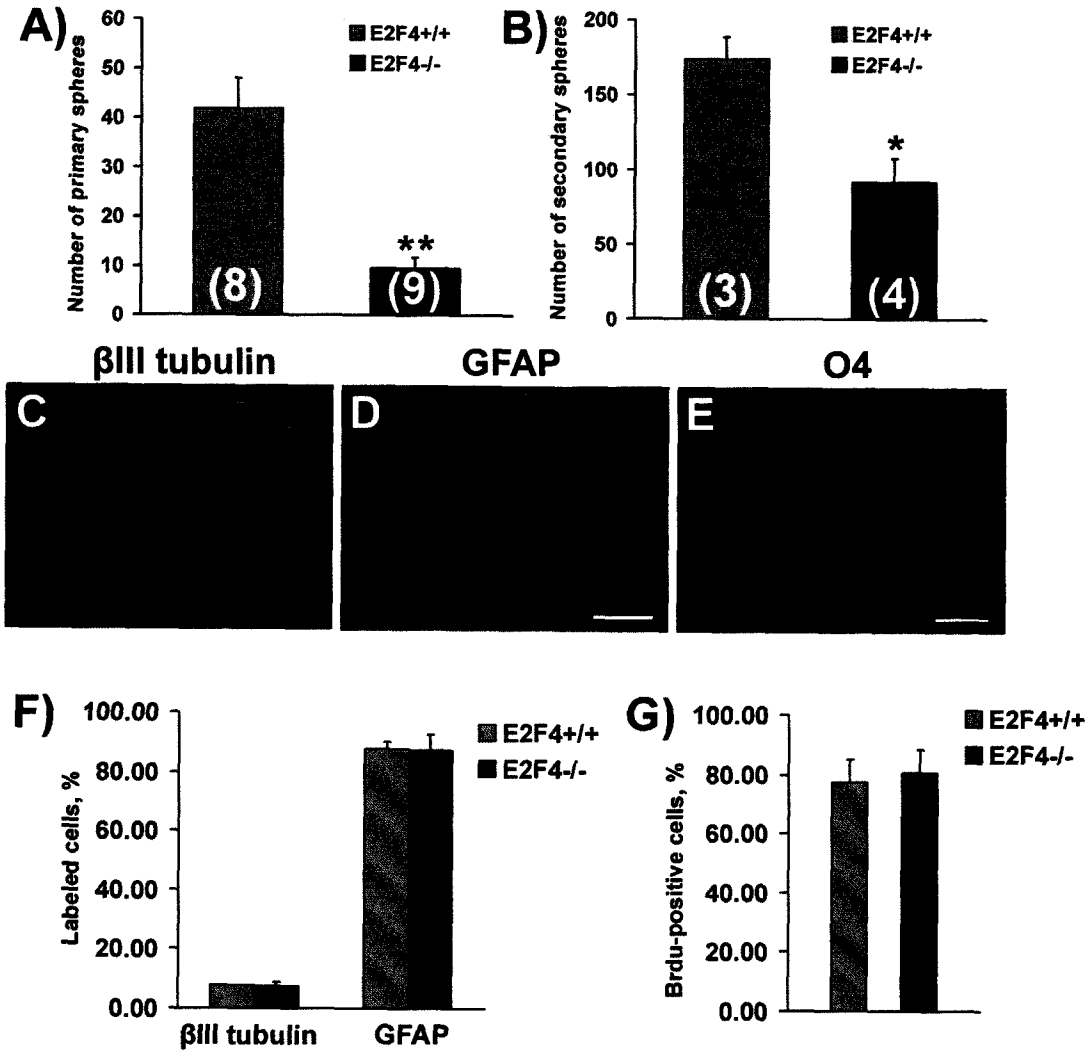
As no defect was found in the rate of progenitor proliferation in E2F4 deficient embryos, we next asked whether the loss of ventral telencephalic structures results from a decrease in the number of neural stem cells. Since there is no unambiguous marker for neural stem cells *in vivo*, we employed an *in vitro* neurosphere assay (Reynolds and Weiss, 1996) to quantify stem cells from the brains of wild type and E2F4 deficient mice. When plated at equal densities, neuroepithelial cells from the telencephalon of E2F4 deficient embryos at E13.5 gave rise to 77% fewer primary neurospheres compared to wild type, indicative of a reduction in the number of sphere forming cells in the mutant

brains (Fig. 2.4A). To confirm that neurospheres exhibited the properties of stem cells, we assessed their self-renewal capacity and multipotentiality. The self-renewal capacity of primary neurospheres from E2F4 null embryos was markedly reduced relative to controls as demonstrated by the number of secondary spheres generated (Fig. 2.4B). Neurospheres from E2F4 deficient embryos were still multipotent, as demonstrated by their ability to differentiate into βIII-tubulin- (early neuronal), GFAP-, and O4- (glial) positive cells (Fig. 2.4C-F). To ask whether E2F4 affects cell cycle regulation in neural precursors, we measured BrdU incorporation in monolayer cultures of neural progenitors *in vitro.* No differences in the proliferation index was detected in cultures derived from wild type and E2F4 deficient mouse embryos, indicating that E2F4 deficiency does not affect the rate of cell proliferation (Fig. 2.4G). Consistent with *in vivo* findings, loss of E2F4 does not affect the rate of progenitor proliferation but instead our results show a reduction in the size and self-renewal capacity of the neural precursor pool in the telencephalon.

Sonic Hedgehog signaling is impaired in the forebrains of E2F4 deficient mice

Since E2F4 deficiency specifically affects ventral telencephalic patterning and stem cell self-renewal we examined the regulation of genes that direct the development of this region. While several candidates were examined, we focused our studies on the components of the Sonic hedgehog (Shh) pathway. Shh was assessed because (a) previous studies revealed a requirement for Shh for specification of the ventral telencephalon (Chiang et al., 1996; Rallu et al., 2002b), (b) Shh promotes stem cell self-renewal (Machold et al., 2003; Palma et al., 2005), and, (c) the E2F4 deficient phenotype

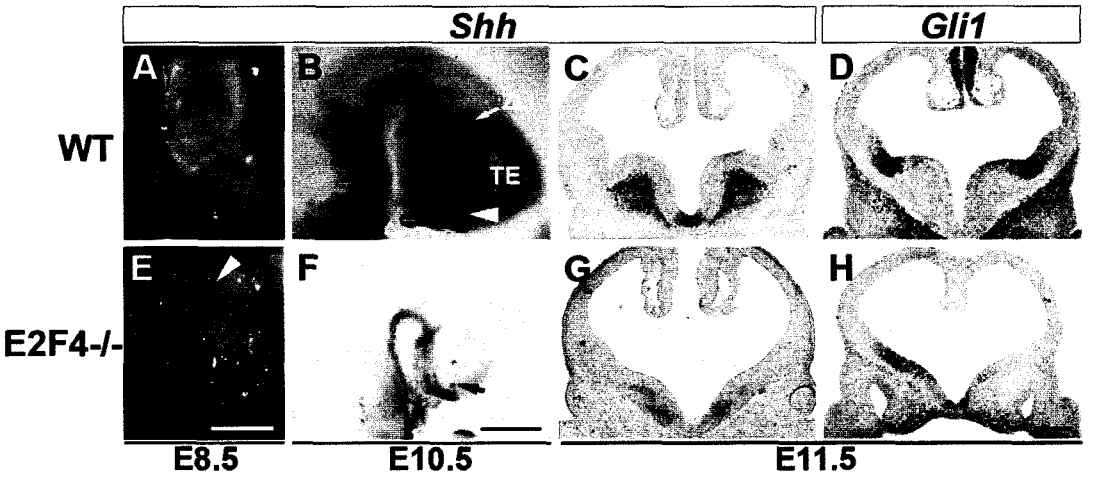
Figure 2.4. Reduced number and self-renewal capacity of neural precursors in E2F4 deficient mouse embryos. Cultures from E2F4-/- embryos generated significantly lower numbers of primary (A) and secondary (B) neurospheres versus their WT control littermates. (A) Dissociated telencephalic neuroepithelial cells from E13.5 mouse embryos were cultured in 500 μl of serum-free medium containing 25 ng/ml bFGF at a density of 10 cells/μl. (B) Single primary neurospheres were dissociated and cultured in serum-free medium with bFGF. Primary and secondary spheres were counted after 7 days in vitro. Numbers on the graph bars indicate the number of embryos per genotype analyzed. Error bars indicate s.e.m. *- $p< 0.02$, ** - $p< 0.0001$ (t-test, two-tail). (C-E) Neurons, astrocytes and oligodendrocytes in differentiating neurospheres derived from telencephalic neuroepithelia of E13.5 E2F4-/- mouse embryos. After 7 days in vitro in differentiating medium with FBS cells were fixed with 4% PFA in 1XPBS and processed for βIII-tubulin (C, neurons, green), Glial Fibrillary Acidic Protein (GFAP) (D, astrocytes, green) and O4 (E, oligodendrocytes, red) immunocytochemistry. Cell nuclei were stained with Hoechst (blue). Scale bars: 25 μm (C,D); 50 μm (E) . (F) Percentage of βIII-tubulin- and GFAP-positive cells out of the total number of cells (Hoechst-labelled nuclei) in differentiated neurospheres. Neurospheres from two embryos per genotype were analysed. Data are presented as means±range. (G) Proliferation analysis in neural progenitor cultures from wild type and E2F4 deficient embryos. Cells from wild type (n=4) and E2F4-/- (n=5) mouse embryos were fixed on Day 3 following induction of differentiation. 10 μM BrdU was added to the culture medium 24 hours before the end of the culture period. The proportion of proliferating cells is estimated as a percentage of the total number of cells (Hoechst-positive nuclei). Data are presented as means±s.e.m.

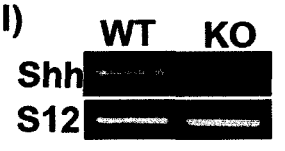

A)
Number of primary spheres
60
50
40
30
20
10
0
E2F4+/+
E2F4-/-
**
(8)
(9)
B)
Number of secondary spheres
200
150
100
50
0
E2F4+/+
E2F4-/-
*
(3)
(4)
βIII tubulin
GFAP
O4
C
D
E
F)
Labeled cells, %
100.00
80.00
60.00
40.00
20.00
0.00
E2F4+/+
E2F4-/-
βIII tubulin
GFAP
G)
Brdu-positive cells, %
100.00
80.00
60.00
40.00
20.00
0.00
E2F4+/+
E2F4-/-

was strikingly similar to the phenotype of the Shh pathway mutants (Fuccillo et al., 2004). To ask if there was a deregulation in Shh signaling, we examined Shh regulation in the telencephalon of E2F4 deficient and wild type littermates. We first asked if loss of E2F4 disrupted Shh expression at earlier developmental timepoints. At E7-8.5, the time of appearance of Shh in the forebrain anlage (Shimamura and Rubenstein, 1997; Rallu et al., 2002b), there was no difference in the levels of Shh mRNA in the rostral neural plate (Fig. 2.5A,E; data not shown). By E10.5 however, there was a decrease in Shh mRNA rostral to the zona limitans interthalamica (ZLI) and an absence of the signal in the ventral telencephalon (Fig. 2.5F). In other areas of the developing embryos such as the ventral spinal cord and notochord, there was no detectable difference in the expression of Shh at E11.5 (Supp. Fig. 2.3). Thus, findings from whole mount in situ demonstrate that Shh disruption occurs after E8 and specifically in the ventral telencephalon and rostral diencephalon of E2F4 null embryos. The timing of Shh disruption occurs after the division of the eyefields and telencephalic hemispheres consistent with the absence of a cyclopia and holoprosencephaly (characteristic features of Shh null phenotype (Chiang et al., 1996)) in E2F4 deficient embryos. We therefore examined Shh regulation specifically at the time when ventral telencephalic structures are developing.

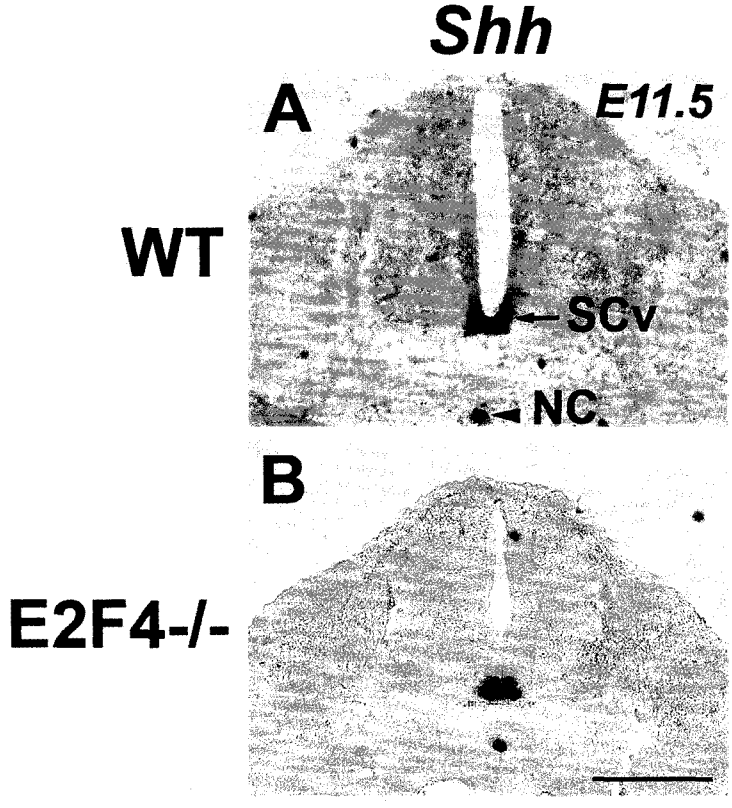
At E11.5, Shh is normally expressed in the ventral telencephalic midline, as well as in the base of MGE (Fig. 2.5C). In E2F4 deficient embryos, Shh was absent from the ventral midline and greatly reduced in the mantle region of the MGE (Fig. 2.5G). To address whether the reduced expression of Shh in E2F4 mutants correlated with reduced Shh pathway activity, we examined the expression of Gli1, a primary target gene of the

Figure 2.5. Deregulation of Shh and Gli1 expression in the ventral telencephalon and in neurosphere cultures from E2F4 deficient mice. (A,B,E,F) Whole-mount in situ hybridization for *Shh* mRNA of E8.5 (A,E) and E10.5 (B,F) WT (A,B) and E2F4-/- (E,F) mouse embryos. Note Shh mRNA expression is present in the rostral neural plate ventral midline of E2F4 deficient embryo at E8.5 (E, white arrowhead) similar to the control littermate. At E10.5 Shh is detected in the ventral telencephalon of WT embryos (B, white arrowhead), whereas it is absent in E2F4 deficient mice (F, black arrow). TE – telencephalon; ZLI – zona limitans interthalamica (indicated by white arrow). Scale bar 500 μm. (C,D,G,H) Coronal sections through the telencephalon of WT (C,D) and E2F4-/- (G,H) E11.5 embryos were analyzed for Shh (C,G) and Gli1 (D,H) mRNA expression (in situ hybridization). Scale bar 250 μm. (I) RT-PCR analysis of mRNA extracted from WT and E2F4-/- secondary neurospheres. Shh mRNA level is reduced in neurospheres from E2F4 deficient mouse embryos (n=3). S12 was used as loading control.


Shh
Gli1
A
B
C
D
WT
TE
E
F
G
H
E2F4-/-
E8.5
E10.5
E11.5


I)
WT
KO
Shh
S12

Supplementary figure 2.3. E2F4 deletion does not affect Shh expression in the notochord and spinal cord at E11.5. In situ hybridization analysis for Shh mRNA expression in the ventral spinal cord (A, SCv, black arrow) and notochord (A, NC, black arrowhead) of E11.5 wild type (WT) and E2F4-/- mouse embryos. Scale bar 250 μm.


Shh
A
E11.5
WT
SCv
NC
B
E2F4-/-

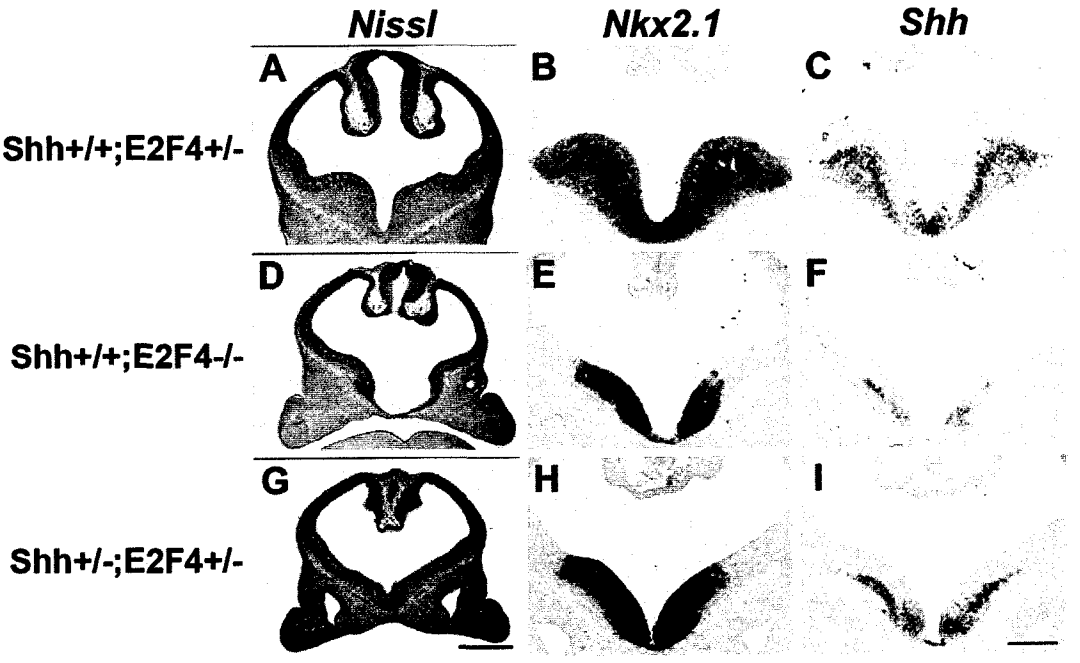
pathway (Lee et al., 1997). In wild type embryos, Gli1 was strongly expressed in the ventricular and subventricular zones of ventral telencephalon between the MGE and LGE (Fig. 2.5D). In contrast, Gli1 expression was greatly diminished in E2F4 deficient littermates, consistent with decreased Shh activity in this region of the brain (Fig. 2.5H).

To determine whether the disruption of Shh signaling results from a cell autonomous defect in E2F4 deficient neural precursor cells, we asked whether Shh was also reduced in neurospheres cultured from E2F4 deficient brains. RT-PCR revealed a reduction in the levels of Shh mRNA in E2F4 deficient neurospheres (Fig. 2.5I). Decreased Shh expression in neurosphere cultures is consistent with an impairment of self-renewal capacity found in E2F4 deficient precursors. Taken together these data indicate that E2F4 deficiency leads to a cell autonomous impairment of Shh signaling in the developing ventral telencephalon.

Genetic interaction between Shh and E2F4 in ventral telencephalic patterning

As Shh is disrupted in E2F4 deficient brains, we asked if there was a genetic interaction between E2F4 and the Shh pathway. To address this question, compound Shh and E2F4 heterozygous mice were generated. Compound heterozygous mice for E2F4 and Shh exhibited a reduction in Shh expression in the ventral telencephalon. Since mice heterozygous for either E2F4 or Shh are similar to wild type, we asked if compound heterozygous mice exhibit an exacerbated phenotype, thus revealing a genetic interaction. In contrast to the single E2F4 (Fig. 2.6A-C) or Shh (data not shown) heterozygous mice, double heterozygous E2F4/Shh mice (Fig. 2.6G-I) displayed a telencephalic phenotype

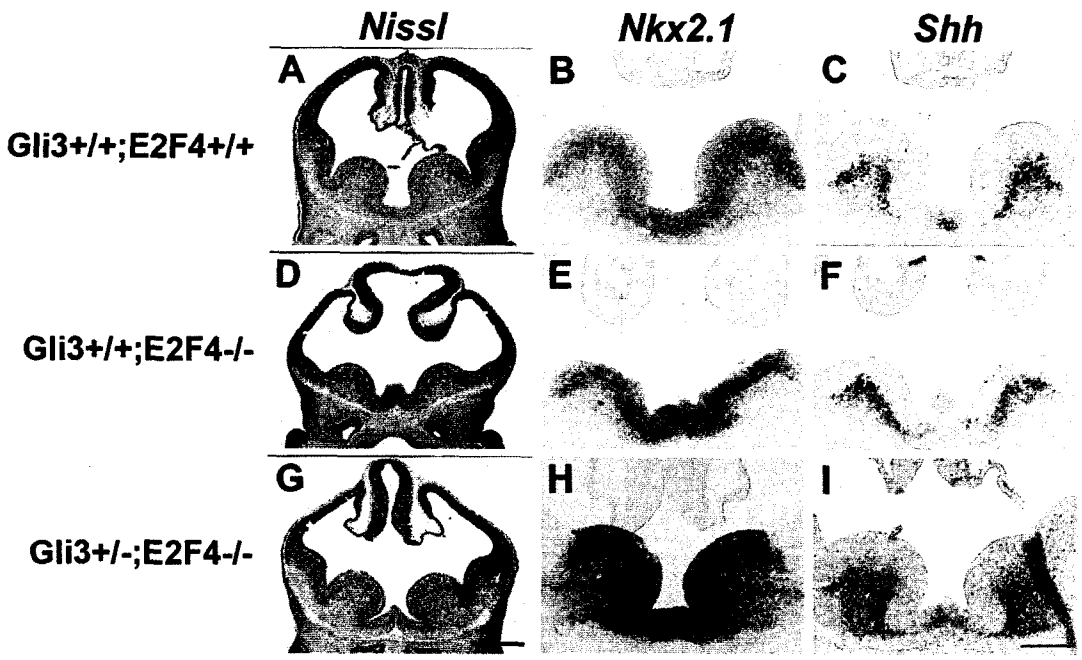
Figure 2.6. Altered telencephalic patterning in combined double heterozygous (Shh+/-;E2F4+/-) mouse embryos. (A,D,G) Cresyl violet staining of coronal sections through the telencephalon of E2F4 heterozygous (Shh+/+;E2F4+/-), E2F4 deficient (Shh+/+;E2F4-/-), and Shh;E2F4 double heterozygous (Shh+/-;E2F4+/-) E11.5 embryonic brains. A minimum of four embryos per genotype were examined. Scale bar 500 μm. (B,C,E,F,H,I) In situ hybridization analysis of Nkx2.1 and Shh mRNA expression in the MGE and ventral telencephalic midline of E11.5 embryos. Scale bar 200 μm.


Nissl
Nkx2.1
Shh
A
B
C
D
E
F
G
H
I
Shh+/+;E2F4+/-
Shh+/+;E2F4-/-
Shh+/-;E2F4+/-

similar to that found in E2F4 deficiency (Fig. 2.6D-F). Shh/E2F4 double deficient embryos could not be recovered, suggesting that the combined null mutation is lethal before E11 and that the loss of E2F4 exacerbates Shh deficiency. These studies demonstrate a novel genetic interaction between E2F4 and Shh.

Another member of the Shh signaling pathway with a known role in dorsoventral telencephalic patterning is the Gli3 transcription factor, which functions as an antagonist of Shh signaling (Rallu et al., 2002a; Rallu et al., 2002b). Gli3 functions as a transcriptional repressor in the absence of Shh activity, and its repressive effects are relieved upon activation of the Shh pathway (Cayuso and Marti, 2005). Spontaneous mutant extra-toes (Xt) heterozygous Gli3 mice ($Gli3^{Xt/+}$) display normal telencephalic structures, whereas $Gli3^{Xt/Xt}$ homozygous embryos have severe craniofacial abnormalities often combined with the loss of the dorsal midline, exencephaly and perinatal lethality (Buscher et al., 1997). Since removal of Gli3 rescues the Shh deficient phenotype in the forebrain (Rallu et al., 2002b), we asked if the phenotype we observed in the telencephalon of E2F4 deficient mice could also be rescued by disruption of Gli3 activity. Analysis of E11.5 mouse embryos demonstrates that heterozygosity for Gli3 restores the size of ventral telencephalic structures and the expression of Nkx2.1 and Shh in E2F4 deficient embryos (Fig. 2.7G-I). These results highlight the importance of E2F4 in normal dorsoventral patterning during early telencephalic development.

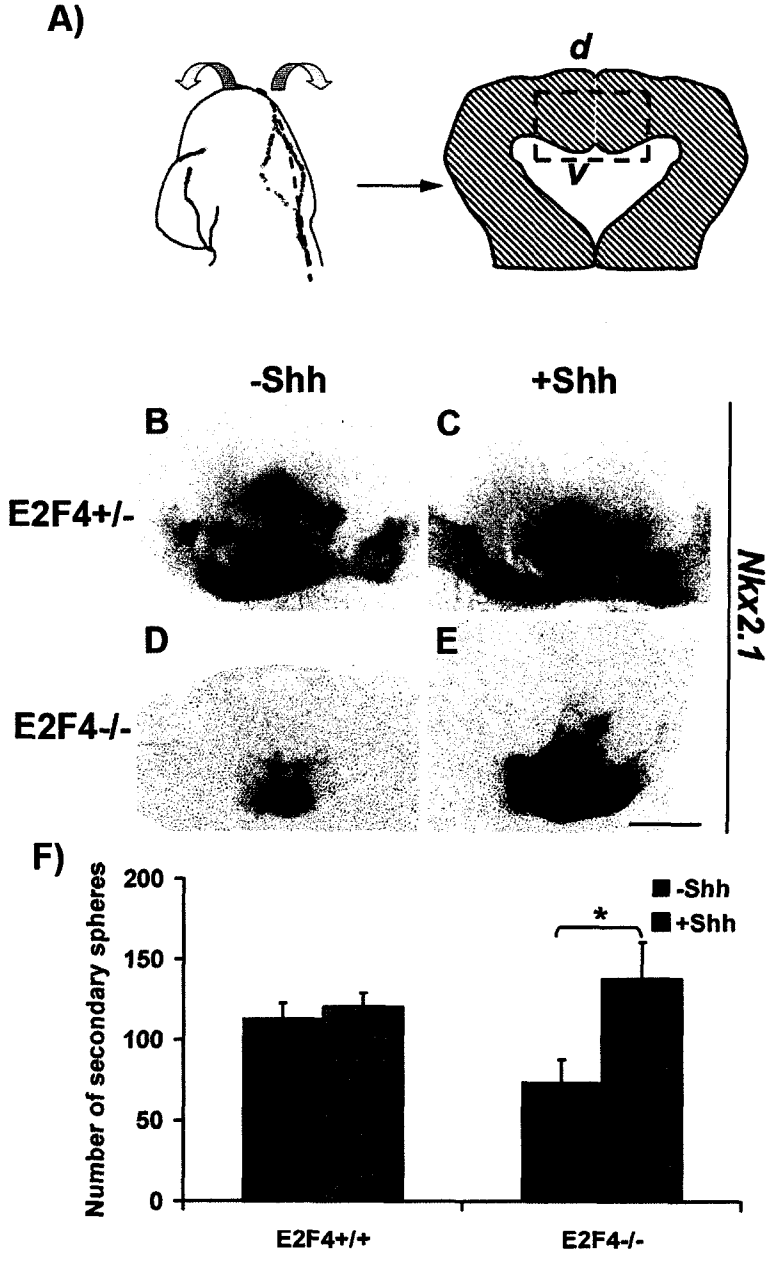
Figure 2.7. Partial removal of Gli3 restores ventral telencephalic development in Gli3+/-;E2F4-/- compound mutant mice. Cresyl violet staining (A,D,G) and in situ hybridization for Nkx2.1 (B,E,H) and Shh (C,F,I) mRNA of WT (Gli3+/+;E2F4+/+), E2F4 deficient (Gli3+/+;E2F4-/-), and combined Gli3 heterozygous;E2F4 deficient (Gli3+/-;E2F4-/-), (n=4/5) of E11.5 embryonic brains coronal sections. Scale bars: A,D,G – 500 μm; B,C,E,F,H,I – 200 μm.


Nissl
Nkx2.1
Shh
A
B
C
D
E
F
G
H
I
Gli3+/+;E2F4+/+
Gli3+/+;E2F4-/-
Gli3+/-;E2F4-/-

Shh agonist rescues defective ventral telencephalic patterning and self-renewal capacity of neural precursors in E2F4 deficient embryos

Since Shh signaling activity is decreased in the absence of E2F4, we next asked whether replacing Shh could rescue the development of ventral structures. To address this question we treated E9.5 embryonic neural tube explants (Echevarria et al., 2001) from E2F4 deficient mice with a Smo receptor agonist (Frank-Kamenetsky et al., 2002) and evaluated whether Nkx2.1, which is known to be induced by Shh (Gaiano et al., 1999; Wilson and Rubenstein, 2000), could be restored. Activation of Shh signaling by the exogenous agonist rescued the expression of Nkx2.1 in explants from E2F4 deficient mice (Fig. 2.8E). These findings indicate that Shh signaling downstream of the Smo receptor is preserved in the absence of E2F4 and activation of the Shh pathway is sufficient to partially restore cells that express homeodomain genes that specify ventral telencephalic structures in E2F4 deficient mice. We also asked if replacing Shh could restore stem cell self-renewal in E2F4 deficient neural precursors. Addition of the Hh pathway agonist rescues the self-renewal capacity of E2F4 deficient neural precursor cells (Fig. 2.8F). Although the Hh agonist has no significant effect on the self-renewal capacity of wild type neurospheres, Shh dependency was demonstrated by the inhibition of neurosphere formation following the addition of the Hh pathway antagonist cyclopamine (data not shown). In conclusion, these results establish that the Shh pathway is functional in E2F4 deficient embryos and that replacement of Hh agonists can restore precursor self renewal and cells expressing ventrally derived markers in tissue explants. Since replacing Hh agonist partially rescues defects in E2F4 deficient tissue, we next

Figure 2.8. Shh agonist partially restores neurosphere self-renewal capacity and cells expressing Nkx2.1 in the E2F4-/- ventral telencephalon. (A) Diagram of the explant culture experiment. Postero-lateral view of the head of the developing E9.5 embryo (left). Dashed black line indicates the cut along the dorsal midline. Blue arrows show the directions the neural tube is opened. Diagram of the opened neural tube explant (right); filled area represents luminal (ventricular) surface of neural tube. Dashed black box indicates the area represented in the B-E. d – dorsal, v – ventral, TE – telencephalon. (B-E) In situ hybridization for Nkx2.1 mRNA of E9.5 explants cultured with or without 20 nM of Shh agonist for 48 hours. n=3. Scale bar 250 μm. (F) Self-renewal capacity of E2F4-/- neurospheres is restored with the addition of Shh agonist. n=3. Error bars indicate s.e.m. *-$p<0.05$ (t-test, two-tail).

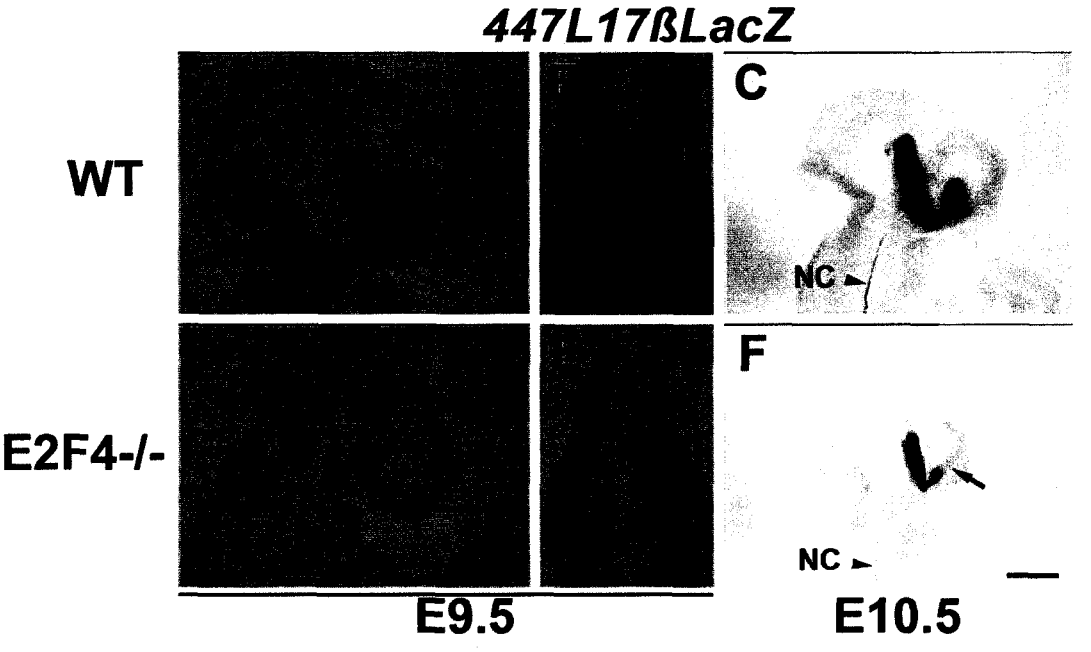

A)
d
V
-Shh
+Shh
B
C
E2F4+/-
D
E
E2F4-/-
Nkx2.1
F)
Number of secondary spheres
200
150
100
50
0
-Shh
+Shh
*
E2F4+/+
E2F4-/-

asked whether E2F4 may be required for the regulation of Shh gene expression in the ventral telencephalon.

E2F4 deficiency leads to aberrant activation of Shh brain enhancers

Given the widespread E2F4 expression (Supp. Fig. 2.1; (Dagnino et al., 1997; Callaghan et al., 1999)), we questioned how Shh regulation could be disrupted specifically in a spatial and temporal manner in E2F4 deficient embryos. One of the mechanisms to achieve differential regulation of *Shh* expression along the rostro-caudal axis of the neural tube is through the regulation of region-specific Shh enhancers (Epstein et al., 1999; Jeong et al., 2006). As the expression of *Shh* in the rostral diencephalon and ventral telencephalon is controlled by *Shh* brain enhancer 2 (SBE2), SBE3, and SBE4 (Jeong, et al., 2006), we asked whether the activity of these enhancers is deregulated in the absence of E2F4. To answer this question we interbred E2F4 mutant mice with transgenic mice carrying a 447L17ßlacZ reporter construct, which contains the SBE2, SBE3, and SBE4 regulatory sequences. The activity of these enhancers specifically targets reporter gene expression to the sites of *Shh* transcription in the developing forebrain (Jeong et al., 2006). First, consistent with the unaffected regulation of Shh in other areas, we observed similar reporter activity in the notochord of wild type and E2F4 deficient transgenic embryos (Fig. 2.9C,F arrowheads). At E9.5 strongly-expressing *lacZ* regions were present in the wild type ventral forebrain from the middle of diencephalon and converging at the midline rostrally at the level of the optic vesicle (Fig. 2.9A,B). In the absence of E2F4, the reporter activity is barely detectable in the region of the optic vesicle (Fig. 2.9D,E). By E10.5 the intensity of X-gal staining in the ventral forebrain of

Figure 2.9. Aberrant activity of *Shh* regulatory elements in the ventral forebrain of E2F4 deficient mouse embryos. Whole-mount *lacZ* expression (X-gal) of E9.5 (A,B,D,E) and E10.5 (C,F) WT (A-C) and E2F4-/- (D-F) embryos carrying 447L17ßlacZ reporter construct. Note the reduced reporter activity in E2F4 deficient embryos at E9.5 (D,E) and failure to expand into the ventral telencephalon at E10.5 (F, black arrow), whereas reporter activity is present in the notochord (NC) (C, F black arrowheads). Scale bars: A,D – 250 μm; B,E – 125 μm; C,F – 500 μm.


447L17ßLacZ
WT
E2F4-/-
C
NC
F
NC
E9.5
E10.5

E2F4 deficient embryos is increased but still failed to extend into the ventral telencephalon (Fig. 2.9C,F). These results demonstrate that there is a prominent developmental delay in the activation of the Shh enhancer activity in the absence of E2F4. Thus, the impairment in the activation of the brain specific enhancers can account for the region specific disruption of Shh activity seen in E2F4 deficient embryos. In conclusion, the results of our studies examining Shh gene expression combined with Shh enhancer activity in E2F4 null embryos demonstrate that E2F4 is required for the development of the ventral telencephalon by affecting the region specific regulation of Shh.

DISCUSSION

The results of these studies show for the first time that the cell cycle regulatory protein E2F4 is required for normal development of the ventral telencephalon. Animals lacking E2F4 exhibit a dramatic loss of ventral telencephalic structures characterized by the absence or reduction of the lateral and medial ganglionic eminences. Homeodomain proteins including Nkx2.1 and Dlx2, which specify these regions, are absent or expressed at very low levels. While a cell cycle defect was not detected in existing progenitor cells, there was an impairment in the self-renewal capacity of neural stem cells. Examination of the mechanism underlying the telencephalic defect found in E2F4 deficiency revealed a striking reduction in Shh, a morphogen essential for the development of ventral structures. These studies define a novel requirement for E2F4 in regulating the specification of the ventral telencephalon.

E2F4 is a regulatory target for Rb family members and has been found to interact with all three proteins, namely, p107, p130 and Rb (Trimarchi and Lees, 2002). While the Rb family of cell cycle proteins are well known for their role in the regulation of proliferation, new evidence is emerging that reveals novel functions for these proteins beyond cell cycle control (McClellan and Slack, 2006). For example, p107 has been shown to regulate the neural precursor pool, specifically by controlling the self-renewal capacity of neural stem cells (Vanderluit et al., 2004). The mechanism by which p107 mediates this effect was through the repression of the Notch signaling pathway since p107 deficient brains exhibit enhanced levels of the Notch1 intercellular domain and elevated Hes1. Loss of only one Hes1 allele restores the neural precursor pool back to wild type levels. As with p107, studies examining the role of Rb have also identified cell

cycle independent functions. It is well established that Rb is required for the regulation of terminal mitosis in multiple cell types (Callaghan et al., 1999; Lipinski and Jacks, 1999); however, more recently Rb has been shown to regulate neuronal migration (Ferguson et al., 2005). Conditional mutants, in which Rb has been deleted in the telencephalon, have revealed defects associated with migration that could not be reconciled with ectopic proliferation. In the Rb deficient ventral telencephalon several neuronal populations arising from this region fail to migrate to their final destinations within the cortical plate. A search for E2F targets which may be deregulated in Rb null mice has revealed genes known to play important roles in controlling neuronal migration (McClellan et al., submitted). Given that Rb deficiency exhibits a striking defect in ventral telencephalic development, we asked if the loss of Rb interacting partners, such as the E2F transcription factors affected this same region. We therefore examined development of the brain in E2F4 null mice.

E2F4 in early brain development

While previous studies have revealed that E2F4 is important during embryogenesis the mechanisms by which it impacts development remain poorly understood. E2F4 is required for proliferation and maturation of erythroid lineage and for the normal development of the intestinal epithelium and craniofacial structures (Humbert et al., 2000b; Rempel et al., 2000; Deschenes et al., 2004; Kinross et al., 2006). Closer examination of the intestinal epithelium of E2F4 deficient mice suggests that E2F4 has a role in the regulation of differentiation (Deschenes et al., 2004). Here we report a novel previously unidentified requirement for E2F4 in early patterning of the ventral

telencephalon. Specifically, E2F4 deficient mouse embryos display a severe phenotype characterized by the loss of the ventral telencephalic structures. Analysis of region specific patterning markers revealed a dramatic reduction in their expression in the ganglionic eminences and telencephalic ventral midline of E2F4 deficient embryos. While Rb is known to regulate progenitor proliferation and migration (Ferguson et al., 2002; Ferguson et al., 2005), and the mechanism by which this occurs is through the E2F pathway (McClellan et al.,submitted), our present findings are the first to reveal a requirement for E2F4 in early patterning of the ventral forebrain.

E2F4 mutant embryos exhibit aberrant Shh signaling

The results of the secondary neurosphere assay indicate that in the absence of E2F4 the self-renewal capacity of neural stem cells is significantly reduced. Consistent with the known role of the Shh pathway in regulation of embryonic and adult stem cell compartments (Lai et al., 2003; Palma et al., 2005), we have observed reduced levels of Shh transcripts in neural precursor cells of E2F4 deficient mice. The dependence of the self-renewal capacity of E2F4 deficient neural stem cells on Shh is further supported by the ability of the exogenous Shh agonist to rescue the number of secondary spheres in E2F4 deficient cultures. These data demonstrate that E2F4 is required to regulate the self-renewal capacity of neural precursor cells during early telencephalic development by modulating Shh signaling.

Our *in vivo* studies reveal that defective telencephalic development in E2F4 deficient embryos results from a temporal and region specific reduction of Shh signaling. The importance of Shh in patterning of the ventral telencephalon is demonstrated by

holoprosencephaly and cyclopia, a phenotype exhibited by whole embryo Shh knockouts (Chiang et al., 1996; Wallis and Muenke, 1999). Unlike animals lacking Shh, E2F4 deficient mice do not develop cyclopia and holoprosencephaly, suggesting that patterning defects occur after the division of the eye field and separation of telencephalic hemispheres. The ventral phenotype exhibited by E2F4 deficiency is strikingly similar to that found in conditional mutants of Smo, the obligate transducer of Shh signaling. Complete deletion of Smo in the mouse telencephalon at E9 results in the loss of ganglionic eminences and a reduction of Nkx2.1 and Mash1 expression, with the ventral expansion of dorsal markers Pax6, Ngn2, and Emx2 (Fuccillo et al., 2004). E2F4 deficiency results in impaired Shh expression in the ventral telencephalon after E8.5 when the division of the telencephalic hemispheres is complete.

As E2F4 is ubiquitously expressed, one might ask why Shh expression is impaired only in the ventral forebrain in E2F4 deficient mice. A number of reasons could account for such a temporal and region specific activation of Shh. One explanation is that the disruption of Shh signaling is not complete in the absence of E2F4. Thus, the reduced activity of Shh may be marginally sufficient for the development of the dorsal pallial structures but not for the induction of the ventral-most patterning genes. Partial restoration of cells expressing Nkx2.1 in response to exogenous Shh agonist supports this interpretation. Alternatively, and most likely, is the more recent finding demonstrating that the temporal and region specific expression of Shh throughout the embryo is regulated by region specific regulatory elements (Epstein et al., 1999; Jeong et al., 2006). As each enhancer is unique, these results would suggest that E2F4 is required for normal activation of the brain specific enhancers that direct Shh expression in the ventral

telencephalon (Jeong et al., 2006). Our results with LacZ reporter mice demonstrating impaired activation of Shh brain enhancers are consistent with this interpretation.

In conclusion, these results demonstrate that the cell cycle regulatory protein E2F4 is essential for ventral telencephalic patterning. The deficit in ventral telencephalic structures is accompanied by a defect in neural stem cell self-renewal. The mechanism underlying aberrant ventral patterning lies in a dramatic loss in Shh expression in this region. Treatment with Hh agonist reveals a rescue of stem cell self-renewal and cells expressing homeodomain proteins that specify the ventral telencephalic structures. Finally, we found that E2F4 deficiency resulted in reduced activity of Shh forebrain specific enhancers. In conclusion, these studies establish a novel genetic interaction between E2F4 and the Shh pathway and identify a novel function for the cell cycle regulatory protein, E2F4, in the development of the ventral telencephalon.

Chapter 3.

Vladimir A. Ruzhynsky, Marosh Furimsky, David S. Park, Valerie A. Wallace and Ruth S. Slack, (2007). E2F4 is required for early eye patterning. (*Developmental Dynamics* in revisions)

E2F4 IS REQUIRED FOR EARLY EYE PATTERNING

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Running Title: E2F4 in eye development
Keywords: E2F; cell cycle; nervous system; morphogenesis

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Funded by:

Canadian Institutes of Health Research

Contributions of the author and collaborators

I performed the experiments in this study and analyzed the data. Dr. Marosh Furimsky assisted with initial analysis of eye phenotype and critical review of the manuscript. Dr. David S. Park provided mice and intellectual input. Dr. Valerie A. Wallace assisted with data analysis of eye phenotype, provided valuable suggestions and some immunohistochemical reagents. The study was conducted under the guidance and with analytical help of Dr. Ruth R. Slack. This manuscript was written by me, and reviewed and edited by Dr. Valerie A. Wallace and Dr. Ruth S. Slack.

ABSTRACT

Increasingly, studies reveal novel functions for cell cycle proteins during development. Here we investigated the role of E2F4 in eye development. E2F4 deficient mouse embryos exhibit severe early eye patterning defects, which are evident from E11.5 and characterized by microphthalmia, coloboma, and aberrant shape of the optic cup. Loss of E2F4 is associated with proximal-distal patterning defects in the optic vesicle. These defects are characterized by the expansion of optic stalk marker gene expression to the optic cup and reduced expression of ventral optic cup markers. These defects are associated with a split of *Shh* expression domain at the ventral midline of the forebrain. Despite these patterning defects, neuronal differentiation and Shh expression in the retina are not affected by *E2F4* deletion. Overall, the results of our studies show a novel role of E2F4 in the early eye development.

INTRODUCTION

Correct cell cycle regulation is essential in a number of cellular processes during development including cell proliferation, differentiation, and survival. The retinoblastoma protein (pRb) is a critical regulator of cell cycle (reviewed in (Khidr and Chen, 2006)). Initially discovered as a tumor suppressor gene (Friend et al., 1986), Rb is also required for regulation of the cell cycle and apoptosis during development in a number of systems, including the eye (Bremner et al., 2004). For example, Rb deficiency in the developing retina results in defects in cell cycle exit of committed progenitors (Chen et al., 2004a) and impairment of rod photoreceptor and horizontal cell differentiation (Donovan and Dyer, 2004; Zhang et al., 2004). Moreover, others reported that conditional *Rb* deletion in the mouse retina results in high levels of apoptosis, the loss of retinal ganglion cells, photoreceptors, and bipolar cells (MacPherson et al., 2004). The results of these studies demonstrated the requirement for Rb in retinogenesis.

Rb executes cell cycle regulation by interacting with and controlling the activity of E2F transcription factors (Trimarchi and Lees, 2002; Khidr and Chen, 2006). The hypophosphorylated Rb binds to E2Fs thus preventing transcriptional activation by free activating E2Fs and/or actively repressing target genes (Stevaux and Dyson, 2002). Hyperphosphorylation of Rb by cyclin-dependent kinases inactivates Rb, thereby releasing E2Fs, thus enabling transcriptional activation or de-repression of target genes (Lipinski and Jacks, 1999).

Eight genes encoding nine E2F family members have been identified (reviewed in (DeGregori and Johnson, 2006)). The involvement of E2F transcription factors in Rb regulation of proliferation and differentiation is evident from the analysis of mouse

specific *Rb* mutants R654W, which are unable to bind E2Fs 1-3 (Sun et al., 2006). These animals display ectopic cell proliferation and rod photoreceptor differentiation defects in the retina, similar to *Rb* null embryos (Sun et al., 2006).

At the same time, a number of studies revealed specific roles E2F family members as targets of Rb in eye development. E2F1, E2F2, and E2F3 contribute equally to the ectopic proliferation in the retinas from Rb deficient mouse embryos, while in the lens Rb controls proliferation primarily through E2F3 (Saavedra et al., 2002). Furthermore, the Rb-mediated control of apoptosis in the lens and retina is mediated by E2F1 (Saavedra et al., 2002). Thus, E2Fs have specific roles in the control of Rb-dependent proliferation and apoptosis in developing eye structures.

A number of reports describe non cell cycle-related functions of Rb/E2F pathway in eye and nervous system development. Deficiency for the Rb family member p107 leads to activation of the Notch signaling pathway and an increase in the number of neural precursors in the embryonic and adult brain (Vanderluit et al., 2004). Rb is also required cell-autonomously for interneuron migration in the developing telencephalon (Ferguson et al., 2005). Similar to the mechanism of Rb/E2F cell cycle control, E2Fs regulate a number of non-proliferating genes associated with development, apoptosis, and differentiation (Muller et al., 2001).

One of the Rb partners is the E2F4 transcription factor. The importance of E2F4 for normal development is evident from the analysis of E2F4 deficient mice. The loss of *E2F4* causes general growth retardation, a high rate of late embryonic and perinatal lethality, and impaired proliferation and maturation of several tissues (Humbert et al., 2000b; Rempel et al., 2000; Kinross et al., 2006). E2F4 deficiency is also associated with

macrocytic anemia due to the impaired erythrocytes proliferation (Kinross et al., 2006), defects in the intestinal epithelium and craniofacial abnormalities (Humbert et al., 2000b; Rempel et al., 2000).

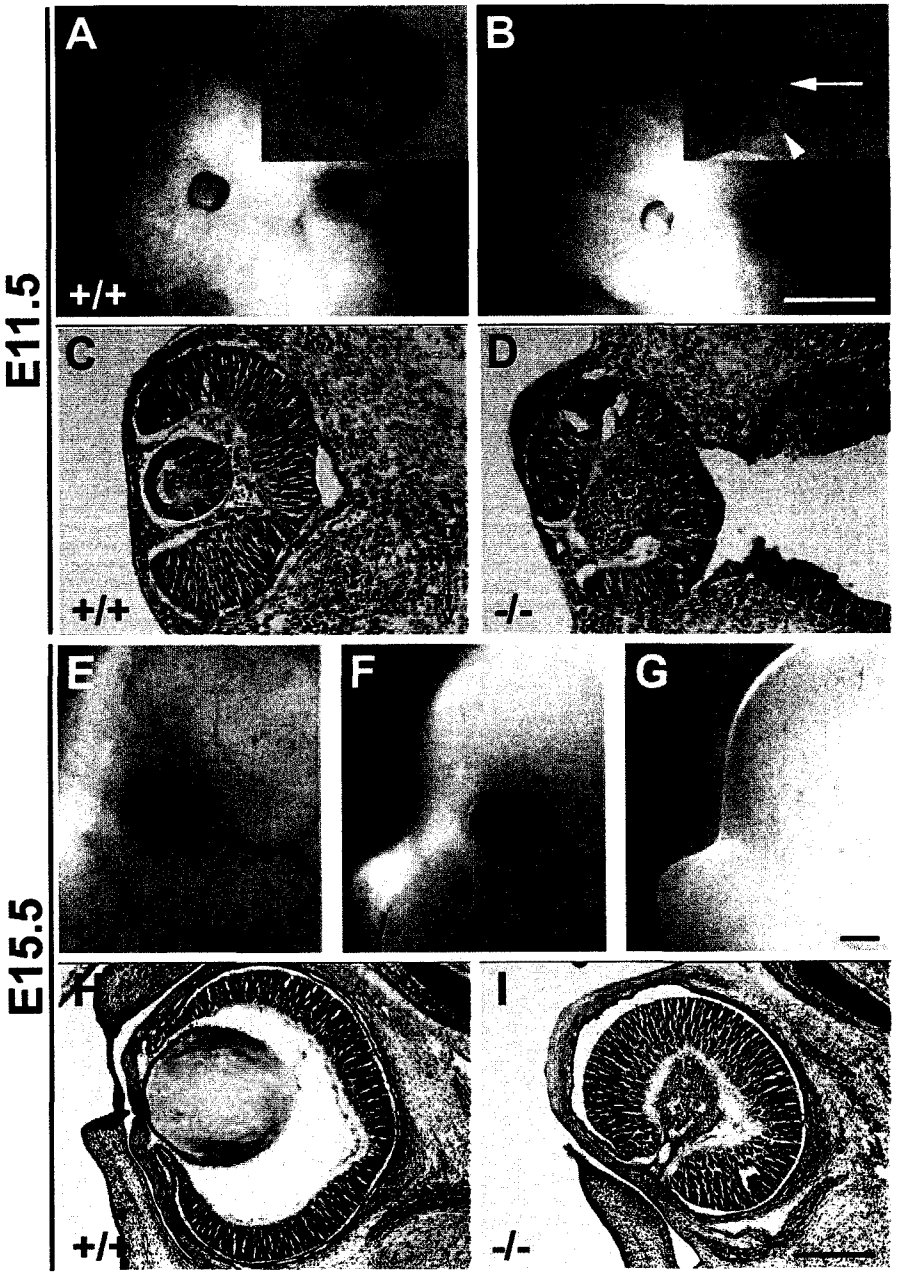
Whereas the roles of E2F1, E2F2, and E2F3 in Rb-mediated control of retinal development have been previously described, the involvement of E2F4 in this process is unknown. Here, we report the novel requirement for E2F4 in early visual system development. The absence of E2F4 causes abnormal eye morphogenesis, which is associated with aberrant expression of region-specific early eye patterning genes. Strikingly, E2F4 deficient mouse embryos exhibit defects in the expression of *Sonic hedgehog (Shh)* in the ventral forebrain. However, later in development E2F4 is dispensable for early retinal differentiation. Overall, these studies suggest a unique role of E2F4 in the control of early eye morphogenesis.

RESULTS AND DISCUSSION

E2F4 deficient mice exhibit eye defects

Since previous studies revealed the important roles of Rb and E2F protein families in eye development (Saavedra et al., 2002; Bremner et al., 2004; Khidr and Chen, 2006), we asked if one of its regulatory targets, E2F4, also played a unique role in this process. To investigate whether E2F4 is required for eye morphogenesis, we examined the phenotype of E2F4 deficient mice. In the absence of E2F4 83.3% (50/60) of the embryos examined between the embryonic day (E) 11.5 and E16.5 displayed gross eye malformation, including microphthalmia, reduced pigmentation in the ventral eye region and ventral displacement of the dorsal limit of the retinal pigmented epithelium (Fig. 3.1B,F). At early (E11.5) and later (E15.5) developmental timepoints we observed coloboma, the failure of the choroid fissure to close (Fig. 3.1F). Analysis of coronal sections revealed aberrant dorsal-ventral development of the optic cup in the E2F4 null mice. Specifically, the ventral optic cup was reduced, while the relative size of the dorsal optic cup was increased (Fig. 3.1D,I). The lens was reduced in size or absent (Fig. 3.1D,I). Moreover, some embryos displayed more severe phenotypes, where eye structures were much reduced or absent altogether (Fig. 3.1G). Thus, the absence of E2F4 leads to aberrant eye morphogenesis, characterized by microphthalmia, coloboma, and reduction in the ventral optic cup territory. These abnormalities are evident from very early stages and persist into the later time points of embryological development. These results suggest that E2F4 may directly or indirectly through regulation of other genes play an important role in the early eye morphogenesis.

Figure 3.1. E2F4 deficient mouse embryos display aberrant eye morphology. (A, B, E-G) Photographs of E11.5 and E15.5 wild type (+/+) and E2F4 deficient (-/-) mouse embryos. Inserts in A and B are magnified images of the eyes in corresponding pictures. Note the reduced pigmentation of the ventral side of the eye (B insert, white arrowhead), whereas the dorsal limit of the retinal pigmented epithelium is shifted to the centre of the eye (B insert, white arrow); coloboma (F, black arrow) and absence of the eye (G) in *E2F4* mutant embryos. Scale bars: 1 mm. (C,D,H,I) 14 μm coronal sections through the eyes of E11.5 and E15.5 wild type (+/+) and E2F4 deficient (-/-) mice stained with cresyl violet. Dorsal (D)-ventral (V) orientation is indicated by the black double-ended arrow (C). NR – neural retina, vOS – ventral optic stalk (D). Scale bar: 250 μm.


E11.5
E15.5
A
B
C
D
E
F
G
H
I
+/+
+/+
-/-
+/+
-/-

Aberrant early eye patterning

Eyes develop from a single eye field located at the rostral end of the neural plate (Chow and Lang, 2001). Following bilateral separation of the of the eye field, the optic vesicles evaginate and extend distally from the midline. After contacting the surface ectoderm, optic vesicles invaginate to form the optic cup. At this stage the proximal (optic stalk) and the distal (optic cup) territories of the developing visual system are delineated by the expression of regional patterning genes, such as the homeobox transcription factors *Pax2* and *Vax1* (proximal) (Torres et al., 1996), and *Pax6* and *Vax2* (distal) (Gehring and Ikeo, 1999; Ohsaki et al., 1999), which specify these structures. To investigate whether E2F4 function is required for the normal regulation of eye patterning, we examined the expression of these genes. At the initial stage of eye morphogenesis *Pax2* expression is normally restricted to the ventral optic vesicle (Nornes et al., 1990). Following invagination of the optic cup *Pax2* is expressed in the developing optic stalk and is required for the closure of the optic fissure, since the loss of *Pax2* results in the development of coloboma (Fig. 3.2A;(Torres et al., 1996)). In the absence of E2F4, *Pax*2 expression was expanded distally into the optic cup (Fig. 3.2B). In wild type embryos Pax6 is uniformly expressed to the optic cup and lens (Fig. 3.2C). In E2F4 deficient embryos *Pax6* expression is reduced in the ventral region of the optic cup (Fig. 3.2D). Consistently, the pattern of *Pax2* and *Pax6* protein expression in the absence of E2F4 recapitulates the results of mRNA analysis, such that Pax2 immunofluorescence appears in the ventral optic cup, whereas Pax6 protein level is reduced in this region (Fig. 3.3A-D). *Vax1* is a ventral anterior homeobox-containing gene (Mui et al., 2005), which

Figure 3.2. Early eye patterning defects in E2F4 deficient embryos at E11.5. Non-radioactive in situ hybridization was performed on 14 μm coronal sections through the eyes of wild-type (WT) and E2F4-/- littermates with antisense riboprobes for *Pax2, Pax6, Vax1*, and *Vax2*. Note the expansion of *Pax2* and *Vax1* into the optic cup (B,F) and simultaneous reduction of *Pax6* expression in the ventral optic cup (C,D, black arrows). Dorsal (D)-ventral (V) and proximal-distal orientation are indicated by the black double-ended arrows (A). L – lens, nr – neural retina of the optic cup, os – optic stalk. Scale bar 250 μm.

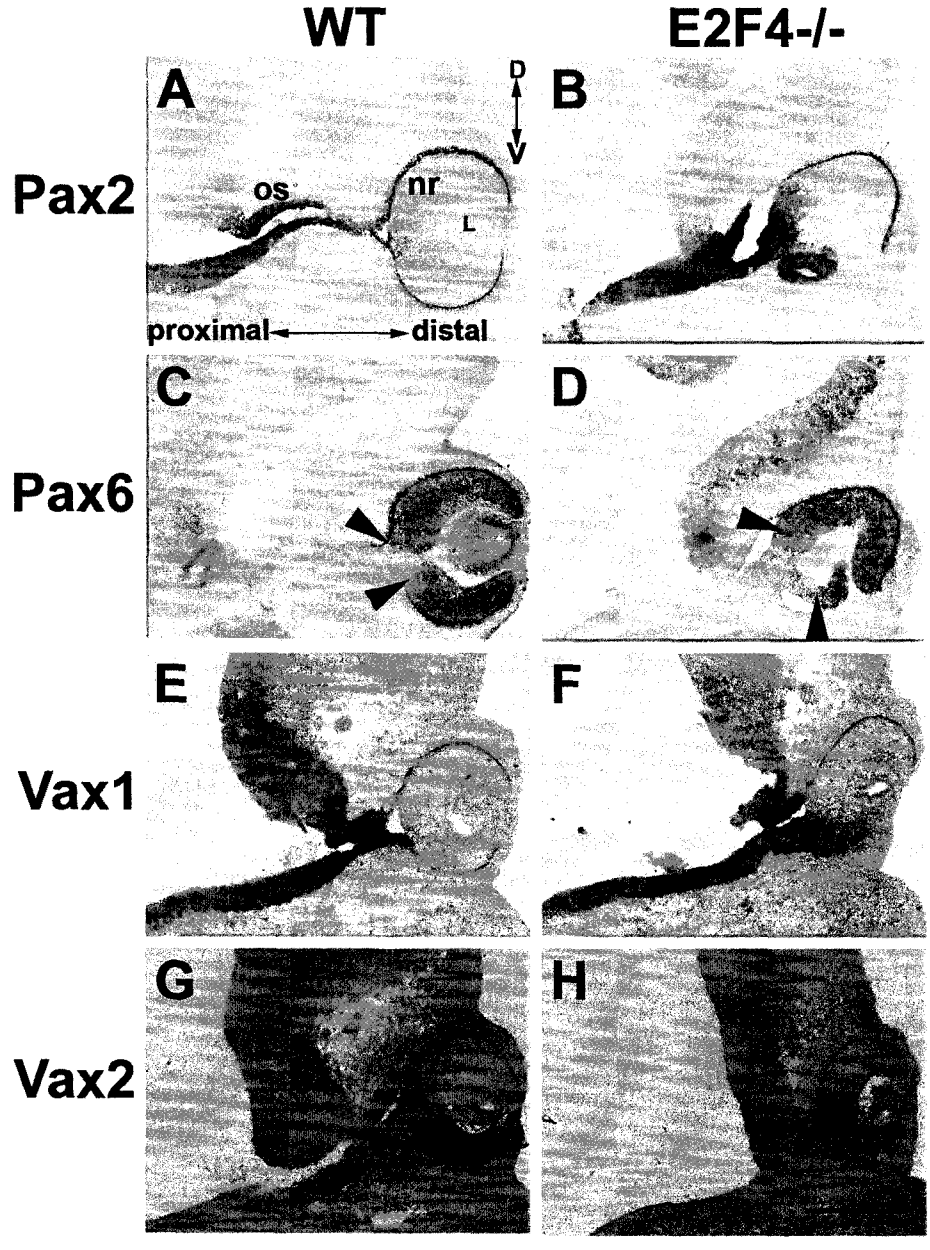
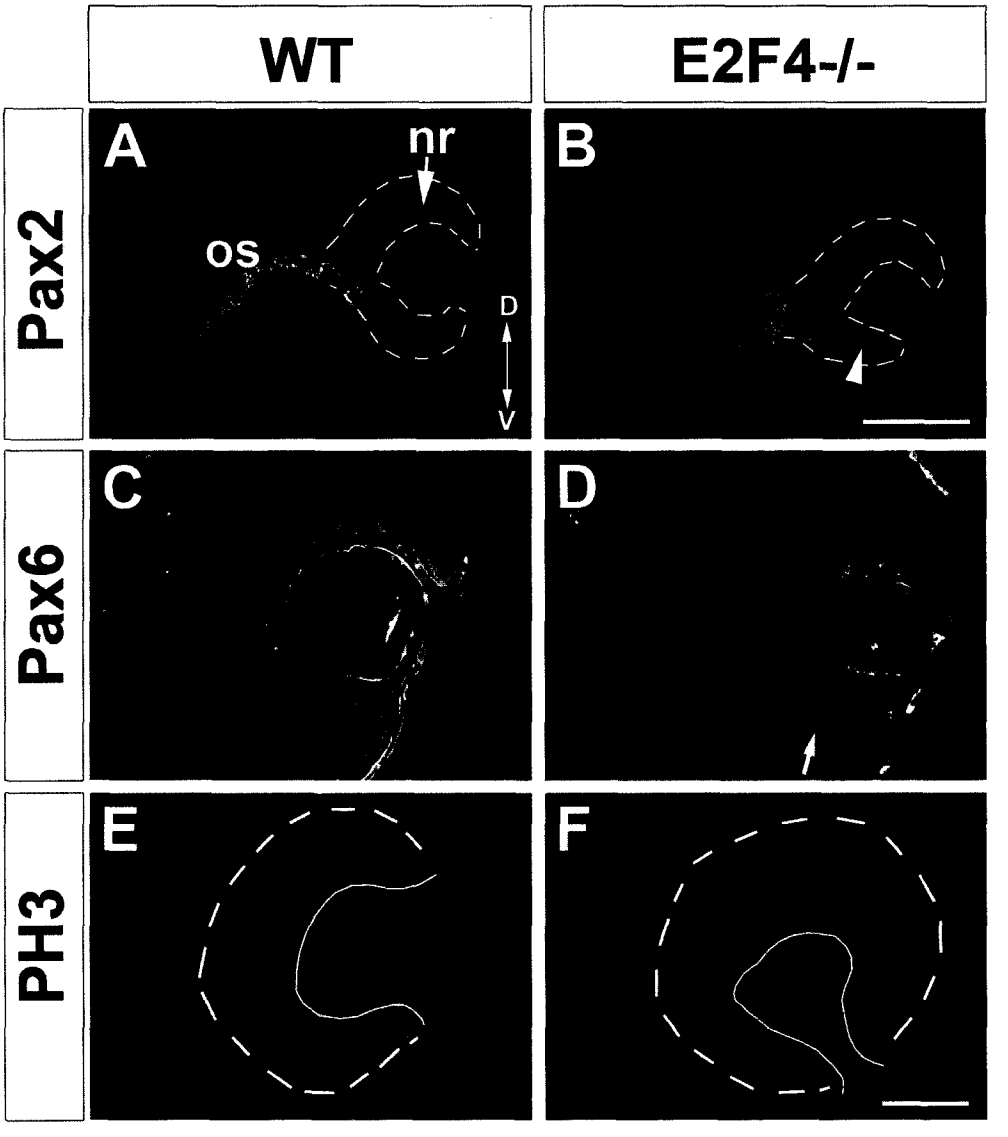

WT
E2F4-/-
Pax2
Pax6
Vax1
Vax2
A
B
C
D
E
F
G
H
D
V
os
nr
L
proximal
distal

Figure 3.3. Patterning and proliferation analysis in E2F4 deficient embryos at E11.5. Immunofluorescent labeling with anti-Pax2 (A, B), anti-Pax6 (C, D), and anti-Phospho-Histone H3 antibodies of the WT and E2F4-/- eyes coronal sections. Note increased Pax2 (B, white arrowhead) and reduced Pax6 (D, white arrow) immunoreactivity in the ventral optic cup. (E, F) No difference in the number of dividing cells within the neural retina is detected. Neural retinal borders are indicated by white dashed line. Dorsal (D)-ventral (V) orientation indicated by white double-ended arrow (A). Abbreviations: os – optic stalk, nr – neural retina. Scale bar: A,B 250 μm; C-F 100 μm.


WT
E2F4-/-
Pax2
Pax6
PH3
A
B
C
D
E
F
nr
os
D
V

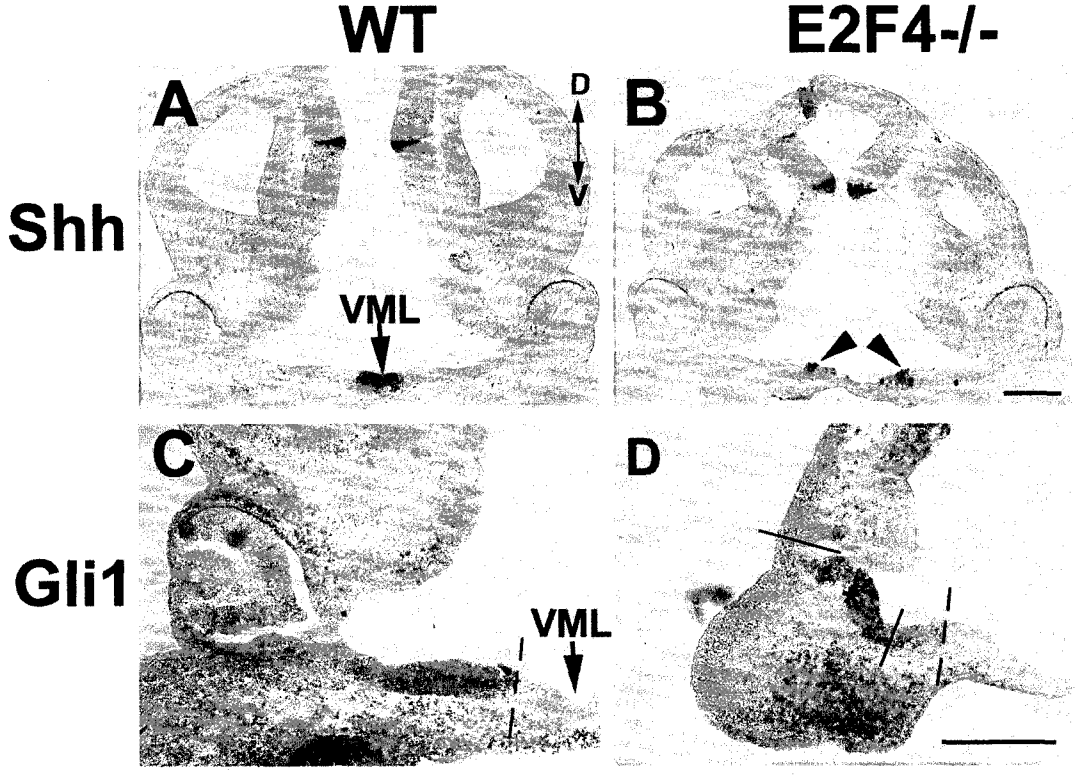
is expressed in the developing optic nerve in wild type embryos (Fig. 3.2E). In *E2F4* mutant embryos, *Vax1* expression is extended distally into the ventral optic cup and is reduced in the diencephalon adjacent to the dorsal part of the optic stalk (Fig. 3.2F). *Vax2*, a homeobox transcription factor closely related to *Vax1*, is normally expressed in the ventral optic cup (Fig. 3.2G; (Schulte et al., 1999)). In the absence of E2F4, however, *Vax2* expression expands into the dorsal region and is decreased in the ventral optic cup (Fig. 3.2H). In summary, these findings indicate that E2F4 deficiency causes early eye patterning defects, characterized by the expansion of proximal region specific genes *Pax2* and *Vax1* into the more distal-ventral area of the optic cup with concomitant reduction of *Pax6* and aberrant expression of *Vax2*. Proliferation analysis with Phospho-histone H3 (mitotic cell marker) reveals that loss of E2F4 does not affect proliferation in the developing retina (20.67±2.89 PH3-positive cells/retina in WT vs 18.33±2.03 cells/retina in E2F4-/-; n=3 embryos, data presented as mean±s.e.m.) (Fig. 3.3 E,F). We also did not detect any change in cell survival in E2F4 deficient neural retinas (data not shown). Therefore, E2F4 plays an important role in the temporal and region specific regulation of early eye patterning.

E2F4 deficiency leads to alteration in Shh signaling

One of the signals that controls early eye morphogenesis is the secreted morphogen Sonic hedgehog (Shh) (Jean et al., 1998; Zhang and Yang, 2001a). Initially, Shh from the prechordal plate and rostrally moving neural midline cells (Wilson and Houart, 2004) is required for the bilateral separation of the cerebral hemispheres and the eye field. Complete elimination of Shh signaling before this stage in mice or even partial

reduction in humans results in holoprosencephaly, which in the most extreme instances is characterized by cyclopia (Chiang et al., 1996). Later, rostral expansion of *Shh* expression in the ventral telencephalon is required for the regulation of the proximal-distal patterning of the developing eye (Jean et al., 1998). Misexpression or inhibition of Shh signaling at this stage causes abnormal eye development (Zhang and Yang, 2001a). Shh from the ventral midline positively regulates *Pax2*, *Vax1*, Vax2, which, in turn, repress *Pax6* expression (Hallonet et al., 1999; Take-uchi et al., 2003; Mui et al., 2005). Considering that E2F4 deficiency causes aberrant expression of proximal-distal markers, we questioned whether Shh signaling was perturbed in the mutant embryos. In wild type embryos *Shh* expression is limited to a single domain in the region of the ventral midline (Fig. 3.4A). In the absence of E2F4, we observed aberrant *Shh* mRNA expression in this region. Compared with the wildtype littermate controls, *Shh* expression was detected in two paramedian strips, which were displaced distally from the midline (Fig. 3.4B). Next, we asked whether the activity of the Shh was affected in E2F4 deficient embryos. To address this question we analyzed the expression of Gli1, which is considered to be a reliable readout of Shh activity (Lee et al., 1997). In wild type embryos *Gli1* mRNA expression is highest in the proximal ventral optic stalk, complementing *Shh* expression, but is practically undetected in the dorsal neural retina (Fig. 3.4C). In the absence of E2F4, *Gli1* expression is present in the neural retina, which is especially evident in severely affected embryos (Fig. 3.4D). Thus, the absence of E2F4 leads to the mispatterning of *Shh* expression in the floor of the optic recess and causes the distal expansion of the Shh pathway activity into the neural retina. These results suggest that

Figure 3.4. Aberrant *Shh* expression and deregulation of Shh activity in the ventral forebrain and neural retina of E2F4 deficient embryos. In situ hybridization analysis of E11.5 wild type (A, C) and E2F4-/- (B, D) embryos for *Shh* and *Gli1* mRNA expression. Wild type embryos have a single *Shh* expressing region in the ventral midline (A: VML, black arrow), whereas E2F4-/- mice have two paramedian areas of *Shh* expression in the ventral forebrain (B, black arrowheads). *Gli1* mRNA is expressed in the neural retina of the E2F4 deficient embryos (D, black asterisk). Black lines limit the neural retina (D). Black dashed line indicates the complementary *Shh* and *Gli1* expression (C,D). Dorsal (D)-ventral (V) orientation indicated by the black double-ended arrow (A). Scale bar 250 μm.


WT
E2F4-/-
A
B
C
D
Shh
Gli1
D
V
VML
VML

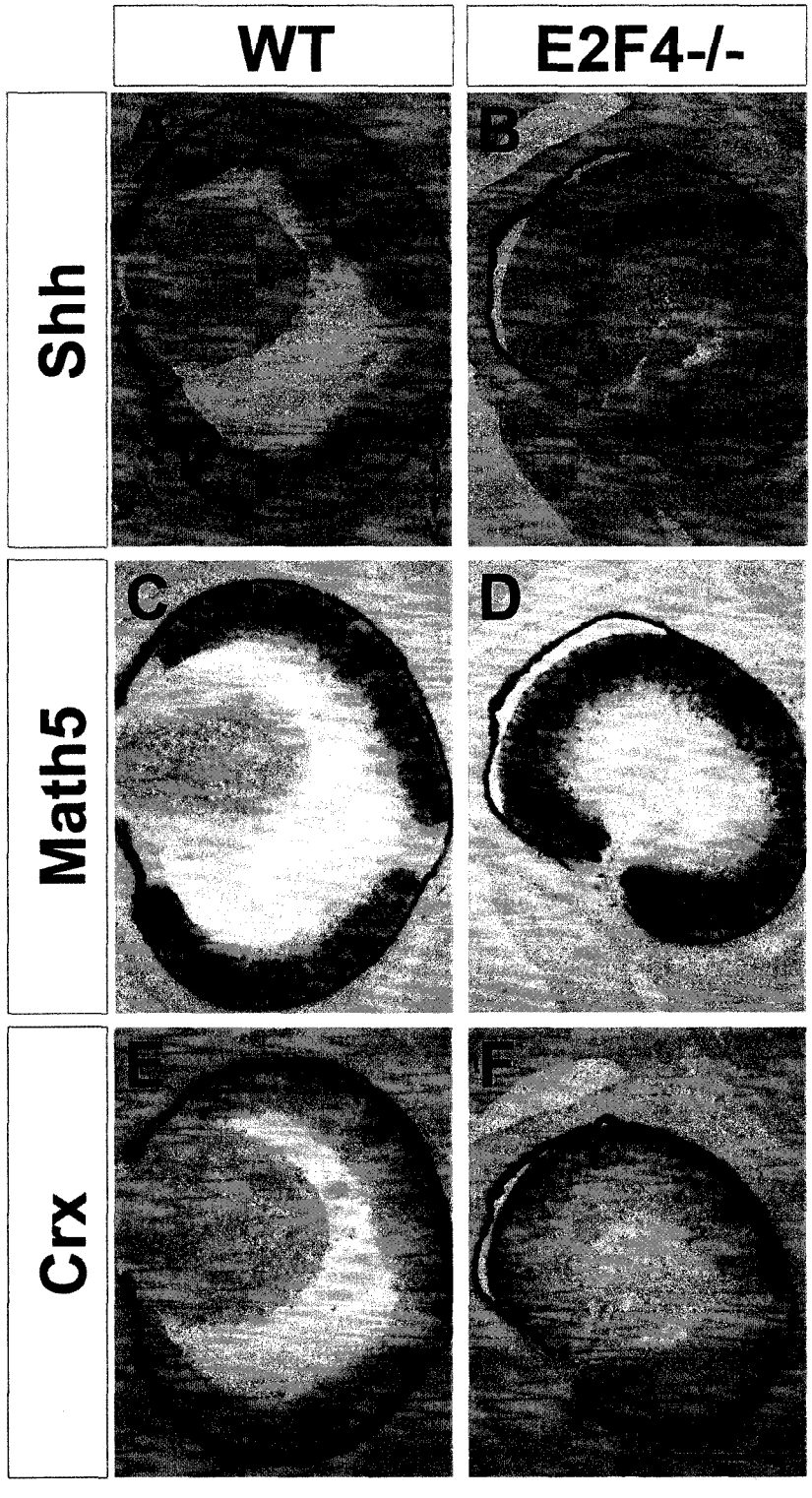
E2F4 is required for the region specific expression of *Shh* during the crucial period of early eye patterning.

Early retinal differentiation appears unaffected by the loss of E2F4

In addition to regulating ventral neural tube development (Shimamura et al., 1995) and early eye morphogenesis, Shh controls proliferation and diversification of progenitor cells in the neural retina at later stages of embryogenesis (Jean et al., 1998; Wang et al., 2005). In the mouse retina the expression of *Shh* begins at E12 in differentiating retinal ganglion cells in the central retina and extends to the periphery, following the central to peripheral wave of RGC differentiation (Wang et al., 2005). Since E2F4 deletion results in aberrant Shh signaling during the early stages of eye development, we questioned whether *Shh* expression in the neural retina was affected by the absence of E2F4 at later stages. We did not detect any difference in the expression of *Shh* mRNA in the neural retina of E2F4 deficient embryos compared to the controls at E15.5 (Fig. 3.5A,B), indicating that E2F4 is not required for regulation of Shh during retinogenesis.

To determine whether E2F4 is required for the specification of retinal neurons, we examined the expression of early neurogenic and differentiation markers. The proneural gene *Math5*, which is transiently upregulated in early differentiating retinal ganglion cells (Brown et al., 1998; Wang et al., 2001), is expressed in a similar pattern in the neuroblastic layer in wild type and E2F4 mutant retinas (Fig. 3.5C,D). The *Crx* homeodomain transcription factor is specifically expressed in postmitotic committed photoreceptor cells in the outer neuroblastic layer of the retina (Fig. 3.5C)

Figure 3.5. *Shh* expression and specification of retinal neurons are unaffected by E2F4 deletion. mRNA in situ hybridization analysis was performed on the coronal eye sections from E15.5 mouse embryos. Wild type (A) and E2F4-/- (B) mouse embryos have comparable levels of *Shh* mRNA (A,B). Early retinal ganglion cell marker *Math5* (C,D) and committed photoreceptor marker *Crx* (E,F) are similarly expressed wild type and E2F4 deficient neural retinas. Dorsal (D)-ventral (V) orientation indicated by the black double-ended arrow (A). Scale bar 250 μm.


WT
E2F4-/-
Shh
Math5
Crx
A
B
C
D
E
F

(Furukawa et al., 1997). We did not detect any difference in *Crx* expression between E2F4 deficient and control embryos (Fig. 3.5E,F).

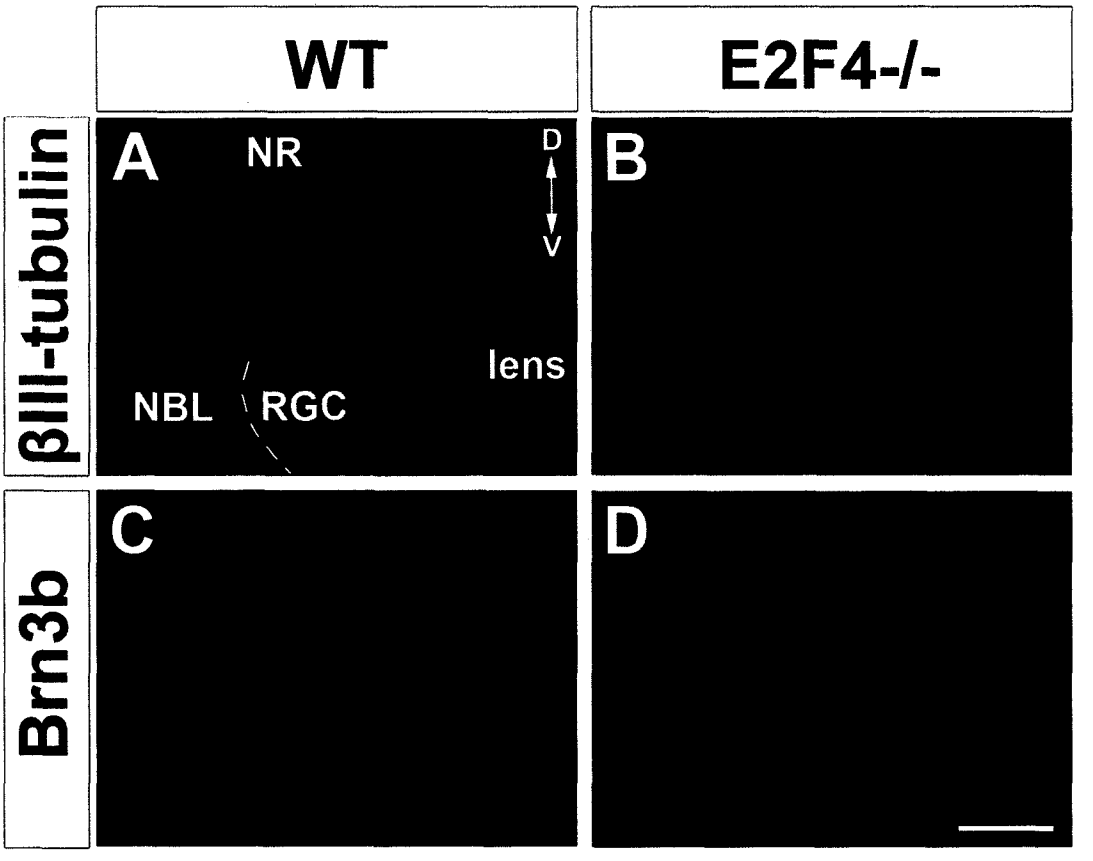
We also asked whether E2F4 deficiency is required for early differentiation in the neural retina. To answer this question we performed immunohistochemical analysis of βIII-tubulin and Brn3b proteins. βIII-tubulin protein, which labels early committed neurons after they exit the cell cycle (Menezes and Luskin, 1994), is equally high in the ganglion cell layer of wild type and E2F4 deficient mouse embryos at E15.5 (Fig. 3.6A,B). The POU domain transcription factor, Brn3b, labels the earliest postmitotic retinal ganglion cell precursors (Xiang, 1998). Brn3b immunolabeled cells are present within differentiating E2F4 deficient retinas similar to wild type controls (Fig. 3.6C,D).

Next, we questioned whether E2F4 was required for regulating proliferation and cell survival in the developing retinas. To address this question we performed analysis of BrdU incorporation (S phase marker of the cell cycle) in the neural retinas of wild type and E2F4 deficient embryos at E15.5. Loss of E2F4 does not significantly change the proportion of BrdU-positive cell in dissociated neural retinas relatively to the wild type controls (25.49±1.85 % of BrdU-positive cells/retina in WT vs 26.44±1.57 % of BrdU-positive cells/retina in E2F4-/-; n=4, data presented as mean±s.e.m.). E2F4 deficiency does not alter cell survival in the differentiating neural retina (data not shown).

Overall, these results demonstrate that E2F4 is not required for regulation of Shh expression and early photoreceptor and ganglion cell specification and differentiation in the retina, but instead E2F4 is required for early eye patterning.

In addition to already reported defects in hematopoiesis, gut and facial development, (Humbert et al., 2000b; Rempel et al., 2000; Kinross et al., 2006), we show

Figure 3.6. Differentiation in the neural retinas of wild type and E2F4 deficient embryos. 14 μm coronal sections through the eye of E15.5 mouse embryos were processed for βIII-tubulin and Brn3b immunohistochemistry. Immunofluorescent analysis reveals similar labeling of (A,B) and Brn3b (C,D) positive cell populations in differentiating inner layer of retinal ganglion cells (RGC) and outer neuroblastic layer (NBL; white dashed line indicates the border between RGC and NBL layers). Dorsal (D)-ventral (V) orientation indicated by the black double-ended arrow (A). NR – neural retina. Scale bar 100 μm.


WT
E2F4-/-
βIII-tubulin
Brn3b
A
NR
D
V
lens
NBL
RGC
B
C
D

here for the first time that E2F4 deficiency is also associated with proximal-distal eye patterning defects. The absence of E2F4 leads to mispatterning of early eye specification genes, so that genes from the proximal structures, such as *Pax2* and *Vax1*, expand into the more distal domains (optic cup), which was paralleled by reduced expression of optic cup specific genes *Pax6* and *Vax2*. These defects were associated with temporal and site specific alterations in Shh expression in the developing ventral forebrain and expansion of Shh signaling activity into the neural retina. Shh is known regulator of eye patterning, proliferation and differentiation (reviewed in (Wallace, 2007)). A similar eye patterning phenotype is described in Gli3 deficient mouse embryos (Hui and Joyner, 1993; Furimsky and Wallace, 2006). Gli3 is a zinc-finger transcription factor, which functions primarily as a repressor and antagonizes the Shh signaling activaty during nervous system, eye, and limb development (reviewed in (Ingham and McMahon, 2001)). In the absence of Gli3 proximal (optic stalk) markers expand distally into the ventral optic, and the expression of neural retina specific markers is reduced in the ventral territory (Furimsky and Wallace, 2006). Therefore, the defects of the early eye morphogenesis and patterning in E2F4 deficient mice are consistent with aberrant Shh signaling in the ventral forebrain. Moreover, these results further expand the essential requirement for E2F4 in the patterning of the ventral forebrain structures. We have recently shown, that the absence of E2F4 leads to abnormal development of the ventral telencephalon in mice, which also correlates with disrupted Shh signaling (Ruzhynsky et al., 2007). Taken together, our studies reveal a unique requirement for cell cycle transcription factor E2F4 in the early eye patterning.

EXPERIMENTAL PROCEDURES

E2F4 mice

Germline E2F4 deficient mice were generated and generously provided by Dr. Jacqueline Lees (Massachusetts Institute of Technology) (Humbert et al., 2000b). Mice were maintained on a C57Bl/6 background (Charles River Laboratories International, Inc., Wilmington, MA, USA). Animals were genotyped according to previously published protocols (Humbert et al., 2000b). Embryonic age was estimated from the time of vaginal plug detection, which was counted as embryonic day 0.5 (E0.5). To analyze retinal progenitor proliferation at E15.5, pregnant mice received intraperitonial injection of 50 mg/kg^{-1} BrdU (Sigma; cat.#B5002) in 0.9% NaCl 1 hour prior to sacrifice. Mice were killed with an injection of 65 mg/ml sodium pentobarbital solution (Somnotol) at indicated time points. All experiments were approved by the University of Ottawa's Animal Care ethics committee adhering to the Guidelines of the Canadian Council on Animal Care.

Phenotypic analysis, immunohistochemistry, and in situ hybridization

E11.5 embryo were dissected and fixed in 4% PFA overnight in phosphate buffer, pH 7.4, cryoproteced overnight in 30% sucrose/1XDulbecco's Phosphate Buffered Saline (DPBS) (Gibco) solution, embedded in 1:1 mix of 30% sucrose in 1XDPBS/OCT (TissueTek 4583, Sakura Finetek, USA), and frozen on liquid nitrogen. E15.5 embryos were processed as previously described (Ferguson 2005). 14 μm coronal sections were cut on a cryostat and mounted on Superfrost slides (Fisher Scientific, Cat. #12-550-15). Cresyl violet staining was performed as per standard protocols. Immunohistochemistry

was performed using anti-ßIII-tubulin (mouse monoclonal hybridoma supernatant, 1:50 (Caccamo et al., 1989)), anti-phosphohistone H3 (1:1000, Upstate; cat.#06570), anti-BrdU (1:50; BD Biosciences; cat.#347580), anti-Pax2 (1:500; Covance; cat.#PRB-276P), anti-Pax6, and anti-Brn3b (Santa Cruz Biotechnology; cat.#sc-31988) primary antibodies. Non-radioactive in situ hybridization (ISH) was performed according to the previously published protocol (Bruhn and Cepko, 1996; Wallace and Raff, 1999). Antisense riboprobes for *Shh* (Echelard et al., 1993), *Gli1* (Hui and Joyner, 1993), *Pax2* (Dressler et al., 1990), *Pax6, Vax1* (Hallonet et al., 1999), *Vax2* (Schulte et al., 1999), *Math5* (Brown et al., 1998), and *Crx* (Furukawa et al., 1997) were prepared from linearized plasmids using DIG RNA Labeling Mix, 10Xconc. (Roche Diagnostics GmbH, Cat.# 1 277 073). Embryos were analyzed using a Zeiss Axioskop 2 (Oberkochen, Germany) upright microscope and images were captured using Sony Power HAD 3CCD color video camera or QiCam digital video camera (QImaging Corporation, Burnaby, BC, Canada) and Northern Eclipse 6.0 software (Empix Imaging Inc., Mississauga, ON, Canada).

Proliferation analysis

Proliferation analysis at E11.5 was performed on eye coronal sections by counting the total number of PH3-positive (mitotic marker) along the total ventricular (outer) length of the neural retina. Three embryos per geneotype were analyzed. The number of proliferating cells at E15.5 was determined in dissociated retinas according to the protocol described in (Jensen and Wallace, 1997). BrdU-positive cells were counted in 2-3 random fields of each dissociated retina (both eyes for each embryo) and expressed as a percentage of the total number of cells estimated by Hoechst labeling (1:250; Sigma).

Four embryos per geneotype were analyzed. Statistical analysis was performed by Student's t-test. Data are expressed as mean±standard error mean (s.e.m.).

ACKNOWLEDGMENTS

We are grateful to Dr. A. P. McMahon, C. L. Cepko, P. Gruss, N. Pringle and T. Glaser for cDNA probes. I would like to thank Dr. Y-P Wang, postdoctoral fellow in Dr.Valerie Wallace laboratory, for teaching guidance with retinal dissections. V. Ruzhynsky is a recipient of OGS and SCN studentships, M. Furimsky is a recipient of a CIHR/CNIB A.E. Baker Foundation Postdoctoral Fellowship.

Chapter 4. General Discussion

Alterations in forebrain development are the underlying causes of numerous neurological and psychiatric disorders. Recent work from our laboratory points to important spatial and temporal roles of the cell cycle regulatory Rb/E2F pathway in diverse neurodevelopmental processes, such as cortical lamination, neural precursor self-renewal, differentiation, and neuronal migration (Ferguson et al., 2002; Vanderluit et al., 2004; Ferguson et al., 2005; McClellan et al., 2007; Vanderluit et al., 2007). This thesis presents new unexpected roles for E2F4 in early nervous system development.

4.1. Summary of the findings

In Chapter 2, I established a requirement for E2F4 in the development of the ventral telencephalon. The earliest developmental time point of the previous survival analysis of these embryos was E13.5, though no detailed morphological description of telencephalic or eye development was provided (Humbert et al., 2000b). I looked at the development of the telencephalon at E11.5, the time when initial regional patterning is established and neurogenesis is just initiated (Rubenstein et al., 1998). *E2F4* expression was reported to be the highest in the brain at this time point (Kusek et al., 2001). E2F4 deficient embryos were smaller than their wild type littermates, and the developing telencephalic vesicles were also relatively smaller to the overall size of the anterior neural tube. Close examination of telencephalic coronal sections revealed the absence or severe reduction of the subpallial structures, namely MGE and LGE. Correspondingly, expression of the ventral patterning genes was also decreased, especially the MGE marker *Nkx2.1*. Analysis of telencephalic structures at mid-neurogenesis (E15.5) showed that E2F4 deficient embryos had subtle defects, characterized by underdeveloped striatal

neuroepithelium and aberrant expression of the interneuronal marker, *Lhx6*, in the ventral regions and developing cortex. I did not detect any defects in relative proliferation or cell survival in vivo at either developmental time point. Loss of *E2F4*, however, caused a decrease in the number of neural stem cells and diminished their self-renewal capacity. Since a primary patterning defect was observed in the ventral telencephalon, I analyzed the expression and activity of Shh, the known regulator of ventral patterning (Lupo et al., 2006). In the absence of *E2F4*, *Shh* expression was lost in the ventral telencephalic midline and the activity of the Shh pathway was diminished. Interbreeding E2F4 deficient and *Shh* or $Gli3^{xt/xt}$ mutant mice revealed the presence of a genetic interaction between E2F4 and the Shh pathway. Moreover, loss of E2F4 resulted in impaired activity of *Shh* forebrain specific enhancers. Finally, activating Shh signaling with a Hh agonist restored neural stem cell self-renewal and *Nkx2.1* expressing cells in neural tube explants.

In Chapter 3, I determined the requirement for E2F4 in early eye development and retinogenesis. Loss of *E2F4* resulted in a high incidence of severe morphological eye defects, such as microphthalmia, coloboma, and abnormal development of the optic cup. Absence of E2F4 led to the expansion of the proximal (optic nerve) specific early eye patterning genes into the optic cup. I did not detect alterations in cell proliferation or cell survival in E2F4 deficient retinas at E11.5. Similar to the defect in the ventral telencephalon, however, Shh signaling was also perturbed in the rostral hypothalamus, which is the regulatory signaling centre for early eye development. Specifically, the solitary domain of *Shh* expression was split and shifted bilaterally from the midline. This correlated with increased *Gli1* expression in the developing ventral optic cup. Finally, E2F4 deficiency did not affect the initial differentiation and proliferation at E15.5.

The findings of this thesis represent a significant step forward in our understanding of the functions of E2F transcription factors, and E2F4 specifically, in early telencephalic and eye development. It has been shown previously that some E2F transcription factors are required in the developing nervous and visual systems during late embryogenesis and postnatally (Lindeman et al., 1998; Saavedra et al., 2002; McClellan et al., 2007). This work presents the first in vivo evidence for a crucial role for E2F4 during the initial stages of nervous system development.

4.2. E2F4 as an integral part of the morphogenetic network

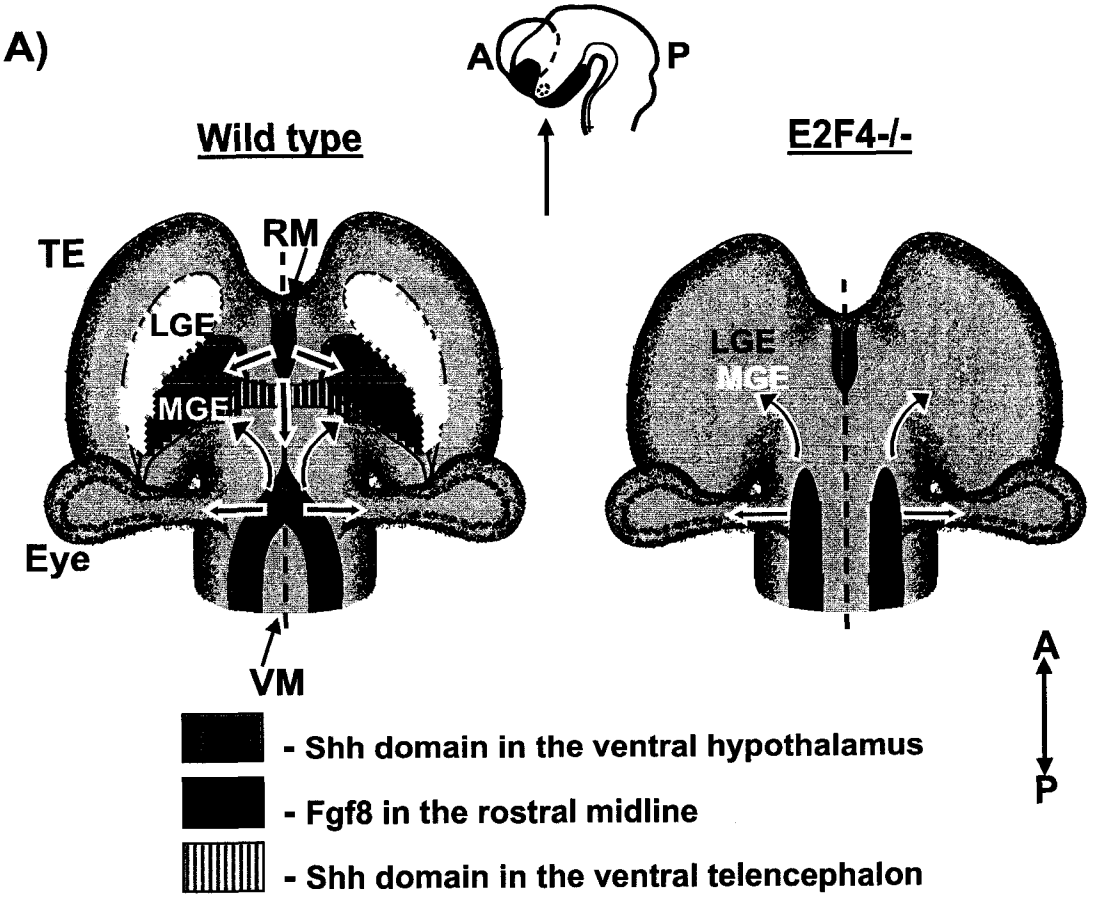
Importantly, the absence of E2F4 disrupts the morphology and activity of Shh signaling centres in the rostral ventral hypothalamus and ventral telencephalon. I also show a novel genetic interaction between E2F4 and Shh signaling pathway. This is a surprising new twist in the known relationship between morphogens and cell cycle regulatory proteins. The Shh pathway is viewed as one of the common denominators of the morphogenetic and proliferative functions (Ruiz i Altaba et al., 2003; Cayuso et al., 2006). Besides regulating specification and patterning, Shh pathway acts as a mitogen and controls the CKI-Cyclin/cdk-Rb-E2F axis in the nervous system, including the stem cell compartments, as well as in the developing retina and other organs (Kenney et al., 2003; Oliver et al., 2003; Mill et al., 2005; Wang et al., 2005). While the results presented here do not rule out the existence of this mechanism during early patterning, they indicate that E2F4 is an integral player of this multilevel morphogenetic network.

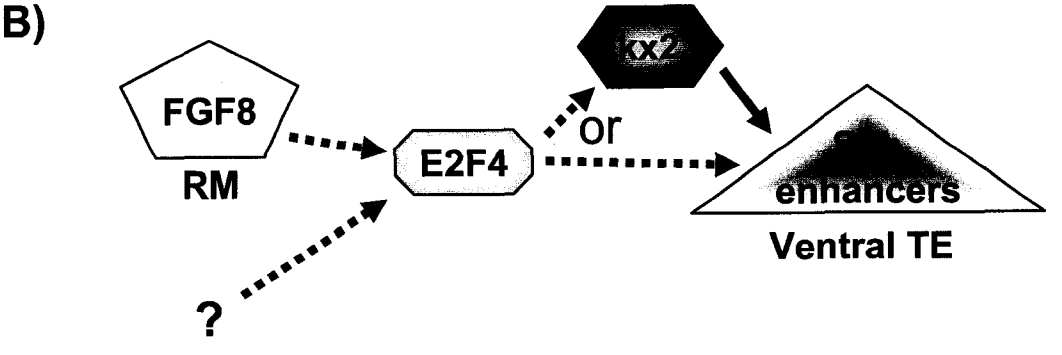
Simultaneous patterning defects in the telencephalon and eye are not unusual, since both of these structures are derivatives of the secondary prosencephalon (Puelles,

2001). Accordingly, early patterning of these structures is regulated by common signaling mechanisms, which emanate from the anterior neural ridge, ventral midline region of the hypothalamus and later from the telencephalic midline (Wilson and Rubenstein, 2000; Ohkubo et al., 2002; Rallu et al., 2002a; Puelles and Rubenstein, 2003; Wilson and Houart, 2004). Cross-regulation between morphogenetic signaling from these centres guides the regional specification of forebrain structures along the anterior-posterior and dorso-ventral axes by controlling the expression of downstream homeobox genes (Ohkubo et al., 2002). These transcription factors establish a complex grid of positional information (transcription code) for future developing structures, and also provide feed-back to the patterning centres to regulate their morphology and activity (Briscoe et al., 2000; Ohkubo et al., 2002; Schuurmans and Guillemot, 2002). While it is known that early telencephalic development is controlled by signaling from the anterior neural ridge (Shimamura and Rubenstein, 1997; Crossley et al., 2001; Storm et al., 2006), the exact mechanism of regulation remains unclear. Therefore, the presented results suggest that E2F4 may represent an essential link between morphogenetic signaling pathways and region-specific transcription factors. Specifically, I propose several possibilities to explain how E2F4 mediates patterning of the ventral forebrain.

One scenario is that E2F4 may mediate Fgf8 or other signaling from the rostral midline to induce the expression of *Nkx2.1* and *Shh* in the ventral telencephalon at E8.5 (Fig. 4.1B). Accordingly, this pathway is disrupted in the absence of E2F4 (Storm et al., 2006). Moreover, aberrant signaling from the rostral midline may also disrupt the convergence of the longitudinal hypothalamic *Shh* expressing domains at the level of

Figure 4.1. Role of E2F4 in early forebrain development. A) Diagram of the ventral view of the developing telencephalon (insert at the top middle with arrow pointing the direction of view). Major signaling centres in the rostral and ventral midline are indicated by colors. MGE and LGE development is severely affected by the absence of E2F4. Single hypothalamic *Shh* domain is split at the level of optic recess in E2F4-/- embryos. B) E2F4 might be required for the inductive signaling from the rostral midline (RM), such as Fgf8, or from other regional patterning centre. E2F4 may directly or indirectly regulate the activity of the Shh brain enhancers in the ventral telencephalon. A – anterior, P – posterior, VM – ventral midline.


A)
A
P
Wild type
E2F4-/-
TE
RM
LGE
MGE
Eye
VM
LGE
MGE
A
P
- Shh domain in the ventral hypothalamus
- Fgf8 in the rostral midline
- Shh domain in the ventral telencephalon


B)
FGF8
RM
E2F4
?
X2
or
enhancers
Ventral TE

optic recess (Shimamura et al., 1995). This leads to the split of the normally single centre (convergence point) of Shh signaling, thus affecting the patterning of the developing eye (Fig. 4.1A). One explanation to resolve the question as to why some E2F4 deficient embryos survive despite this defect and exhibit partially restored telencephalic morphology, is that Shh signaling from the hypothalamic centre may compensate and initiate delayed ventral telencephalic patterning.

Another possibility is that E2F4 may be essential for the regulation of *Shh* enhancers in the developing forebrain and ultimately the expression of *Shh* (Fig. 4.1B). In this case, the ventral telencephalic defect in the absence of E2F4 may be a result of aberrant activation of the *Shh* brain specific enhancers. Future studies are required to distinguish between these possibilities and identify the exact molecular nature of E2F4 interactions with regional patterning signaling in the developing ventral forebrain.

4.3. Ventral telencephalic development and neuropathology

The requirement for E2F4 in ventral forebrain development calls for analysis of other related developmental processes, such as axonal guidance, hypothalamic and pituitary development, and differentiation of the ventrally derived neurons. The disruption of these events could lead to a number of neurodevelopmental disorders (Lewis and Levitt, 2002; Powell et al., 2003; Levitt et al., 2004; Moldin et al., 2006; Di Cristo, 2007).

We show that development Lhx6-positive interneurons is is affected by E2F4 deletion. The majority of the cortical inhibitory interneurons originates from the telencephalic subpallium, which is severely affected by the loss of *E2F4* (Anderson et al.,

1997; Lavdas et al., 1999; Sussel et al., 1999). Reduction of the striatal neuroepithelium and the specific decrease of the Lhx6-positive subpopulation of the ventrally-derived interneurons in E15.5 mouse embryos are consistent with earlier patterning defects caused by E2F4 deficiency. These results also suggest the requirement for E2F4 in interneuron migration or differentiation and would represent a valuable extension of these studies.

Impaired specification and migration of inhibitory interneurons to the cerebral cortex lead to the disruption of the balance between excitation and inhibition in the brain. Such defects may represent an underlying mechanism of numerous psychiatric and seizure disorders, such as epilepsy, schizophrenia and autism (Powell et al., 2003; Hamilton et al., 2005). The MGE also gives rise to the projection neurons of the globus pallidus, which is intimately involved in movement control as part of the basal ganglia (Gerfen, 1992; Kimura et al., 1996; Sussel et al., 1999; Herrero et al., 2002). Abnormal function of the basal ganglia leads to the development of extrapyramidal movement disorders, including chorea, Parkinson's disease, and tremors (Mink, 2003). For example, mutations of the homeobox gene *Nkx2.1*, the expression of which is severely affected by the loss of *E2F4*, leads to the development of an autosomal dominant movement disorder - benign hereditary chorea in humans (Breedveld et al., 2002; Pohlenz et al., 2002; do Carmo Costa et al., 2005; Moya et al., 2006). Therefore, future studies would establish whether E2F4 is required for initial interneuronal differentiation or also during migration and maintenance. Conditional E2F4 inactivation in the developing telencephalon would bypass the high perinatal lethality and help to elucidate specific requirements for E2F4 in interneuronal differentiation, migration or survival. These results would give us new

insights into understanding the neurodevelopmental mechanisms, knowledge which is the crucial for the design of new therapeutic modalities for treatment of neuro- and psychopathological disorders.

4.4. E2F4 and neural stem cells

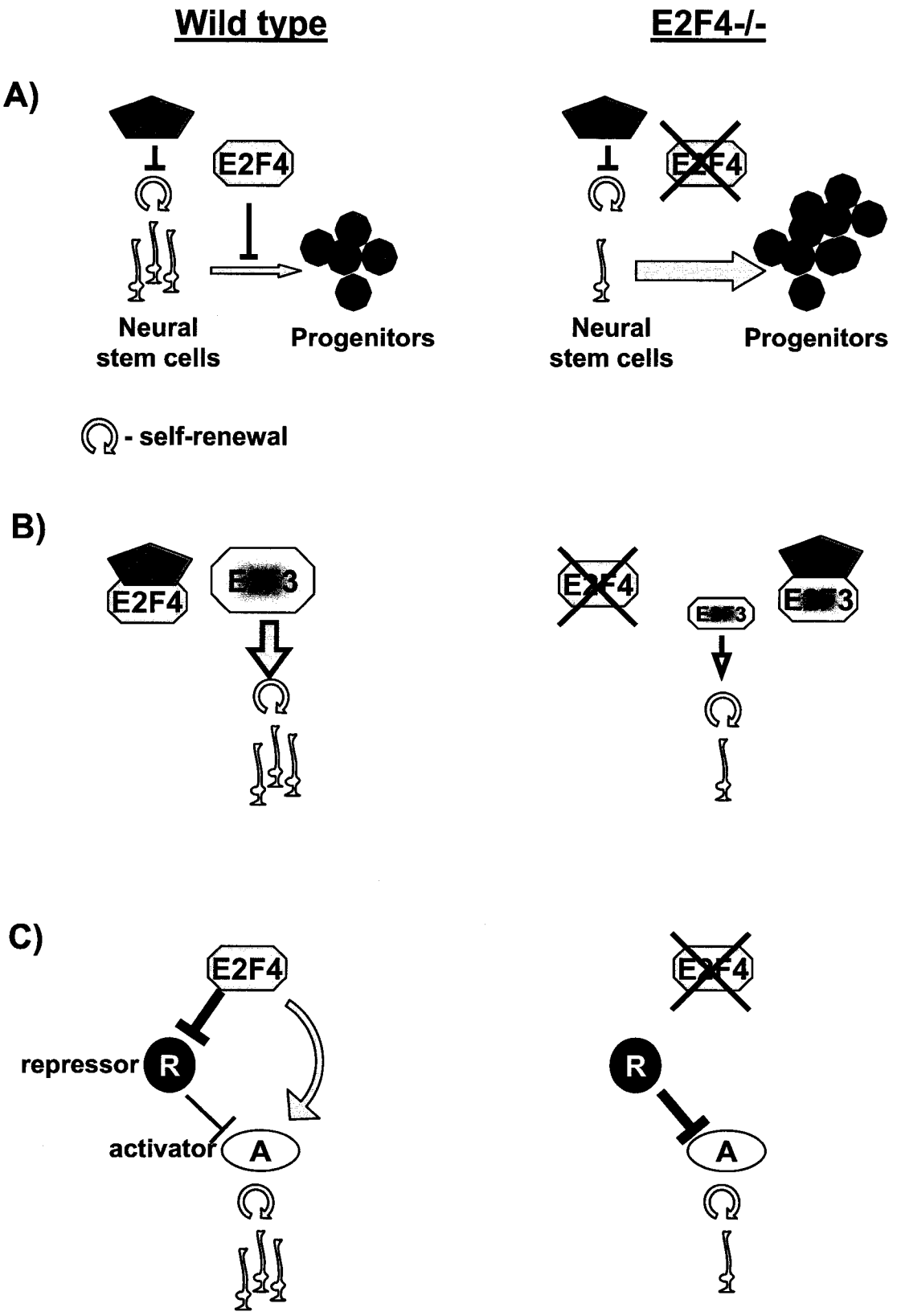
The discovery of stem cells in the adult central nervous system (CNS) provides an opportunity for the development of novel therapeutic approaches for the treatment of numerous acute and chronic degenerative CNS disorders. Activation of endogenous neural stem cells is an attractive strategy, since it exploits the physiological mechanisms of neural precursor regulation. Moreover, it eliminates the potential immunologic reactions, which could be associated with cell transplantation. Finally, this approach resolves ethical dilemmas associated with embryonic/fetal stem cell manipulations. Knowledge of the molecular mechanisms governing stem cell behavior, therefore, is essential for the development of therapies which may facilitate brain regeneration after injury.

It has been previously shown that E2F1 and E2F3 are required for neural precursor proliferation in the adult brain (Cooper-Kuhn et al., 2002; McClellan et al., 2007). This thesis presents a new role for E2F4 as a positive regulator of neural stem cells. At first glance, this discovery challenges the general view of E2F4 as a 'repressive' transcription factor (reviewed in (Stevaux and Dyson, 2002; Trimarchi and Lees, 2002; DeGregori and Johnson, 2006)). Also, the p107 pocket protein, a major E2F4 interacting partner in proliferating cells, represses neural stem cell self-renewal (Vanderluit et al., 2004).

Several possibilities could be suggested to reconcile these facts: one is that E2F4 and p107 might be regulating different sets of target genes in neural stem cells (Fig. 4.2A). p107 is repressing the expression and activity of Notch signaling, which is the positive regulator of stem cell self-renewal (Hitoshi et al., 2002; Vanderluit et al., 2004). E2F4 might be required to repress the genes required for initiation of a differentiation pathway and transition of the stem cell into the restricted progenitor state. For example, activation of *c-myc* promotes differentiation of epidermal stem cells into transit amplifying progenitor cells in the skin (Arnold and Watt, 2001). Notably, E2F4 can mediate transforming growth factor-β repression of the *c-myc* promoter in epithelial cells (Chen et al., 2002). The absence of E2F4, in this case, would lead to the premature activation of the differentiation program in stem cells and their transition into the progenitor stage, which would deplete the neural stem cell compartment. Thus, E2F4 might be required to prevent premature activation of the differentiation program and to maintain neural precursors in the stem cell compartment.

Alternatively, self-renewal of neural stem cell might be stimulated by unbound activating E2Fs, such as E2F3, while E2F4 complexed with pocket proteins prevents sequestration of those free activating E2Fs (Fig. 4.2B). Should this be the case, then loss of E2F4 would lead to reorganization of the pocket protein-E2F complexes and increased sequestration of activating E2Fs by Rb proteins which normally interact with E2F4 (Lee et al., 2002b). The reduced pool of free E2Fs, in turn, would cause impairment of neural

Figure 4.2. Possible mechanisms of neural stem cell regulation by E2F4. A) E2F4 regulates transition from stem cell to progenitor compartments. B) E2F4 prevents sequestration of activating E2Fs by pocket proteins. C) E2F4 directly or indirectly regulates genes controlling neural stem cell self-renewal.


Wild type
E2F4-/-
A)
E2F4
Neural stem cells
Progenitors
Neural stem cells
Progenitors
- self-renewal
B)
E2F4
C)
E2F4
repressor
R
activator
A
R
A

stem cell self-renewal in E2F4 deficient mouse embryos. This would suggest that E2F4 regulates the number of neural stem cells by preventing sequestration of free activating E2Fs.

Another possibility is that E2F4 may control the transcription of genes required for neural stem cell self-renewal (Fig. 4.2C). As a transcriptional repressor, E2F4 may repress another repressor gene that is important in regulation of neural stem self-renewal. The absence of E2F4 would lead to increased repression of stem cell self-renewal. Alternatively, while E2F4 is believed to function as a transcriptional repressor, there are numerous examples where E2F4 can bind and activate transcription. Consistently, the diminished Shh activity in the E2F4 deficient mouse embryos can also lead to a reduced number of neural stem cells, since Shh is a known regulator of the stem cell population in the embryonic and adult brain (Machold et al., 2003; Palma et al., 2005). E2F4, therefore, might be required for direct or indirect transcriptional regulation of the genes which control neural stem cell self-renewal. Finally, the proposed mechanisms may have a cumulative effect on the neural stem cell number.

In conclusion, this thesis provides new *in vivo* evidence for E2F4 requirement for early ventral telencephalon and eye patterning and neural stem cell regulation. Importantly, E2F4 genetically interacts with the known morphogenetic regulator Shh in this process. These studies open the door for future investigations, the results of which will lay the foundation for understanding the complex mechanisms of neurodevelopmental disorders and towards the development of novel therapeutic approaches.

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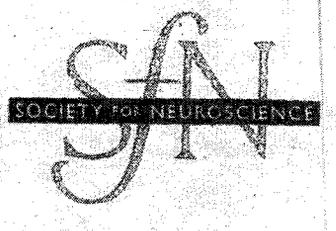

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I am presently writing my doctoral thesis in a format as a collection of manuscripts. I request a permission to include the following manuscript that I authored: Ruzhynsky et al. "Cell cycle regulator E2F4 is essential for the development of the ventral telencephalon." J. Neurosci., May 2007; 27: 5926 - 5935; doi:10.1523/JNEUROSCI.1538-07.2007. Please let me know if I have to send you my request in writing via mail or by fax.

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Sincerely,

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Appendix B. Curriculum Vitae

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EDUCATION

April 2004-present	University of Ottawa, Doctorate of Philosophy Program, Dept. of Cellular and Molecular Medicine, Ottawa, Canada
Aug.2002-April 2004	University of Ottawa, Master of Science Program, Dept. of Cellular and Molecular Medicine Ottawa, Canada
Sept.2000-May 2001	University of Ottawa, Bachelor of Science, Honours (2001) Physiology Program, Dept. of Cellular and Molecular Medicine, Ottawa, Canada
Sept.1994-Sept.1997	National Medical University, General Medicine Kiev, Ukraine
Sept.1988-Feb.1992	Kiev Medical College #2, Feldsher (Physician Assistant) Program, Kiev, Ukraine

RESEARCH EXPERIENCE

September 2002-present	Ph.D/M.Sc. Research Project "E2F4 in Development of Ventral Telencephalon and Visual System". Supervisor: Dr. Ruth S. Slack University of Ottawa
Sept.2000-May 2001	Bachelor of Science Honours Research Project "Role of Cl^- in Muscle Fatigue". University of Ottawa Supervisor: Dr. Jean-Marc Renaud University of Ottawa

WORK EXPERIENCE

January 2003-May 2007	Demonstrator, Division of Clinical and Functional Anatomy, Dept. of Cellular and Molecular Medicine University of Ottawa, Canada
June 2001-July 2007	Registered Nurse, General Surgery, Ottawa Hospital, Civic Campus, Ottawa, Canada

July 1998-June 2001	Patient Care Assistant, General Surgery, Ottawa Hospital, Civic Campus, Ottawa, Canada
Apr.1994-Aug. 1994	Ambulance Feldsher (Physician Assistant), Emergency Medical Service Station, Kiev, Ukraine
June 1992-Jan. 1994	Physician Assistant, Ukrainian Army Kharkov, Ukraine
July 1990-Oct.1991	Ambulance Orderly, Emergency Medical Service Station, Kiev, Ukraine

Publications

1. V. Ruzhynsky, M. Furimsky, D. Park, V. Wallace and R Slack. E2F4 is required for early eye development. (*Developmental Dynamics* in revisions)

2. V. Ruzhynsky, K. McClellan, J. Vanderluit, M. Furimsky, D. Park, V. Wallace and R Slack. Cell cycle regulator E2F4 is a mediator of Shh signaling activity. *J Neurosci.* 2007 May 30;27(22):5926-35

3. McClellan KA, Ruzhynsky VA, Douda DN, Vanderluit JL, Ferguson KL, Chen D, Bremner R, Park DS, Leone G, Slack RS. Unique requirement for Rb/E2F3 in neuronal migration: evidence for cell cycle-independent functions. Mol Cell Biol. 2007 Jul;27(13):4825-43. Epub 2007 Apr 23

4. J. Vanderluit, K. Ferguson, V Nikoletopoulou, M. Parker, V. Ruzhynsky, T. Alexson, S. McNamara, J. MacLaurin, C. McIntosh, D. Park, M. Rudnicki, and R. Slack. P107 regulates the neural precursor cell population in the mammalian brain. *Journal of Cell Biology*, 166:6, Sept. 2004.

5. S. Cairns, V. Ruzhynsky, and J.-M. Renaud. Protective role of extracellular chloride in fatigue of isolated mammalian skeletal muscle. *Am J Physiol Cell Physiol*, 287: C762 – 770, Sept. 2004.

Invited Presentations

1. V. Ruzhynsky, K. McClellan, J. Vanderluit, M. Furimsky, V. Wallace & Ruth S. Slack. Requirement for E2F4 in the development of ventral telencephalon. Platform presentation OHRI Research Day, Ottawa, Ontario. October 24, 2005.

Scienctific Astracts

1. V. Ruzhynsky, K. McClellan, J. Vanderluit, M. Furimsky, V. Wallace & Ruth S. Slack. Crosstalk between cell cycle regulator E2F4 and morphogen Shh. [abstract]. In: Abcam The inaugural meeting Stem Cells; 2006 Dec. 14-17; Cancun, Mexico; 2006. p.117.

2. V. Ruzhynsky, K. McClellan, J. Vanderluit, M. Furimsky, V. Wallace & Ruth S. Slack. Crosstalk between cell cycle regulator E2F4 and morphogen Shh. [abstract]. In: 6th Stem Cell Network Annual General Meeting; 2006 Nov. 13-15; Ottawa, ON, Canada.
3. V. Ruzhynsky, K. McClellan, J. Vanderluit, M. Furimsky, V. Wallace & Ruth S. Slack. Effect of E2F4 deletion in the developing mouse brain. Poster presentation at the 3rd Canadian Developmental Biology Conference, Mont-Tremblant, QC, Canada. April 6-9, 2006.
4. V. Ruzhynsky, K. McClellan, J. Vanderluit, M. Furimsky, V. Wallace & Ruth S. Slack. Requirement for E2F4 in the development of ventral telencephalon. Poster presentation at the Stem Cell Network Annual General Meeting 2005, Calgary, AB, Canada, Nov. 23-26, 2005.
5. V. Ruzhynsky, J. Vanderluit, J. MacLaurin & R.S. Slack. The effects of E2F4 deletion in the developing mouse brain. Poster presentation Keystone Caner & Development Symposium, Banff, AB, Canada, Feb.2005 .
6. V. Ruzhynsky, Jacqueline L. Vanderluit, Jason MacLaurin & Ruth S. Slack. The effects of E2F4 deletion in the developing mouse brain. Poster presentation at the Stem Cell Network Annual General Meeting 2004, Montreal, QC. Nov. 3-5, 2004.
7. V. Ruzhynsky, Jacqueline L. Vanderluit, Jason MacLaurin & Ruth S. Slack. The effects of E2F4 deletion in the developing mouse brain. Poster presentation at the Neuroscience 2004, the Society for Neuroscience 34th Annual Meeting, San Diego, CA, USA. October 23-27, 2004.
8. V. Ruzhynsky, J. Vanderluit, J. MacLaurin & R. Slack. Regulation of neural stem cells by E2F-4 transcription factor during mammalian embryonic development. Poster presented at the Neuroscience 2003, the Society for Neuroscience 33rd Annual Meeting, New Orleans, LA, USA. November 8 - 12, 2003.
9. V. Ruzhynsky, J. Vanderluit, J. MacLaurin & R. Slack The E2F-4 transcription factor positively regulates the number of mammalian neural stem cells. Poster presented at the Stem Cell Network Annual General Meeting, Vancouver, BC, Canada. September 20-23, 2003.
10. V.A. Ruzhynsky, J.L. Vanderluit, & R.S. Slack The role of E2F-4 transcription factor in regulation of mammalian neural stem cells. Poster presented at the Society for Developmental Biology 62nd Annual Meeting Jointly with the International Society of Developmental Biologists, Boston, MA, USA. July 30-August 3, 2003.
11. V. Ruzhynsky, J. Vanderluit, J. MacLaurin & R. Slack. Regulation of neural stem cells by E2F-4 transcription factor during mammalian embryonic development. Poster presented at the OHRI Research Day, Ottawa, Ontario. November 28, 2003.

SCHOLARSHIPS AND ACADEMIC AWARDS.

1. Ontario Graduate Scholarship, May 2006 – April 2007
2. University of Ottawa Excellence Scholarship, May 2006 – April 2007
3. Ontario Graduate Scholarship, May 2005 – April 2006
4. University of Ottawa Excellence Scholarship, May 2005 – April 2006
5. University of Ottawa Travel Award, Sept. 2004.
6. Stem Cell Network Graduate Studentship Award, September 2004.

7. Excellence Award in recognition of outstanding contribution to Anatomy laboratory teaching 2003-2004, June 2004.
8. University of Ottawa Admission Scholarship, May 2004.
9. 1st place Master Students' Poster Competition, Ottawa Health Research Institute Research Day, November, 2003.
10. University of Ottawa Excellence Scholarship, May 2003 – April 2004.
11. Ontario Graduate Scholarship, May 2003 – April 2004.
12. Dept. of Cellular and Molecular Medicine Admission Award, University of Ottawa, September 2002.
13. University of Ottawa Entrance Scholarship, September, 2002.
14. Honorary (Top 10%) Scholarship for Excellence in Studies, Sept.1995-Aug.1997, National Medical University, Kiev, Ukraine.
15. Honorary (Top 10%) Scholarship for Excellence in Studies, Jan.1989-Feb.1992, Kiev Medical College #2, Kiev, Ukraine.

Languages: English, French (beginner), Ukrainian, Russian