Downstream targets of transcription factor Pax6 in cortical development

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This thesis is dedicated to the memory of my beloved mother

Krishna Kumari Boppana

Contents

Abbreviations

ADA	Adenosine deaminase
AP2	Activation protein 2
APC	Adenomatous polyposis coli
arRP	Autosomal recessive retinitis pigmentosa
bHLH	Basic helix-loop-helix
BLBP	Brain lipid-binding protein
BMP	Bone morphogenic protein
BrdU	5-bromo-2-desoxy-uridine
bp	Base pair
BSA	Bovine serum albumin
Cad	Cadherin
cDNA	Complementary DNA
CDS	Coding sequence
ChIP	Chromatin immunoprecipitation assay
CNS	Central nervous system
CP	Cortical plate
CRALBP	Cellular retinaldehyde binding protein
CR	Cajal retzius cells
CUX	Cut domain transcription factor
DAPI	4'-6'-diamidino-2-phenylindole
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotides
DTT	Dithiothreitol
E	Embryonic day
EDTA	Ethylene diamine tetra acetic acid
FCS	Fetal calf serum

FGFR	Fibroblast growth factor receptor 1
Fig.	Figure
G1	G1-phase of cell cycle
G2	G2-phase of cell cycle
GABA	γ-amino butyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
hrs	Hours
HCL	Hydrochloric acid
HD	Homeodomain
HPRT	Hypoxanthine guanine phosphoribosyl transferase
HRP	Horse radish peroxidase
IPC	Intermediate progenitor cell
IHC	Immunohistochemistry
IZ	Intermediate zone
ko	Knock-out
LB	Luria-Bertani
LI	Labeling index
LP	Lens placoid
LRAT	Lecithin-retinol acyltransferase
LRPs	Lipoprotein receptor-related proteins
LTR	Long terminal repeat
mAB	Monoclonal antibody
mRNA	Messenger ribonucleic acid
MZ	Marginal zone
n	Sample number
NaAc	Sodium acetate
NaCl	Natrium chloride
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NE	Neuroepithelium

Ngn	Neurogenin
NR	Neuroretina
NTE	NaCl-tris-EDTA
OC	Optic cup
ORE	Optic recess
OV	Optic vesicle
P	Postnatal day
pAB	Polyclonal antibody
PBS	Phosphate buffered saline
pBS KS	Plasmid bluescript KS
PCR	Polymerase chain reaction
pcDNA	Plasmid-cDNA
PD	Paired domain
PD5a	Paired domain containing exon5a
Pen/Strep	Penicillin/Streptomycin
PFA	Paraformaldehyde
PH3	Phosphorylated histone H3
PRD	Bipartite paired domain
PSPB	Pallial-subpallial boundary
RNA	Ribonucleic acid
RNase	Ribonuclease
RC2	Radial cell 2
rln	Reelin
rpm	Rounds per minute
RT	Room temperature
RPE	Retinal pigmented epithelium
SDS	Sodium dodecyl sulfate
s.d.	Standard deviation
SE	Surface ectoderm
siRNA	Small interfering RNA
SP	Subplate

SP6	Bacteriophage sp6
SEM	Standard error of the mean
SFRP	Secreted frizzled related protein
Shh	Sonic hedgehog
S-phase	DNA-synthesis phase of the cell cycle
SSC	Sodium chloride-Sodium citrate
Svet1	Subventricular tag1
SVZ	Subventricular zone
T7	Bacteriophage T7
TAD	Transactivating domain
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TBS	Tris-buffered saline
Tbr	T-domain transcription factor
TGFß	Transforming growth factor-β
TF	Transcription factor
Tris	Tris-(hydroxymethyl)-aminomethane
Tween 20	Polyoxyethylene sorbitan monolaurate
VZ	Ventricular zone
wt	Wild type

1. Introduction

1.1 Development of the mouse cortex

Specification of a group of cells of the presumptive ectoderm into the neural plate marks the beginning of development of the central nervous system (CNS). The neural plate then invaginates under the influence of signals from the notochord to give rise to the neural tube. After the initial induction of neural tissue, the telencephalon is formed at the rostralmost portion of the neural tube. It subdivides into two cerebral cortices. As development proceeds, signals from the anterior neural ridge in the rostral midline, the roof plate in the dorsal midline, the cortical hem, and the surface ectoderm ensure proper specification and patterning of the cortex.

The neural tube is composed of neuroepithelial cells, progenitors that line the ventricular lumen and form the ventricular zone (VZ). After the onset of cortical neurogenesis, neuroepithelial cells give rise to bipolar radial glial cells, the progenitors of cortical neurons and astrocytes. In contrast, progenitors in the retina and spinal cord mostly maintain neuroepithelial properties and to a lesser extent radial glial properties during neurogenesis. Almost all neurons in the brain are directly or indirectly derived from radial glia [Malatesta et al., 2003]. Neuroepithelial cells show interkinetic nuclear migration, with their nuclei undergoing mitosis at the apical surface of the ventricular zone. Radial glia are also known to exhibit astroglial properties [Kriegstein and Gotz, 2003] and express markers like glial fibrillary acidic protein (GFAP), vimentin, brain-

lipid-binding protein (BLBP) and the calcium binding protein S100β during neurogenesis [Campbell and Gotz, 2002; Gotz, 2003; Kriegstein and Gotz, 2003].

Besides radial glial cells, basal progenitors are another class of neuronal progenitors that appear at the onset of neurogenesis [Miyata et al., 2004; Noctor et al., 2004; Smart, 1973]. VZ progenitors divide asymmetrically at the ventricular surface to self-renew and produce a rounded daughter cell called the Intermediate progenitor cell (IPC). These IPCs migrate away from the VZ to form the SVZ, where they extend multiple short processes. They are also called basal progenitors, and express markers like subventricular tag1 (Svet1), T-domain transcription factor (TF; Tbr2), and the homeobox proteins Cux1 and 2 [Englund et al., 2005; Nieto et al., 2004; Tarabykin et al., 2001; Zimmer et al., 2004]. Multipolar IPCs divide symmetrically at a nonsurface position to produce two immature multipolar neurons [Cai et al., 2002]. These immature neurons migrate into the cortical plate [Noctor et al., 2004] and differentiate into projection neurons. IPC's appear to produce the majority of neurons during early neurogenesis when deep layers are produced [Haubensak et al., 2004].

1.2 Specification of the dorsal telencephalon

After initial induction by extrinsic signals, the telencephalon is specified into dorsal (pallial) and ventral (subpallial) regions. This is mediated by basic helix-loop-helix (bHLH) transcription factors (TFs) like Ngn1 and Ngn2. Neurogenins promote cortical development by suppressing Mash1-dependent ventral fates [Fode et al., 2000]. The LIM homeobox gene Lhx2 further specifies the dorsal telencephalon into choroid plexus

epithelium, cortical hem and cortical VZ. Signals from bone morphogenetic proteins (BMPs) in the dorsal midline roof further expand the cortical VZ [Monuki et al., 2001] and may also regulate patterning.

Before the onset of neurogenesis, transcription factors in the cortical neuroepithelium promote forebrain regionalization, patterning and progenitor proliferation, and suppress neuronal differentiation. The homeodomain (HD) TFs Emx1 and 2, and the paired and homeodomain TFs Pax6, Tlx, Ngn1 and 2 are among those contributing to regionalization [Muzio et al., 2002].

The cerebral cortex is composed of projection neurons that are glutamatergic and excitatory, and interneurons that are GABAergic and inhibitory. Cortical VZ progenitors become glutamatergic projection neurons of the cortex [Parnavelas, 2000] and often lack GABAergic markers. Cortical neurons are arranged in a cytoarchitecturally and functionally defined pattern with different layers exhibiting specific molecular properties. For instance, Er81 is expressed in some progenitor cells and, subsequently in a subset of layer 5 projection neurons, suggesting that Er81 may specify layer 5 fate [Hasegawa et al., 2004; Yoneshima et al., 2006]. Otx1 is also a deep layer marker and is expressed in neurons of layers 5/6 [Frantz et al., 1994]. Lmo4, a LIM homeodomain protein known to be expressed in layers 2, 3 and 5 [Bulchand et al., 2003], is a specific marker for type II pyramidal neurons [Arlotta et al., 2005]. Similarly, Cux2 is expressed in mitotically active SVZ cells and in upper layer neurons suggesting that it may specify upper layer fate [Nieto et al., 2004].

The Cajal-Retzius (CR) cells in the marginal zone (MZ) of the cortical primordium produce Reelin [Ogawa et al., 1995]. Reelin signals to radial glial cells and their daughter neurons [Magdaleno et al., 2002], and provides guidance for young neurons to migrate into the cortical plate.

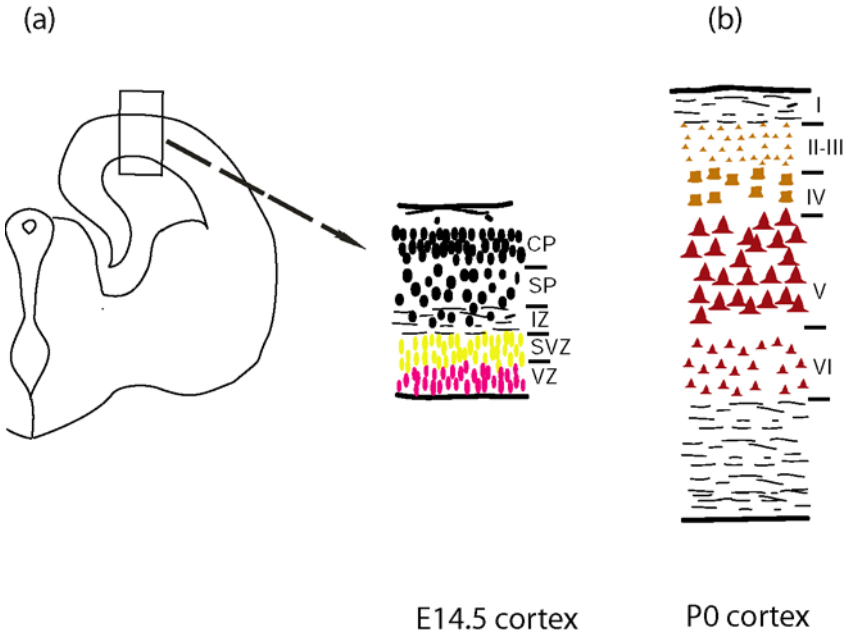

Fig 1: Cortical layer formation in mice. Layering of the cortex during embryonic (E14.5) and postnatal (P0) stages is shown. CP, Cortical plate; SP, Subplate; IZ, intermediate zone; SVZ, Subventricular zone; VZ, Ventricular Zone and I-VI layers. (adapted from Zoltan & Henry, 2005).

1.3 Signaling centers in patterning of cerebral cortex

Regulation of gene expression during early development relies almost entirely on five pathways, namely Notch, transforming growth factor-β (TGF-β), wingless/WNT, Sonic hedgehog (Shh) and receptor tyrosine kinase [Gerhart, 1999].

The cell surface receptor Notch has pleiotropic functions during development. Its signals seem to be involved in brain morphogenesis, neuronal progenitor maintenance, and neuronal migration. During gliogenesis, Notch promotes the differentiation of glial subtypes, with the exception of oligodendrocytes. In neurospheres Notch promotes differentiation of glia at the expense of neurons, followed by more specific differentiation into astrocytes, while repressing oligodendrocyte fate [Grandbarbe et al., 2003]. Thereby, Notch controls cell fate in a stepwise manner.

BMPs belong to the superfamily of TGF-β and transduce their signal via Smad proteins. BMPs are involved in establishing dorsal and ventral cell fates in the embryonic mesoderm [Graff, 1997]. BMP-4 and OP-1 are both expressed in the epidermal ectoderm [Liem et al., 1995] and drive differentiation of dorsal cells in the nervous system. BMPs are important in dorso-ventral specification of cell fates and regulate differentiation of astrocytes from SVZ progenitors [Mehler et al., 1997].

Wnt signaling occurs through three major pathways; the canonical/β-catenin, the planar cell polarity/JNK and the Ca2+/Protein kinase C pathways [Jones and Jomary, 2002].

Canonical Wnt signaling depends on β-catenin and is the best characterized of the three known pathways. Binding of Wnt ligand to Fzd receptor leads to the activation of Dishevelled, which ultimately leads to the stabilization of β-catenin via dephosphorylation. Regulation of canonical/β-catenin Wnt signaling is achieved by cadherin-mediated cell adhesion. Cadherins are able to reduce the free cytoplasmic pool of β-catenin by sequestering it at the plasma membrane. Therefore, by regulating the levels of free β-catenin in the cytoplasm, the threshold for Wnt-activation may be dynamically modulated [Nelson and Nusse, 2004].

Non-canonical pathways are, by definition, independent of β-catenin. Binding of Wnt to Frizzled receptors in the absence of Low density lipoprotein receptor-related proteins (LRPs) activates Dishevelled, leading to activation of c-Jun NH2-terminal kinase (JNK) cascade through small GTP-binding proteins. Wnt signaling through Dishevelled, Rac and JNK controls dendritic development in cultured hippocampal cells [Rosso et al., 2005].

The Ca2+/Protein kinase C (PKC) Wnt pathway, like the PCP, is dependent on Dishevelled and independent of β-catenin and LRPs. Some Wnt signals can raise the levels of intracellular Ca2+ leading to the activation of PKC and Ca2+ - dependent calmodulin kinase II (CamKII).

Shh has a morphogen-like behaviour and has a pivotal role in generating a diverse array of neuronal subtypes that are required for the assembly and function of neuronal circuits [Briscoe and Ericson, 1999] especially within the vertebrate neural tube. Shh is initially

expressed in the notochord, that lies below the ventral neural tube, but is rapidly induced within the ventral midline of the neural tube [Echelard et al., 1993]. Graded activity of Shh establishes distinct domains of homeobox gene expression along the dorsoventral axis of the neural tube [Briscoe et al., 2000]. Shh also has a direct role in regulating proliferation of neural progenitor cells. In the anterior neural tube, a number of structures like cerebellum, the neocortex and the tectum, depend on the mitogenic effect of Shh signaling [Dahmane et al., 2001].

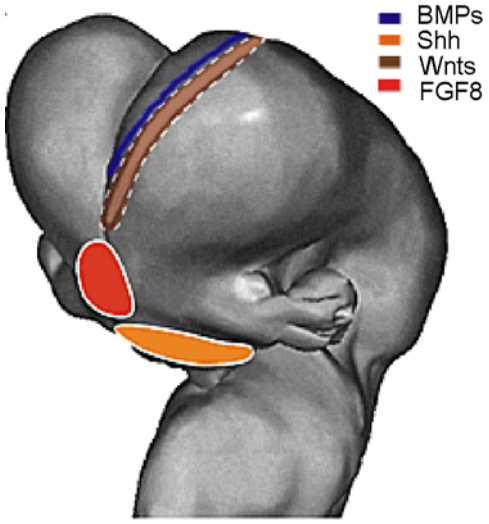

Fig 2: Signaling centers in the cerebral cortex. Medially, the dorsal roof plate produces BMPs; the cortical hem is a source of Wnts. Sonic hedgehog (Shh) is ventrally secreted from the prechordal mesoderm. FGF8 is mainly released from the anterior neural ridge. (adapted from Monuki & Walsh, 2001).

1.4 Transcription factor Pax6 in cortical development

1.4.1 Structural and functional domains of Pax6

Pax6 belongs to the Pax family of transcription factors and encodes a 422 amino acid protein that contains two highly conserved DNA-binding domains- a bipartite paired domain (PRD) and a homeodomain (HD), and a C-terminal region that functions in transcriptional activation [Bopp et al., 1986; Epstein et al., 1994b; Treisman et al., 1991; Walther and Gruss, 1991]. Mammalian Pax6 genes encode predominantly two forms of the Pax6 protein, Pax6 and Pax6(5a) [Carriere et al., 1993]. The 128 amino acid paired domain (PD) [Epstein et al., 1994a] can be separated structurally and functionally into two independent DNA binding subdomains, PAI and RED [Czerny et al., 1993; Epstein et al., 1994b]. Pax6(5a) contains a 14 amino acid insertion within the PAI domain forming a distinct paired domain, PD5a. This insertion destructs the DNA-binding capacity of the PAI subdomain, leaving the RED subdomain and the HD for DNA recognition [Epstein et al., 1994b]. The different DNA binding domains of Pax6 and Pax6(5a) can interact independently with DNA. Interaction between these domains is also crucial for the proper activation of a wide variety of target genes.

1.4.2 Role of Pax6 in patterning of the brain

Pax6 is involved in the dorsoventral specification of telencephalic progenitors. Expression of Pax6 is first detected in the neuroepithelium of the forebrain at around E8.0, and continues during neurogenesis. It shows a regionalized expression in the dorsal telencephalic primordium [Walther and Gruss, 1991], thus specifying the pallial-

subpallial boundary (PSPB). Disruption of this boundary in the *Pax6*/*Small eye* mutant [Hill et al., 1991] leads to the invasion of cells from the subpallium into the neocortex [Chapouton et al., 1999]. It has also been shown that the molecular ventralization of pallial progenitors in *Pax6* mutants leads to respecification of a subset of cortical glutamatergic neurons into subpallial GABAergic interneurons [Kroll and O'Leary, 2005]. Pax6 is expressed in the mitotically active ventricular zone and has previously been shown to control specification, regionalization and arealization of the cerebral cortex. In the wild type (wt) cortex, Pax6 is expressed in a high rostro- lateral to low caudo- medial gradient in apposition to the expression of transcription factors Emx1 and Emx2. Loss of Pax6 leads to the expansion of Emx2 expressing primary visual area, while loss of Emx2 leads to the expansion of caudal Pax6 domains containing somatosensory and motor areas [Bishop et al., 2002]. This was elucidated through studies using several cortical markers such as cad6, Cad8, Id2, RZRß, p75, EphA7 and ephrin-A5 [Bishop et al., 2002].

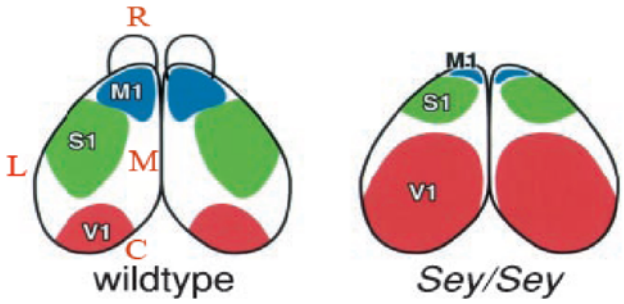

Fig 3: Arealization of mouse neocortex. The diagrams depict the disproportionate changes in area size and positioning in Pax6 mutants. The predicted organization is suggested by analysis of gene markers. In Pax6 mutants, rostro-lateral areas are reduced and the caudo-medial areas are expanded. The visual (V1), motor (M1), and somatosensory (S1) areas are shown. C, Caudal; L, lateral; M, medial; R, rostral; Sey, small eye mutant. (adapted from Bishop & O' Leary, 2002).

In Pax6 mutants, dorsoventral patterning in the spinal cord is also lost [Ericson et al., 1997]. For example, the expression of Ngn2 in ventral spinal cord, which is also known to play a role in patterning, is missing in Pax6 mutants [Scardigli et al., 2001].

1.4.3 Regulation of neurogenesis by Pax6

Loss- and gain of function studies have showed that Pax6 is involved in the regulation of neurogenesis in the developing forebrain. Expression analysis using molecular markers of distinct layers of the neocortex have suggested that in Pax6 mutants, the upper cortical layers are missing [Tarabykin et al., 2001]. In the diencephalon and spinal cord too, certain subpopulations of neurons are lacking [Ericson et al., 1997; Mastick and Andrews, 2001].

1.4.4 Pax6 in eye development

Pax6 is a key regulator of vertebrate eye development and upon misexpression, is sufficient to induce ectopic eyes [Chow et al., 1999]. Mice strains carrying mutations in the Pax6 gene have a characteristic small eye [Hill et al., 1991], which is analogous to human aniridia. The predominant isoform of Pax6 expressed in the eye lacks exon 5a [Jaworski et al., 1997]. Pax6 plays multiple distinct roles in both lens [Ashery-Padan et al., 2000; Lang, 2004] and retinal [Marquardt et al., 2001] development, involving different interactions with other transcription factors. A single wild type Pax6 allele is not sufficient for normal eye development in humans and rodents, implying haploinsufficiency.

Deletion of Pax6 affects the expression of a number of structural eye genes like keratins and crystallins. The sine oculis family gene Six3 [Ashery-Padan et al., 2000; Goudreau et al., 2002], and Maf [Sakai et al., 2001], a member of the v- Maf oncogene family that plays important roles in cellular differentiation, are known to be regulated by Pax6 during eye development [Blank and Andrews, 1997]. Mice heterozygous for Pax6 show small eye (Sey) phenotype whereas homozygote Pax6 mutants completely lack the entire eye structure [Hill et al., 1991].

1.5 Cellular retinaldehyde-binding protein (CRALBP)

Cellular retinaldehyde- binding protein (CRALBP) is a water-soluble retinoid-binding protein of 316 amino acids and belongs to the CRAL- Trio family of proteins. It is expressed in cornea, pineal gland, optic nerve, and abundantly in Muller cells and retinal pigment epithelial (RPE) cells [Bunt-Milam and Saari, 1983], where many of the reactions of the rod visual cycle take place. CRALBP is also expressed by oligodendrocytes of the optic nerve and brain [Saari et al., 1997]. It is a carrier of 11-*cis*-retinol and 11-*cis*-retinaldehyde, that are known to function in vision [Saari et al., 1982]. It can interact with 11-*cis* retinoldehydrogenase in RPE resulting in the reduction of bound 1l-*cis*-retinaldehyde [Saari and Bredberg, 1982].

Photoisomerization of 11-*cis*- to all-trans-retinal occurs in rod photoreceptor cells, whereas enzymatic isomerization of all-*trans*- to 11-*cis*-retinol occurs in adjacent retinal pigment epithelium (RPE). These two reactions occurring in different cellular

compartments are linked into a cycle by the directed flow of retinoids through the intercellular matrix [Dowling, 1960; Wald, 1968; Zimmerman, 1974]. First, photoisomerization converts 11-*cis*-retinal to all-*trans*-retinal. All-*trans*-retinal is reduced to all-*trans*-retinol by reduced nicotinamide adenine dinucleotide phosphate (NADPH) and photoreceptor retinol dehydrogenase which is found within the rod photoreceptor outer segment. All-*trans*-retinol enters retinal pigment epithelium from rod photoreceptor cells, and is sequentially esterified by lecithin-retinol acyltransferase (LRAT). This ester is then converted to 11-*cis*-retinol by an isomerohydrolase, and oxidized to 11-*cis*-retinal by NAD (P) or with other short-chain dehydrogenase-reductases. 11- *cis*-retinal then diffuses back into the rod photoreceptor cell, where it regenerates rhodopsin and completes the visual cycle.

In CRALBP mutant mice, isomerization of all-*trans*- to 11-*cis*-retinol in the visual cycle is substantially impaired [Saari et al., 2001]. Mutations in the human gene encoding CRALBP cause autosomal recessive retinitis pigmentosa (arRP), a condition characterized by progressive photoreceptor degeneration and night blindness [Morimura et al., 1999]. It is also known to cause stationary night blindness, or other related conditions [Thompson and Gal, 2003], one characteristic of which is delayed dark adaptation [Burstedt et al., 2001].

1.6 Transcription factor AP-2 gamma

The Activating protein-2 (AP2) family of transcription factors consists of five members in humans and mice (AP-2α, β,γ δ and ε), that share a highly conserved helix-span-helix dimerization motif at the carboxyl terminal, and a less conserved proline and glutamine rich domain at the amino terminal. The embryonic lethal phenotype in mice lacking AP2 genes shows the importance of these genes during development [Moser et al., 1997a; Schorle et al., 1996; Werling and Schorle, 2002a; Zhang et al., 1996]. It has been shown that AP2 genes might play a role in promoting proliferation and repressing differentiation [Pfisterer et al., 2002].

AP-2 transcription factors are predominantly localized in the nucleus and have also been shown to interfere with signal transduction pathways downstream of Wnt by associating with APC (Adenomatous polyposis coli) tumor suppressor protein in the nucleus [Li and Dashwood, 2004]. In mice, AP-2α, β, and γ are coexpressed in neural-crest cells, the peripheral nervous system and extraembryonic trophoectoderm [Moser et al., 1997b; Zhao et al., 2003], and have been implicated in breast cancer [Jager et al., 2005; Turner et al., 1998]. The mouse AP2γ gene is expressed in the central and peripheral nervous system, ectoderm, face, limbs and mammary glands during mouse development, similar to the other AP2 family members [Chazaud et al., 1996; Mitchell et al., 1991; Moser et al., 1997b]. AP2γ is expressed in trophoblast cells as early as E3.5 of murine development. Its expression in all derivatives of the trophoblast lineage during chorioallantoic fusion [Sapin et al., 2000; Shi and Kellems, 1998] suggests that *AP-2*γ might play a role in regulating trophoblast gene expression programs. It is also essential

for embryonic survival during early postimplantation period, and expression of AP2γ in the extra-embryonic membranes of AP2γ mutant embryos can rescue improper development [Auman et al., 2002]. AP2γ also regulates the expression of genes for human placental lactogen, chorionic gonadotropin-β and adenosine deaminase (*ADA*) [Johnson and Jameson, 1999; Richardson et al., 2000; Shi and Kellems, 1998].

AP2γ was first identified in human breast cancer cell lines due to its ability to recognize critical regulatory element within the erbB2 promoter [Bosher et al., 1996]. Later it was also found to interact with regulatory elements associated with the estrogen receptor gene, involved in oncogenesis [McPherson et al., 1997]. Moreover, tumor progression in a murine breast-cancer model is enhanced after transgenic overexpression of AP2γ [Jager et al., 2005]. These data show a direct role of AP2γ in regulating expression of molecules associated with cell proliferation, especially in breast cancer.

Heterozygous AP2γ mutant mice are viable and display reduced body size at birth. In homozygous mutants, proliferation rate is reduced in the extraembryonic ectoderm eventually leading to a failure in labyrinth layer formation. The embryo is deprived of nutrients resulting in growth retardation and eventual death [Werling and Schorle, 2002a].

2. Materials and Methods

2.1 Animals

Mouse strains carrying floxed alleles for exon 5 of AP2 gamma [Werling and Schorle, 2002b] were crossed with mice that express Cre recombinase under the Emx1^{IRESCre} promoter [Gorski et al., 2002]. LoxP sites that flank exon 5 of AP2 gamma gene are recognized by the Cre- recombinase enzyme that mediates a loop out reaction of the 5th exon resulting in a truncated and nonfunctional protein. Heterozygous mice (AP2γ flox/cre) were crossed to obtain conditional knockout mutants for AP2 γ.

CRALBP knock out mice in which Neo^{r} was inserted into exon 3 of the coding sequence [Saari et al., 2001] was used for the project.

2.2 Molecular biology procedures

2.3 Mouse genotyping

2.3.1 DNA Isolation

Tail or yolk sac (from young mice or embryo's respectively) was incubated in 0.5ml PK-lysis buffer (100mM Tris- HCl pH8.5, 5mM EDTA, 200mM NaCl, 0.2% SDS, 100µg/ml Proteinase K), shaking at 55°C overnight. After a 10 min centrifugation at 13,000 rpm, the DNA in the supernatant was precipitated by the addition of isopropanol to a final concentration of 50%. Genomic DNA was collected by centrifugation, washed twice in 80% ethanol and resuspended in water at 40°C for 1 hr.

2.3.2 Polymerase chain reaction (PCR)

All PCR reactions were carried out in an end volume of 20µl that contained:

10x Buffer (Genecraft)	2µl
10mM dNTP's (Invitrogen)	0.4µl (20pmol/ml)
Primer1/Primer2 (IBA)	0.8µl (40pmol/ml) each
TAQ polymerase (Genecraft)	0.4µl (0.5 units)
Template DNA	1µl
dH2O	13µl

To detect wt and floxed AP2γ alleles, mice were genotyped using specific primers;

A forward primer against exon 5

5'- CAATTTTGTCCAACTTCTCCCTCAA- 3'

and a reverse primer that recognizes intron 4

5'- AACAGGTTATCATTTGGTTGGGATT- 3' were used.

In wt mice, the PCR generated a fragment of ~300 bp whereas floxed alleles generated a fragment of ~340 bp [Werling and Schorle, 2002b].

To identify Cre recombinase gene, a 500 bp fragment was amplified (94°C 10sec, 55°C 30sec, 72°C 40sec; 30 cycles) using the following primers:

5'-TCGATGCAACGAGTGATGAG- 3' (forward)

5'-TTCGGCTATACGTAACAGGG- 3' (reverse).

To identify wt and CRALBP knockout mice, genotyping was done using the following primers [Saari et al., 2001]: For wt exon 3,

5’ -TTA GAC TCA CAG GGG CCA ACA- 3’ (forward)

5’ -ATG ATC CTT GGT TGT GAG CTG CTC- 3’ (reverse);

For neomycin^{r},

5’-ATT TTG AAT GGA AGG ATT GGA GCT A-3 (forward)

5-ATG ATC CTT GGT TGT GAG CTG CTC-3 (reverse)

To detect putative Pax6 binding sites on AP2γ and CRALBP promoter, Chromatin immunoprecipitation assay (ChIP) was performed. As a positive control, the following primers were used against Pax6 promoter region (~300 bp)

5’- CCCACCCTCAGGCTTCCA- 3’ (forward)

5’-CTCCCGAGCCTGCCCATT- 3’ (reverse).

Five primer pairs flanking putative Pax6 consensus sequences upstream of exon1 of AP2γ were designed as follows:

1. -623 to -985 bp upstream (362 bp fragment)

    5’-GGACAGGTTGAGCTAGAGTT -3’ (forward)

    5’-CCGGTGTAGAGAAGGAATGA -3’ (reverse);

2. -1855 to -2188 bp upstream (380 bp fragment)

    5’-GAGTTGAGACACCCTGGAAT-3’ (forward)

    5’-GGGGACCCGAACGCCTTAAT-3’ (reverse);

3. -3329 to -3644 bp upstream (315 bp fragment)

5’-TGAGGAAAGGCGAATGCAGA-3’ (forward)

5’-TGGAATTAGCGGGAGAAGAG-3’ (reverse);

4. -5405 to -5686 bp upstream (281 bp fragment)

5’-CTTCTGTCTTTAACCCCTGC-3’ (forward)

5’-TACTAGGCTTCCACACACCT-3’ (reverse);

5. -6839 to -7187 bp upstream (348 bp fragment)

5’-TCCTCGGACTCGATTGTACA-3’ (forward)

5’-GTCACTTCTGGGGCAAAGAT-3’ (reverse);

Five primer pairs flanking putative Pax6 consensus sequences upstream of exon1 of CRALBP gene were designed as follows:

1. -2009 to -1671 bp upstream (338 bp fragment)

5’-GACACACTAATCCCAGCTTG- 3’ (forward)

5’-GAGTATTTGATCACCTGCCAT- 3’ (reverse);

2. -2998 to -2727 bp upstream (271 bp fragment)

5’-TGTGGCTGGATTTCAGAGAA3’ (forward)

5’ -CAGGCTAAGGGAATGCATC- 3’ (reverse);

3. -3453 to -3128 bp upstream (325 bp fragment)

5’ -AGGTAGGTGGCCAACAGTA- 3’ (forward)

5’ –TATGCCCAAAGAACCTCCCT- 3’ (reverse);

4. -4660 to -4336 bp upstream (324 bp fragment)

5’-TCTGATACCTCTGCACCAG 3’ (forward)

5’-CTTTCTTGACTGCGATTTCCC3’ (reverse);

5. -5713 to -5441 bp upstream (272 bp fragment)

5’-TGCTAAGGTGTTAACAGGCC- 3’ (forward)

5’ -CTGGCACTGGGATTACAAATG- 3’ (reverse);

PCR conditions were as follows: 2min at 94 °C followed by 34 cycles of 30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C.

All PCR products were separated by 1.5% agarose gel electrophoresis at 5V/cm (chamber length). Agarose gels were prepared in TAE buffer (40mMTris- acetate, 1mMEDTA, pH 8) containing 0.5µg/ml ethidium bromide (Fulka) and visualized under ultra violet light. DNA was loaded using OrangeG buffer, and 100 bp or 1kb plus DNA markers (Invitrogen) were used at a concentration of 50ng/µl.

2.4 Preparation of Probes for In situ hybridization

2.4.1 Transformation

Amplification of the desired cDNA plasmid was carried out using competent cells (DH5α- E. Coli). An aliquot (about 20µl) of E. Coli was defrosted on ice for 30 mins. Plasmid of interest (1 µl) was added to the bacterial cells and incubated on ice for 10 mins. Cells were transformed by heat shock (42°C., 30 sec) and placed on ice for 5 mins. They were then incubated in LB medium for 1hr with slight agitation and finally plated on selective LB- agar plates containing appropriate antibiotics (penicillin 100mg/ml). Such plates were incubated at 37°C overnight for the growth of individual colonies.

2.4.2 Plasmid isolation (mini prep)

Individual colonies developed on agar plates were inoculated in 3 ml LB medium containing appropriate antibiotics for 10- 16 hrs at 37°C, 220rpm. The bacterial pellet was obtained by centrifugation (10 mins, 3000rpm). Plasmid isolation was performed using a Macherey- Nagel NucleoSpin™ plasmid Kit, according to the manufacturer's specifications.

2.4.3 Plasmid linearization and purification

Purified plasmids were linearized using specific restriction enzymes (New England biolabs), according to the orientation of the cDNA fragment and the characteristics of the vector. Plasmid DNA was diluted in dH2O to a concentration of 50ng/µl and the following components were added:

1:10 of 10x Buffer, 1- 5µl/ml of restriction enzyme and 1:100 of 100x BSA (if required). Reactions were normally at 37°C for few hours or even overnight until complete restriction, as verified by gel electrophoresis.

To purify DNA from proteins, an equal volume of Tris- saturated phenol-chloroform/isoamyl alcohol pH8 (Invitrogen) was added to the complete reaction mixture. This mixture was then vortexed gently and centrifuged (10 min, 13000rpm). The upper aqueous phase was transferred to a new tube and 0.1 volume of 3M sodium acetate (pH5.5) was added. After vortexing, DNA was precipitated with 3 volumes of 100% ethanol for 1 hr at -20°C, washed twice in 70%ethanol and resuspended in H2O to a final concentration of 0.1- 1µg/µl.

2.4.4 Synthesis of radioactive riboprobes

In vitro transcription of the linearized cDNA was carried out by incubating at 37°C for 1.5 hrs with the following reagents:

Linearized DNA (>0.25µg/µl)	1- 3µl (0.5- 1µg)
Transcription Buffer 10x (Boehringer)	1µl
-U dNTPs (Boehringer)	1µl
RNase inhibitor (Promega)	0.5µl (1U/µl)
T3/T7/SP6 RNA polymerase (Promega)	0.5µl (0.5U/µl)
[α]35 S- UTP (Amersham)	2µl (10mCi/ml)
DEPC- H2O	up to 10µl

Riboprobes bearing a sequence complementary to the mRNA of interest (antisense) were synthesized using the following cDNA templates:

cDNA	Size (bp)	Vector	Enzyme	Pol	Provider
Cad8	400	pGEM-Teasy	SpeI	T7	Lab stock
Cralbp	310	pGEM-T	SpeI	T7	Lab stock
Cux2	530	pGEM-T	SpeI	T7	Lab stock
Er81	300	pGEM-Teasy	SacII	SP6	Lab stock
Id2	350	pGEM-Teasy	SpeI	T7	Lab stock
Lmo4	600	pGEM	SpeI	T7	Lab stock
Pax6	260	pBluescriptKS+	EcoRI	T3	P. Gruss
Svet1	900	pBluescript	XhoI	T7	P. Gruss

2.4.5 In situ Hybridization

Dewaxing of paraffin embedded tissue (sectioned cortex and whole embryo) was done using histoclear (twice for 10 mins). The tissue was rehydrated in a series of ethanol dilutions (100%, 100%, 95%, 90%, 80%, 70%, 50%, 30%, for 2 mins each) and rinsed in saline (0.86% NaCl in DEPC autoclaved water) and PBS- DEPC. The sections were then fixed in cold 4% PFA/PBS and washed twice in PBS- DEPC for 5 mins. The sections were then Proteinase K treated (50mMTris- HCl; 5mM EDTA; 20µg/ml Proteinase K) followed by a PBS- DEPC wash step. These sections were treated with freshly prepared acetylation buffer (0.1M triethanolamine; 0.05M acetic anhydride in DEPC- H2O), twice for 15 mins and washed in PBS- DEPC followed by dehydrating ethanol wash steps.

Hybridization buffer was used for diluting the radioactive RNA probe:

50%	Deionized Formamide (Fulka)
10%	Hybridization salt stock (0.2% polyvinylpyrrolidone; 0.2% Ficoll; 0.1M NaH2PO4; 50mM EDTA pH 6.8; 3M NaCl; 0.1M Tris- HCl pH8 in DEPC- H_2O.
10%	1M DTT (Sigma/Promega)
20%	Dextran sulfate 50% (Amersham)
500µg/ml	tRNA (Sigma)
200µg/ml	αSPthio- ATP (Roche)

Denaturation of the diluted radiolabelled RNA probes was done at 80% for 2 mins and placed on ice for 5 mins. About 12- 18µl of the diluted probe was applied on each section and covered with 15x20mm coverslips that were previously siliconized with SurfaSil™, according to the manufacturer's instructions. Sections were allowed to hybridize with the probe at 55°C in a completely sealed humid chamber containing 50% formamide in 2xSSC.

Hybridized sections were transferred to 2xSSC at 55°C and coverslips were removed with gentle agitation for about 5- 10 mins. sections were then washed in 50% Formamide/2xSSC at 75°C, then at 65°C (both in a shaking water bath). This wash step was again carried out in a fresh aliquot of the same solution for 30min- 2hr at 37°C with slight agitation. Sections were then incubated twice in NTE buffer (0.5M NaCl; 10mM Tris- HCl; 5mM EDTA pH8) for 5 and 15 mins. The unbound radiolabelled RNA probe

was digested with 20µg/ml RNase A (Boehringer) in NTE buffer at 37°C for 30 mins. Sections were then incubated at 37°C in NTE buffer (shaking for 15 mins) to wash out the RNase. They were washed in preheated 50% Formaminde/2xSSC at 65°C then at 37°C for 30 mins each. To decrease salt concentration, sections were first washed in 2xSSC and then in 0.1xSSC for 15 mins each. They were finally dehydrated with the ethanol wash steps and allowed to dry. A preview of the signal was obtained by exposing the sections to Biomax X- ray film (Kodak) overnight.

Autoradiography with the hybridized sections was performed in complete darkness by embedding the slides in NTB- 2 emulsion (Kodak) pre- warmed at 42°C. Sections were allowed to dry and exposed overnight at RT then transferred to 4°C for 5- 10 days depending on the strength of the signal obtained on the X- ray film. After exposition, the emulsion was developed in total darkness by dipping in cold 16% (w/v) Kodak D- 19 developer solution for 4 mins, washed in 1% (v/v) acetic acid for 1 min and fixed in 30% (w/v) sodium thiosulfate (Sigma) for 3 mins. Slides were finally washed several times in water and counterstained in a solution containing 4% Giemsa stock (0.85% Giemsa; 50% Glycerol; 50% Methanol) and 2% 0.2Msodium phosphate buffer (pH6). The excess of stain was washed out in running tap water. These sections were air- dried and mounted with Eukitt®.

2.5 Chromatin Immunoprecipitation (ChIP) Assay

Mouse embryonic cortex (E14.5 or E 15.5) was used as a tissue source of chromatin. Cortex tissues were homogenized in 1x phosphate-buffered saline (10ml for 10-14 hemispheres of cortex tissue) with protease inhibitors (Roche Applied Science). Proteins were cross-linked in 1% formaldehyde for 10 min at 37 C in a water bath incubator. Cross-linking was terminated with three washes in 1x phosphate-buffered saline. Samples were then processed using a ChIP assay kit, essentially as described by the manufacturer (Upstate biotechnology, Lake Placid, NY). In brief, the cells were lysed in SDS lysis buffer with protease inhibitors, then sonicated using a waterbath sonicator, super RK 103H from Schütt labortechnik (Goettingen, Germany) to shear DNA to fragments with a length of 100–1000 bp. To reduce nonspecific background, the cell lysates were precleared by incubation with salmon sperm DNA/protein A-agarose slurry. The agarose beads were pretreated with 2% BSA before the preclearing step as suggested by the company. Supernatants from the preclearing step were incubated with (1: 500) rabbit anti-Pax6 polyclonal IgG (Covance), at 4 °C overnight. Cortex from Pax6 mutant mice were used as a negative control. Chromatin-antibody complexes were precipitated by incubation with Protein A-agarose beads. Chromatin was eluted from the beads after washes in several buffers provided with the kit. The DNA-protein cross-links in all samples were reversed by incubation for 4 h at 65 °C followed by incubation with proteinase K for 1 h at 45 °C. DNA was isolated by phenol/chloroform extraction and ethanol precipitation. PCR was performed with primers that recognize known and putative Pax6 binding sites (refer to ChIP primers in PCR).

2.6 Immunohistochemistry

Immunohistochemistry was performed on 10μM thick cryosections of E14.5 wild-type and pax6 mutant brains. Freshly isolated brains were fixed in 4% PFA for 2-4 hrs at 4°C, soaked in 30% sucrose overnight and cryopreserved in tissue-tek (Sakura Finetek Zoeterwoude, NL). Immunohistochemistry was carried out with rabbit anti-CRALBP (kindly provided by Prof. John Saari, University of Washington, Seattle; 1:500) and mouse monoclonal Pax6 (Developmental Studies Hybridoma Bank, Iowa; 1:100) antibodies. Sections were blocked in 1% BSA and 0.5% Tween in PBS for 1hr, rinsed in PBS and incubated for 2 hrs with primary antibodies at RT. Primary antibodies were washed in PBS and detected using fluorescent secondary antibody (1:1000) for 1 hr. Sections were rinsed in PBS and visualized using a fluorescent microscope after mounting with Dako.

2.7 Plasmid constructs and luciferase reporter assay

Chinese hamster ovarian (CHO) cells were cultured in neurobasal medium in 10% FCS and 1% Pen/Strep. These cells were plated in a 96 well plate at a density of 30,000 cells /well and 12 hrs later, they were transfected with Pax6 cDNA plasmid [Walther and Gruss, 1991] and luciferase cDNA plasmid under the CRALBP promoter as previously described [Intres et al., 1994; Kennedy et al., 1998]. Lipofectamin was used to increase the efficiency of transfection. 24 hrs after transfection, cells were washed twice in cold PBS and lysed using lysis buffer. The firefly and *Renilla* luciferase activities were measured using a dual luciferase assay system kit (Promega UK) and a Microlumat Plus

LB 96V luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbag, Germany).

2.8 Histology

Freshly isolated brains were dehydrated with a series of ethanol wash steps (30%, 50%, 70%, 80%, 90%, 95% and 100%) for atleast 2 hrs each, transferred to toluol for 6 hrs, soaked in fresh paraplast, twice, overnight and then embedded.

2.9 Image acquisition

Bright and dark field images were obtained with a light microscope (Olympus). To obtain fluorescent images, a Zeiss Confocal microscope was used (Zeiss LSM 510 META). Images obtained were edited using Adobe Photoshop $CS2^{+}$.

3. Aim of the project

We investigated the regulatory role of transcription factor Pax6 in cortical development. It is well known that Pax6 regulates cell proliferation, differentiation and migration in various tissues during development. In this study, we focus on CRALBP and AP2γ, two genes that were downregulated in Pax6 mutants as seen through microarray based comparison of gene expression.

CRALBP is a carrier of 11-*cis* retinol, a derivative of vitamin A. Knowing the importance of vitamin A in neuronal development; we wanted to investigate if Pax6 mediates some of its functions through CRALBP. We wanted to study the expression of CRALBP in various tissues and analyzed the cortical phenotype in CRALBP mutants.

Loss of Pax6 has been show to abolish, downregulate and misexpress many cortical markers. We wanted to conduct expression analysis in AP2γ mutants and investigate if Pax6 regulates some of its target genes through AP2γ. Transcription factor AP2γ is known to play roles in proliferation and repress differentiation. We wanted to study possible alterations in proliferation domains and lamination problems in AP2γ mutants.

4. Results

To identify potential target genes activated by the transcription factor Pax6, we performed a microarray analysis with E 14.5 wt and Pax6 mutant cortex and found several genes that were downregulated in Pax6 mutants (data not shown). Two of the genes that were downregulated in Pax6 mutants encode for AP2γ and CRALBP proteins. CRALBP is a carrier protein involved in the transport of 11- cis retinal and has a role in the visual cycle. AP2γ belongs to AP2 family of transcription factors that play important roles in development. In wt cortex, during embryonic development, both genes were expressed in the VZ; this proliferative layer of the cortex also shows strong Pax6 expression.

4.1 Analysis of CRALBP function in the cortex

We investigated the role of CRALBP, a 11- cis retinal binding protein, in cortical development. CRALBP was very strongly downregulated in Pax6 mutants as seen through microarray analysis. CRALBP is known to play an important role as a carrier protein for 11- cis retinal in the visual cycle of the eye [Saari et al., 2001]. The expression of CRALBP in the cortex at earlier stages of development indicated that it could have a role in coticogenesis. This motivated us to investigate the role of CRALBP in the cortex. Since, Pax6 is a pleiotropic player in development it could mediate some of its functions through CRALBP. We wanted to find out whether *CRALBP* is a direct downstream target of Pax6.

4.1.1 Pax6 and CRALBP are coexpressed in several regions of the CNS

To study mRNA expression of CRALBP in wt and Pax6 mutants we performed *in situ* hybridization with radiolabelled antisense-RNA probes. The expression of CRALBP during embryonic stages (E14.5) in wt brain was almost identical to the expression of Pax6. CRALBP was expressed in the neocortical VZ, cortical hem and in the hypothalamus (Fig: 4c). In Pax6 mutant cortex the expression of CRALBP was abolished in all these regions (Fig: 4d). Since Pax6 is also expressed in the spinal cord, we investigated the expression of CRALBP in the spinal cord using *in situ* hybridization. In wt spinal cord, Pax6 and CRALBP are expressed in the VZ (Fig: 5b and c) of the spinal cord. No expression of CRALBP was detected in the spinal cord in Pax6 mutants (Fig: 5d).

Next, we addressed the question of whether Pax6 and CRALBP are expressed in the same cells. To study protein expression, a double immunohistochemistry (IHC) was performed on E14.5 wt and Pax6 mutant cryosections. In wt brain, CRALBP was expressed in all Pax6 expressing regions. These regions included cortical hem, pallial-sub-pallial boundary (PSPB) of the cortex (Fig: 6) and regions around the third ventricle in the thalamus (Fig: 7). In all these regions CRALBP showed sharp boundaries of expression which coincided with those of Pax6. We did not find any CRALBP expressing regions that lack Pax6 expression. However, in the neocortex as opposed to Pax6, expression of CRALBP was not only detected in the ventricular zone but also in the cortical plate (Fig: 6e). In Pax6 mutant brains, we did not find any expression of CRALBP protein (data not shown). The complete abolishment of CRALBP expression

(mRNA and protein) in Pax6 mutants suggested that *CRALBP* could be a direct downstream target of Pax6.

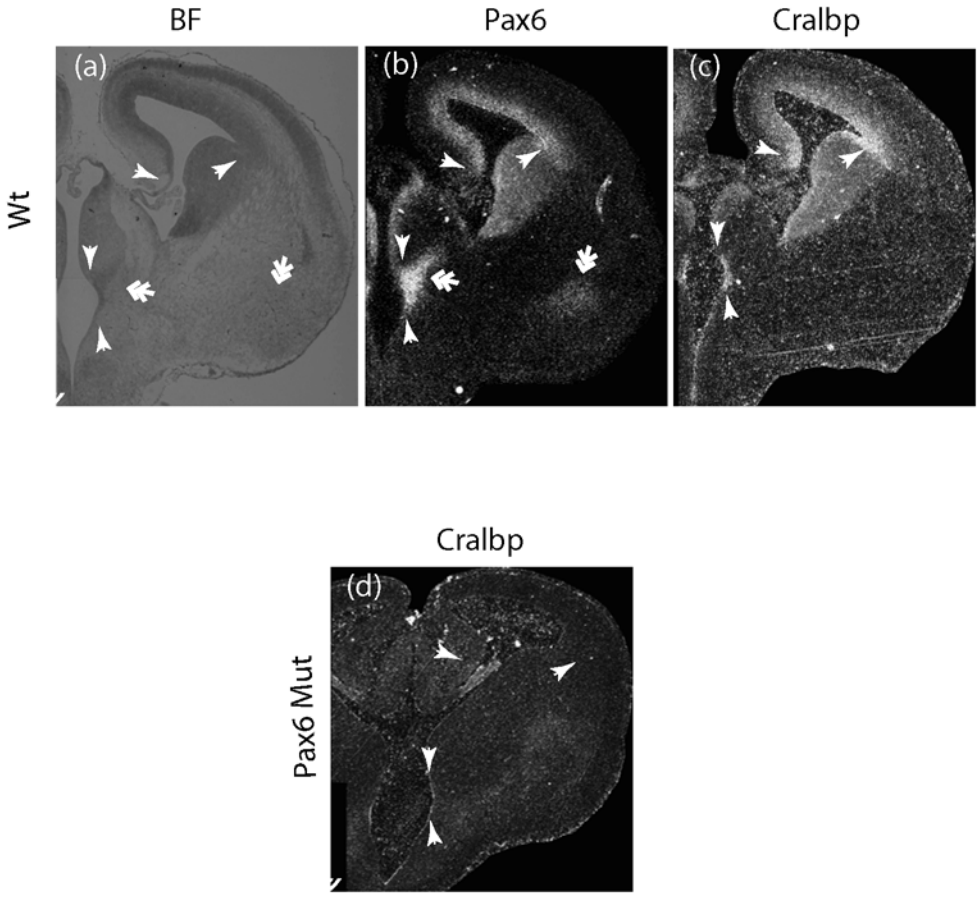

Fig 4: *In situ* hybridization with Pax6 and CRALBP probes in Wt and Pax6 mutant cortex. Pax6 and CRALBP are expressed in the cortical hem, neocortical VZ, PSPB, and around the third ventricle as shown by single arrowheads (b and c). Pax6, and not CRALBP, is also expressed in some postmitotic areas as shown by double arrow heads (b). In Pax6 mutants, expression of CRALBP is completely abolished (d).

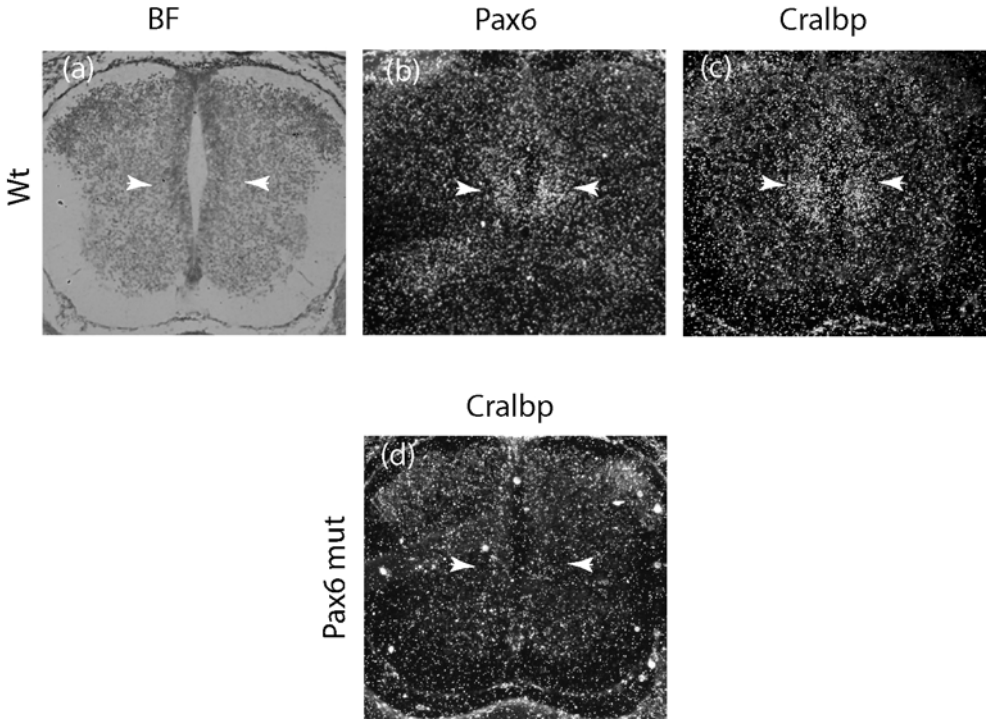

Fig 5: *In situ* hybridization with Pax6 and CRALBP probes in the spinal cord of Wt and Pax6 mutant. Pax6 and CRALBP are expressed in the VZ of spinal cord in wt (b and c) as indicated by arrowheads. In Pax6 mutants, expression of CRALBP is completely abolished (d). The corresponding bright field image is also shown (a).

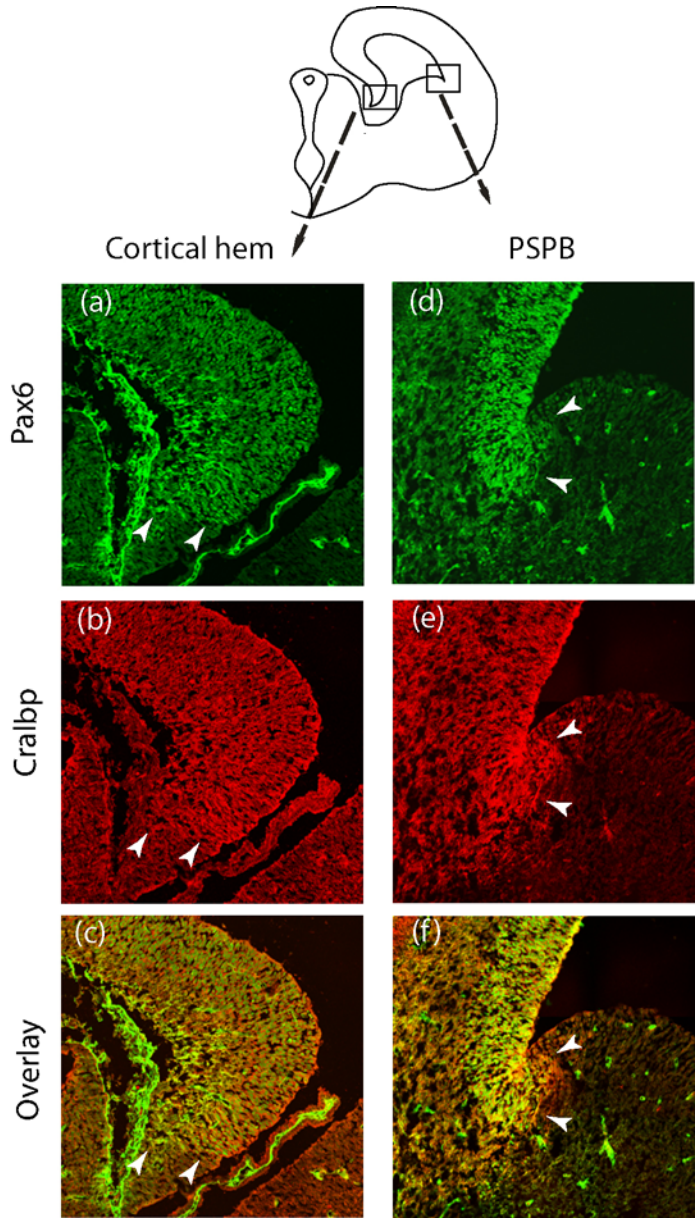

Fig 6: Comparison of Pax6 and CRALBP expression in the cortex by Immunohistochemistry. Pax6 and CRALBP share similar borders of expression in the cortical hem (a, b and c) and the pallial- subpallial boundary (PSPB; d, e and f). Both proteins have common expression domains (as indicated by arrows).

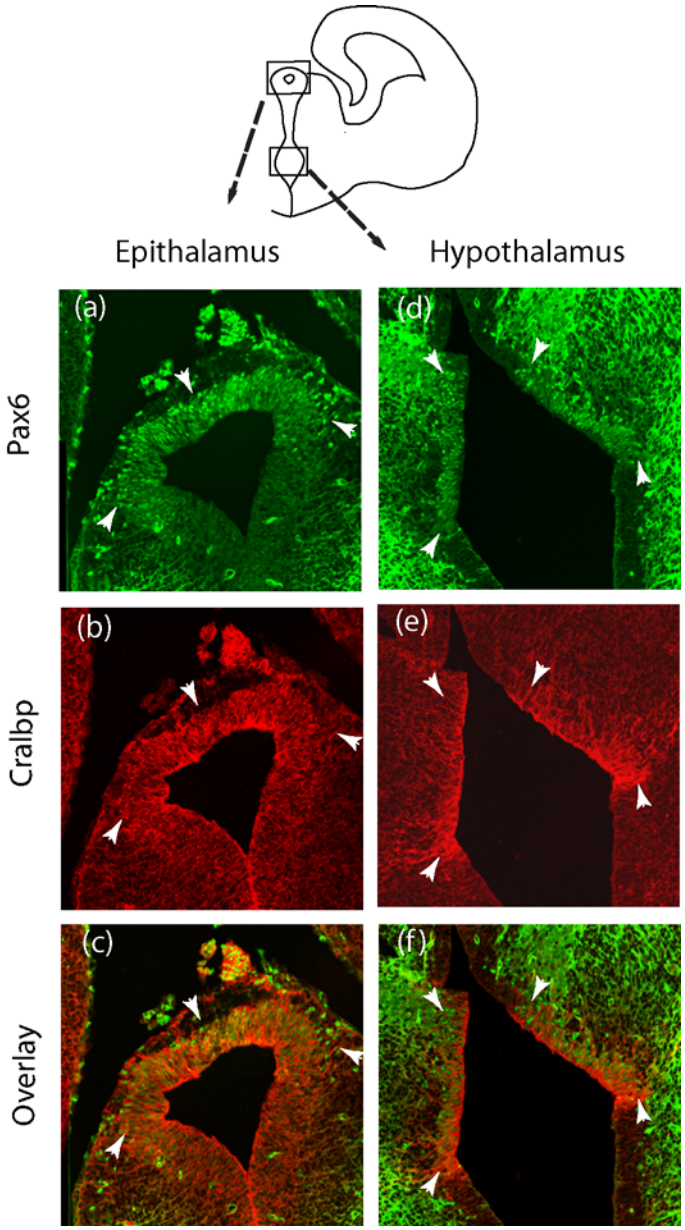

Fig 7: Comparison of Pax6 and CRALBP expression in the thalamus by Immunohistochemistry. Pax6 and CRALBP share similar boundaries of expression around the third ventricle in the epithalamus (a, b and c) and the hypothalamus (d, e and f). Both proteins have common expression domains (as indicated by arrows).

4.1.2 Mutually exclusive expression domains of Pax6 and CRALBP in the eye

Pax6 is a key regulator of vertebrate eye development and upon misexpression is sufficient to induce ectopic eyes [Chow et al., 1999]. Pax6 mutants lack entire eye structure. These findings led to the proposal that Pax6 could be an 'eye selector gene'. CRALBP also plays an important role as a carrier protein in the eye. Since both proteins are known to play important roles in the eye, we wanted to investigate the expression patterns of Pax6 and CRALBP through IHC. Our experiments have shown that Pax6 is expressed in the neuroretina (NR) whereas CRALBP is expressed in the retinal pigment epithelium (RPE) that surrounds the neuroretina (Fig: 8a and b). Our results indicate that Pax6 and CRALBP are not co-localized in the eye (Fig: 8c). We could not investigate the expression of CRALBP in Pax6 mutants since Pax6 mutants are devoid of eyes [Hill et al., 1991].

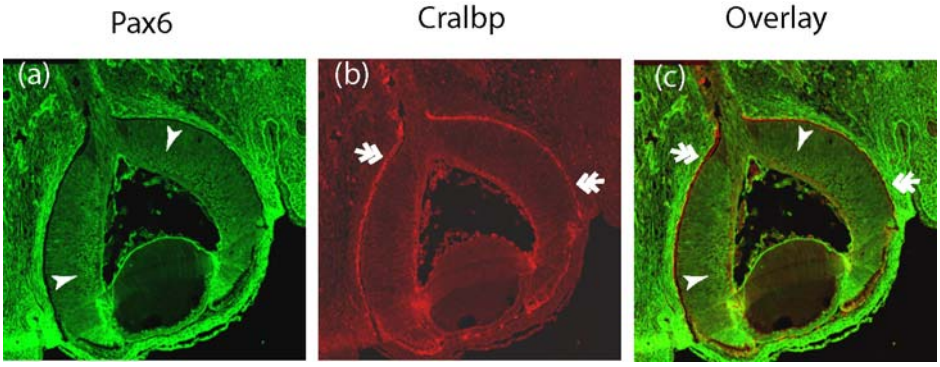

Fig 8: Comparison of Pax6 and CRALBP expression in the eye. Pax6 and CRALBP are expressed in different regions of the eye. Pax6 is expressed in the neuroretina (a) and CRALBP is expressed in the retinal pigment epithelium (b). They do not share overlapping domains of expression (c).

4.1.3 Chromatin immunoprecipitation assay (ChIP) reveals Pax6 binding sites on CRALBP promoter.

Our expression data from *in situ* hybridization and IHC (Fig: 4, 5, 6 and 7) suggested that Pax6 directly regulates the expression of CRALBP. Since putative Pax6 binding sites are known [Epstein et al., 1994a; Morgan, 2004; Sander et al., 1997], we inspected the 5' upstream region of exon1 of the mouse *CRALBP* gene. We were able to identify 6 putative Pax6 binding sites within 6 kb upstream. The consensus sequence "CACGTG" [Morgan, 2004] was located at 5.7 kb, 4.6 kb and 2 kb upstream (Fig: 9a), and the consensus sequence "GGTGGAA" [Sander et al., 1997] was located at 3.4 kb, 2.9 kb and 2 kb (Fig: 9a) upstream of CRALBP exon1. In order to perform Chromatin immunoprecipitation assay (ChIP), we chose a time point during development where Pax6 is known to be highly active. By E14.5, expression of Pax6 can be detected throughout the anterior- posterior axis of the dorsal telencephalon in the ventricular zone and the subventricular zone [Warren et al., 1999]. DNA-protein complexes isolated from E14.5 cortex were precipitated with Pax6 antibody and amplified using primers against Pax6 binding sites on *CRALBP* promoter. Pax6 seems to bind two putative sites on *CRALBP* promoter, at 2kb and 2.9kb upstream of exon1 (Fig. 9c). Pax6 also had a weak affinity for a putative site at 3.4 kb upstream of *CRALBP*, but this could not be reproduced. In addition to the mentioned Pax6 putative binding sites we were able to locate few other binding sites with one or two nucleotide mismatches which we have not investigated further. This experiment was also repeated by precipitating DNA-protein complexes using Pax6 antibody from Pax6 mutant cortex tissue. As predicted, we did not

amplify any DNA in the PCR reaction (data not shown). Our *in vivo* results indicate that Pax6 binds the putative promoter region of *CRALBP*.

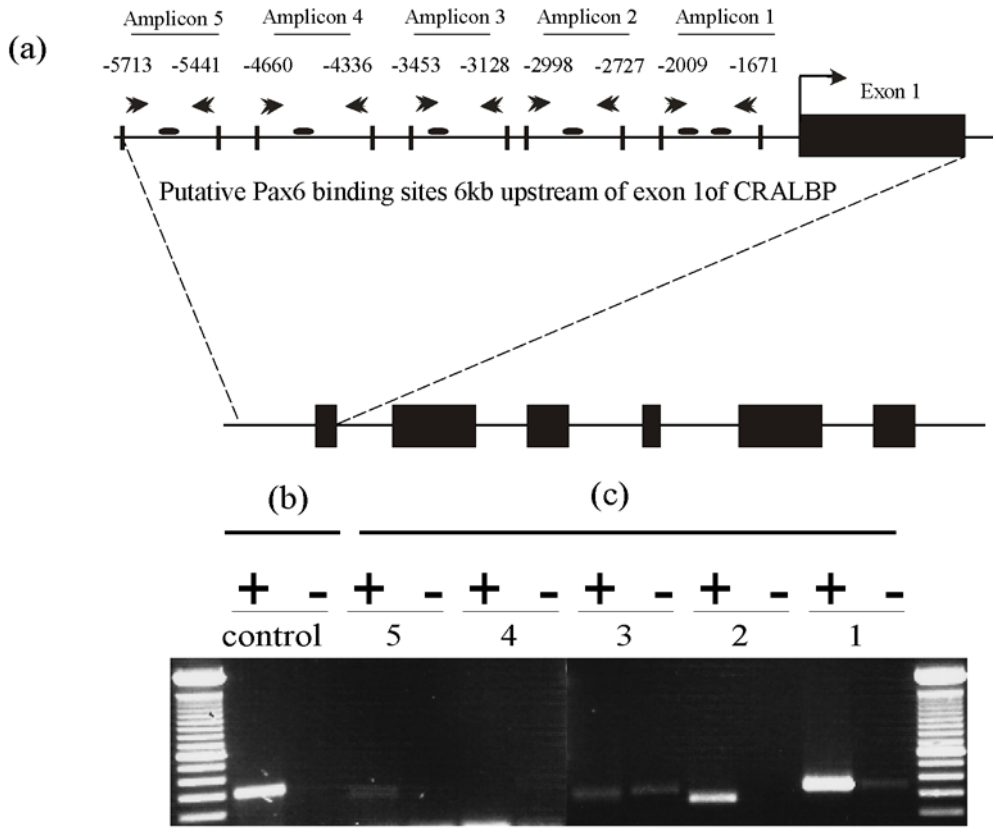

Fig 9: Identification of putative Pax6 binding sites using Chromatin Immunoprecipitation (ChIP) assay. Six putative Pax6 binding sites and the corresponding primers on *CRALBP* promoter are indicated (a). A known Pax6 binding site on its own promoter was used as a positive control for the PCR (b). PCR amplification for the putative Pax6 binding sites are shown (c). DNA-protein complexes precipitated with Pax6 Ab added (+) and without Ab (-) are indicated. Two Pax6 binding sites were identified.

4.1.4 Pax6 activates CRALBP promoter *in vitro*

To evaluate whether binding of Pax6 on *CRALBP* promoter induces expression of CRALBP, a luciferase-reporter assay was performed. CHO cells were transiently cotransfected with Pax6 cDNA plasmids [Walther and Gruss, 1991] and with plasmids that contain luciferase cDNA under the CRALBP promoter [Kennedy et al., 1998]. Co-transfected cells were harvested 16hrs after transfection and luciferase activity was measured. The reporter activity was significantly increased with increasing concentrations of Pax6 cDNA plasmid, whereas the control G5-luciferase reporter activity showed only a basal level of expression (Fig: 10). This *in vitro* experiment shows that Pax6 can positively regulate the activity of *CRALBP* promoter.

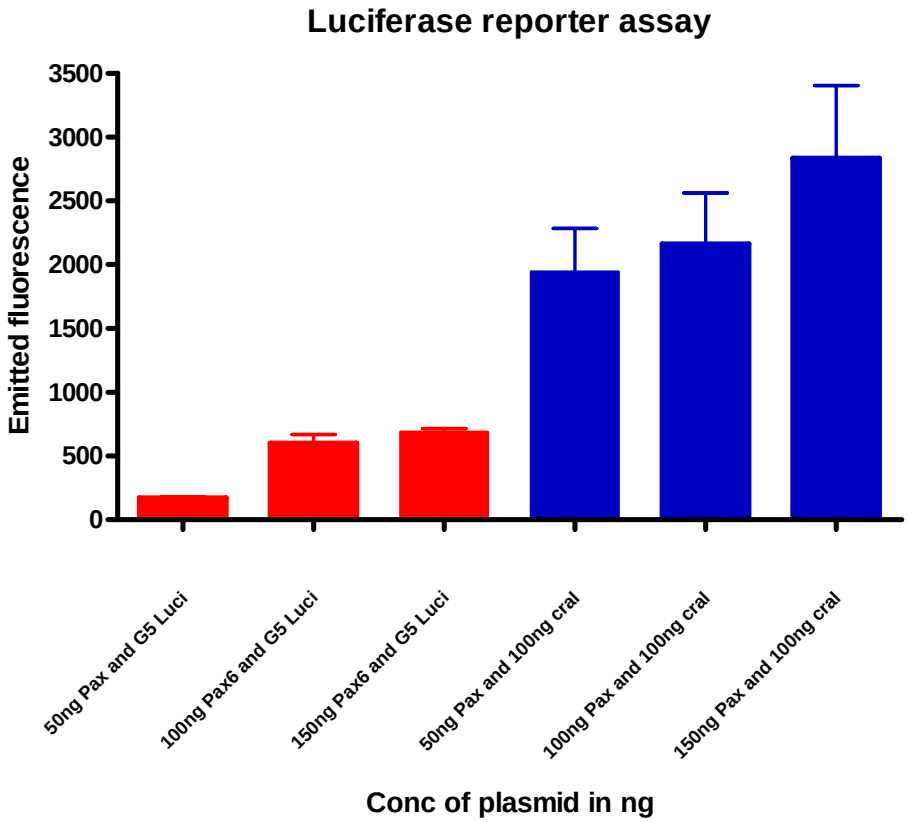

Fig 10: Co-transfection of CHO cells with Pax6 and CRALBP plasmids. Reporter activity of luciferase cDNA under the CRALBP promoter was measured in cells by transfecting them along with Pax6 cDNA plasmid. Increasing concentrations of the latter increases CRALBP-luciferase reporter activity (blue bars). The controls do not show significant increase in activity with increasing concentrations of Pax6 cDNA plasmid (red bars).

4.1.5 Expression of layer-specific markers indicates no lamination defects in CRALBP mutants

Vitamin A (retinol) and its metabolites (retinaldehyde and retinoic acid) are crucial for development [Goodman, 1984]. Retinoic acid has been shown to induce differentiation of neurons and glia [Tibbles and Wiley, 1988]. Since CRALBP is a carrier of 11-*cis*-retinol, this could imply that CRALBP might also be involved in similar functions. Hence, we

expected to find possible lamination problems in CRALBP mutant cortex. For this purpose we looked at the expression of layer specific markers like Satb2 and Ctip2.

Satb2 is a transcription factor mostly expressed in upper layer neurons of the neocortex starting from E13.5 [Britanova et al., 2005]. It regulates gene expression by binding to matrix attachment DNA regions and thus influences chromatin structure [Britanova et al., 2005].

A high majority of $Satb2^{+}$ neurons are born between E14.5 and E15.5 in the dorsal telencephalon [Britanova et al., 2006]. At P0 most cells of layers 2-4 express Satb2. Ctip2 is expressed in almost all cortical plate neurons during early stages of cortical development. At later stages, its expression is confined to type I pyramidal neurons of layer 5 [Arlotta et al., 2005].

At E15.5, in both wt and CRALBP mutants, we detected expression of Satb2 and Ctip2 in some migrating neurons and a subpopulation of post migratory neurons in the cortical plate (Fig: 11a, b; d, e). We found atleast 2 populations of neurons- while the majority of cortical plate neurons express only Ctip2, a minority of cells express both Satb2 and Ctip2. We did not observe any difference in the pattern of expression of either marker (Fig: 11c and f) in wt and mutants.

At E17.5, we continued to observe expression of Satb2 and Ctip2 in different subpopulations of migrating neurons (Fig: 11g, j; h, k). In the cortical plate, differentiated neurons occupying layers 2 to 4 express only Satb2 (Fig: 11g, j), while those of layer 5

express either Ctip2 or both Ctip2 and Satb2 (Fig: 11i, l). Weak expression of Ctip2 is also seen in some cells of layer 6 (Fig: 11h, k). In CRALBP mutants, the relative proportions of these neuronal types does not seem to be altered (Fig: 11l).

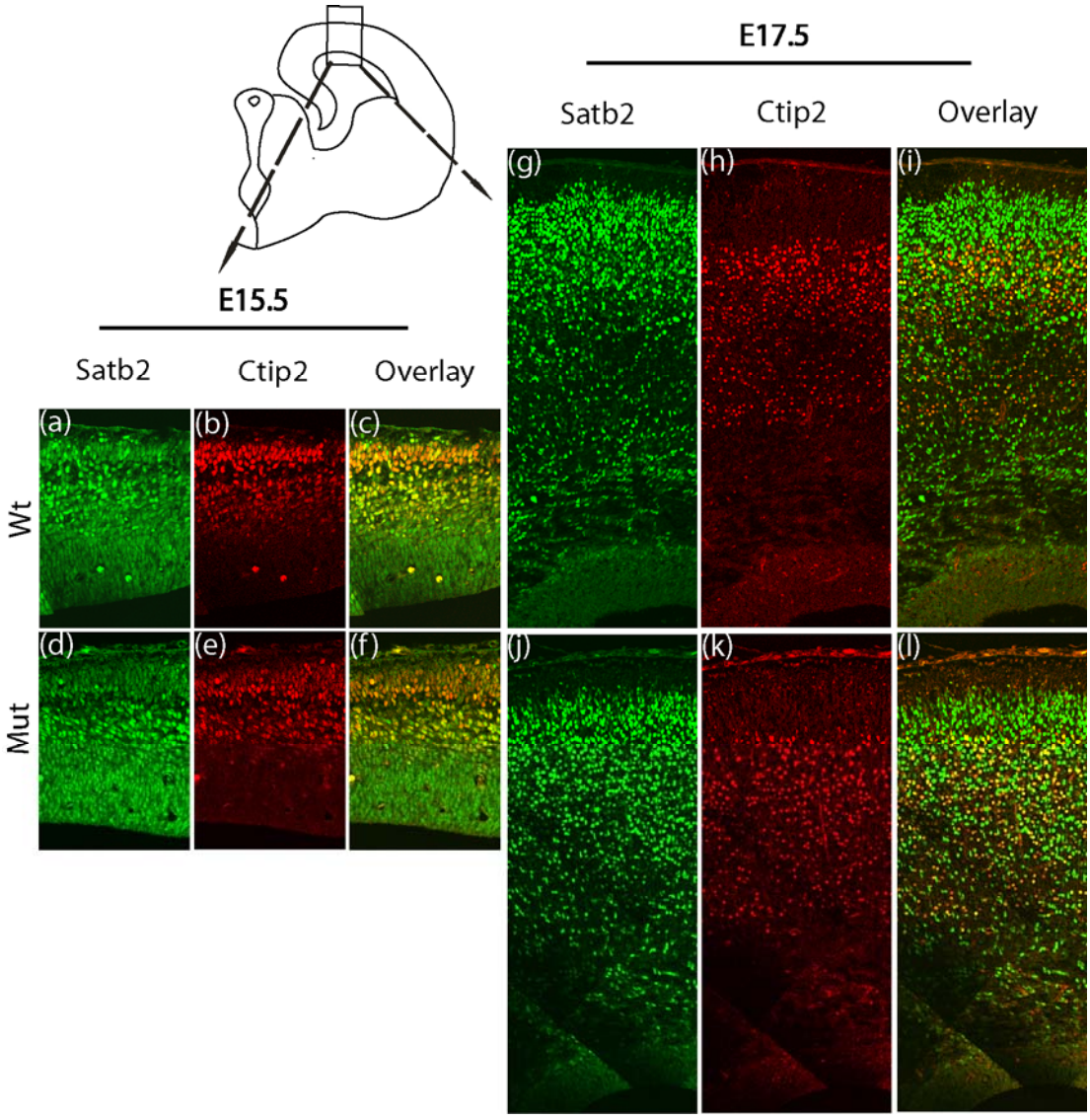

Fig 11: Lamination in wt and CRALBP mutants at E15.5 and E17.5. To determine possible defects in lamination, immunostaining for Satb2 and Ctip2 was done. No differences were observed between wt (a, b, c; g, h and i) and mutant sections (d, e, f; j, k and l).

4.1.6 BLBP expressing radial glial processes show a 'wavy' morphology in mutants

We then wanted to elucidate the function of CRALBP in neocortical VZ, where it was found to be expressed as early as E14.5. For this, we stained radial glial cells with specific markers such as BLBP and Nestin. BLBP is a lipid-binding protein, expressed in radial glial processes early during development. It is known to have a role in neuronal differentiation. Loss of BLBP results in rounding of neuroepithelial cells [Arai et al., 2005]. Nestin is an intermediate filament known to be expressed in all cortical VZ progenitors. At both E15.5 and E17.5, we detected expression of BLBP and Nestin throughout the radial glial processes, extending from ventricular to pial surface (Fig: 12, 13 and 14). We did not find any differences in BLBP staining between wt and mutant at E15.5 (Fig: 12). However, at E17.5, staining with BLBP showed that the radial glial processes possess a wavy morphology in the mutants (Fig: 13). Staining with Nestin failed to recapitulate the same phenotype. (Fig: 14).

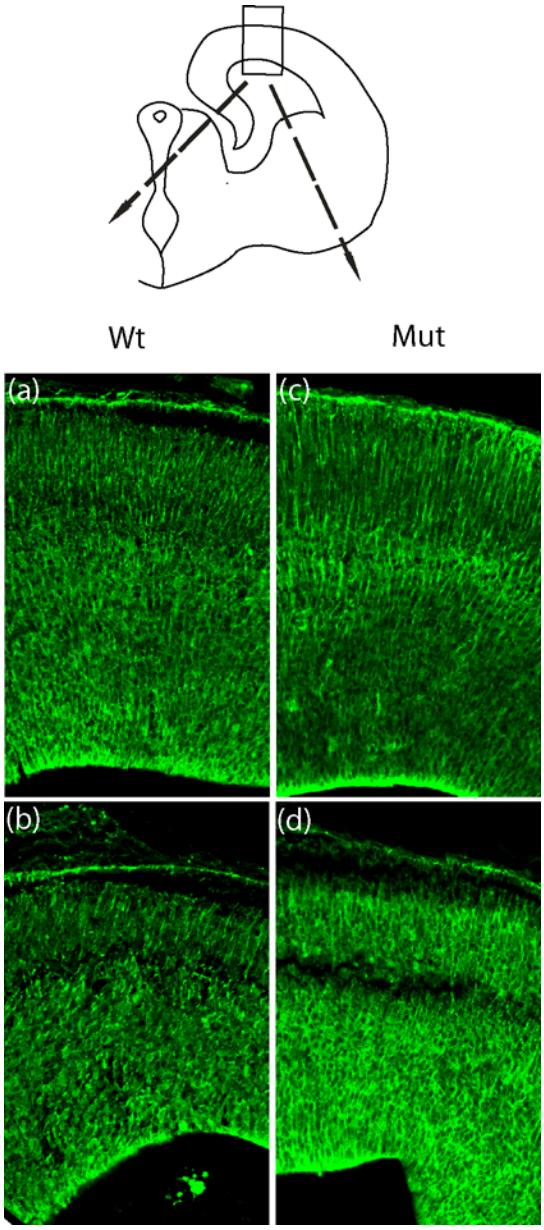

Fig 12: Expression of BLBP in wt and CRALBP mutants at E15.5. Immunostaining for BLBP indicated no difference in expression in mutant cortex (c and d) as compared to wt (a and b).

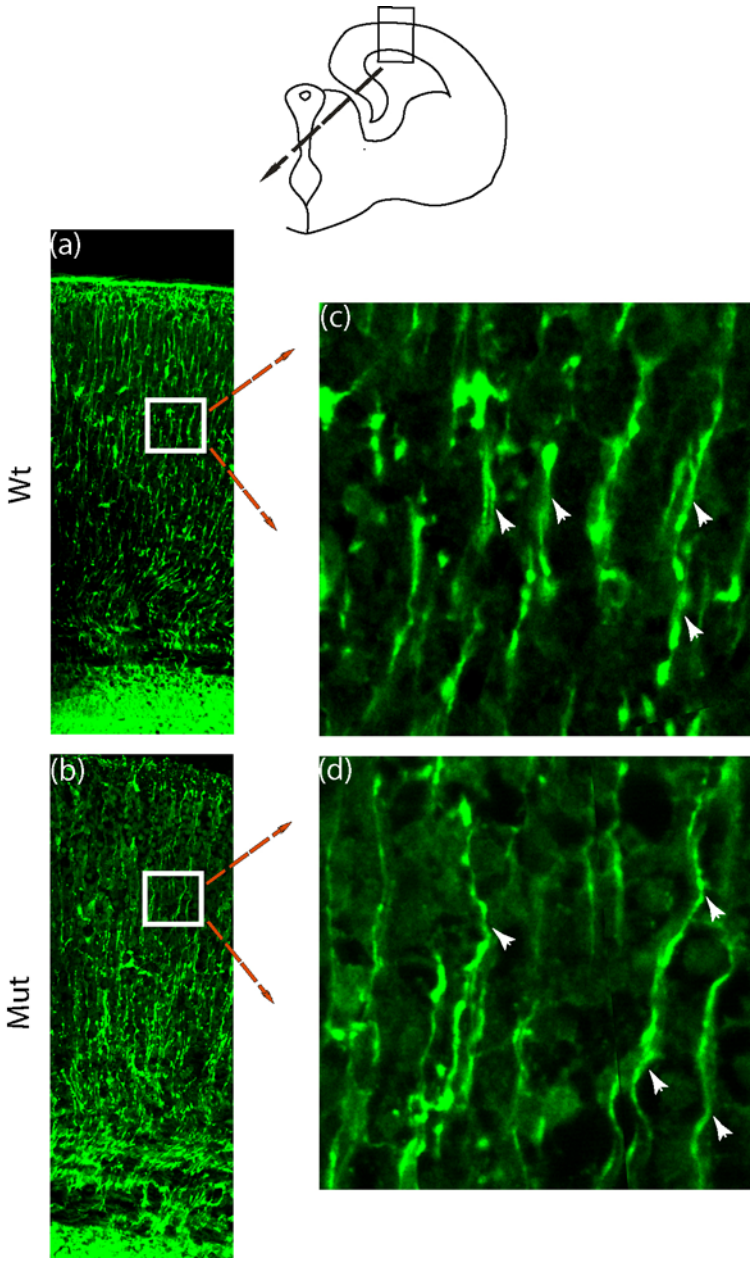

Fig 13: Expression of BLBP in wt and CRALBP mutants at E17.5. Immunostaining for BLBP indicated a "wavy" morphology of radial glial processes in mutant cortex (b; magnified in d) as compared to wt (a; magnified in c).

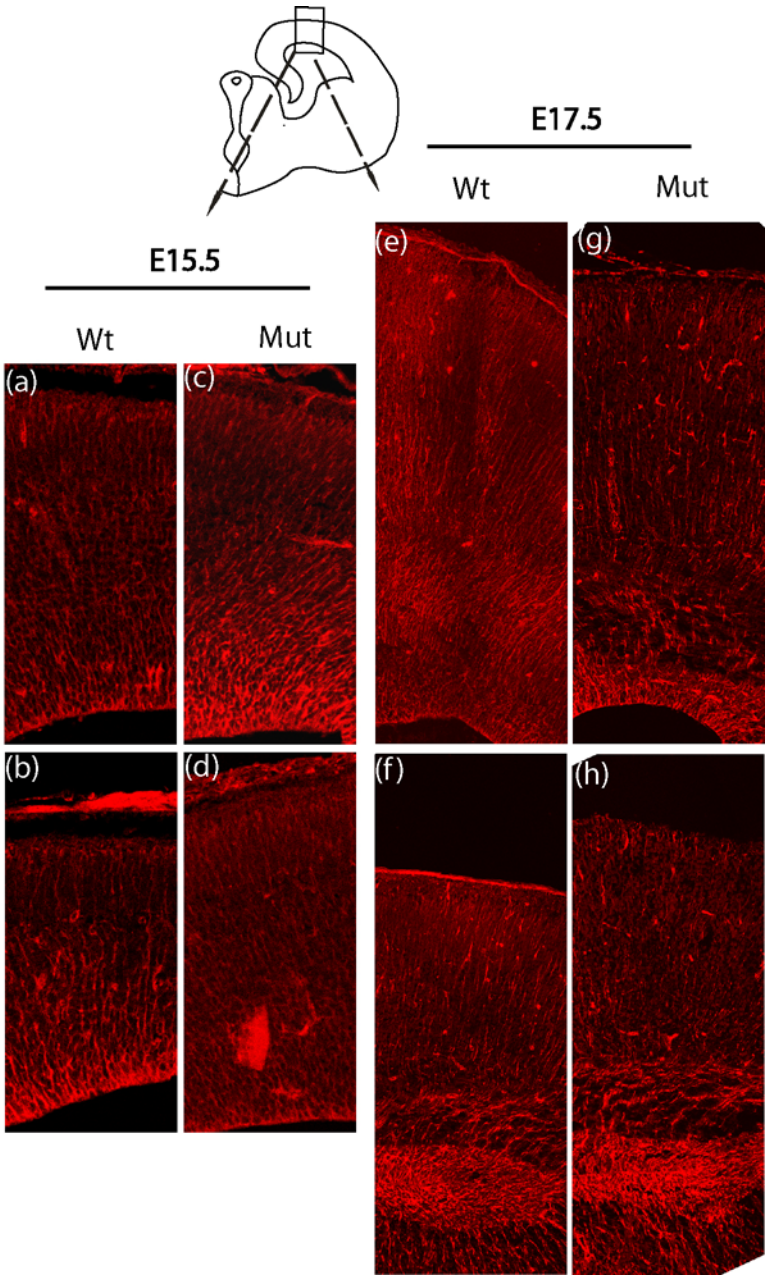

Fig 14: Expression of Nestin in wt and CRALBP mutants. Immunostaining for Nestin at E15.5 and E17.5 indicated no difference in expression in mutant cortex (c, d; g and h) as compared to wt (a, b; e and f).

4.2 Analysis of AP2γ function in the cortex

The expression of AP2γ in Pax6 mutants was downregulated, although not completely abolished. Since Pax6 is known to regulate the expression of many genes during development [Simpson and Price, 2002], we wanted to find out if some effects seen in Pax6 mutants are recapitulated in AP2γ knock out mice. To analyze the expression of some transcription factors (Cux2, Er-81, Id2, LMO4), cell adhesion molecules (Cad8) and the newly described SVZ marker Svet1 [Tarabykin et al., 2001], we performed *in situ* hybridization. Most of the markers that we selected for *in situ* analysis were known to be missing, misexpressed, or downregulated in Pax6 mutants.

4.2.1 Analysis of Cad8 and Id2 expression indicates a shift in Id2 expression boundary

Cadherin8 (Cad8) is a cell-cell adhesion molecule that shows a distinct pattern of expression across the embryonic neocortex. It is used as a marker of positional identity to study arealization [Bishop et al., 2002]. The caudal boundary of Cad8 expression corresponds to the boundary between motor and somatosensory areas [Suzuki et al., 1997]. In Pax6 mutants, the superficial rostral expression domain of Cad8 is absent.

In wt mice, Id2 is expressed in layer 5 strongly in the somatosensory cortex and weakly in the motor cortex. This change in expression level takes place abruptly at the boundary between these two areas [Rubenstein et al., 1999]. This boundary between strong and weak layer 5 expression is shifted rostrally in Pax6 mutants. In addition to layer 5, Id2 is strongly expressed in layers 2 and 3 of rostral neocortex, the expression level declines

towards the intermediate area (somatosensory cortex) and finally becomes undetectable towards the caudal neocortex. In Pax6 mutants, this domain is absent.

In AP2γ mutants, we observed expression of Cad8 in layers 2-5 of rostral neocortex and layer 5 of caudal neocortex (Fig: 15c). This pattern of expression was similar to the expression in the wt (Fig: 15b) as previously reported [Nakagawa et al., 1999]. The expression of Id2 in layers 2 and 3 did not change in AP2γ mutants, but a moderate rostral shift of expression in layer 5 was observed (Fig: 15f).

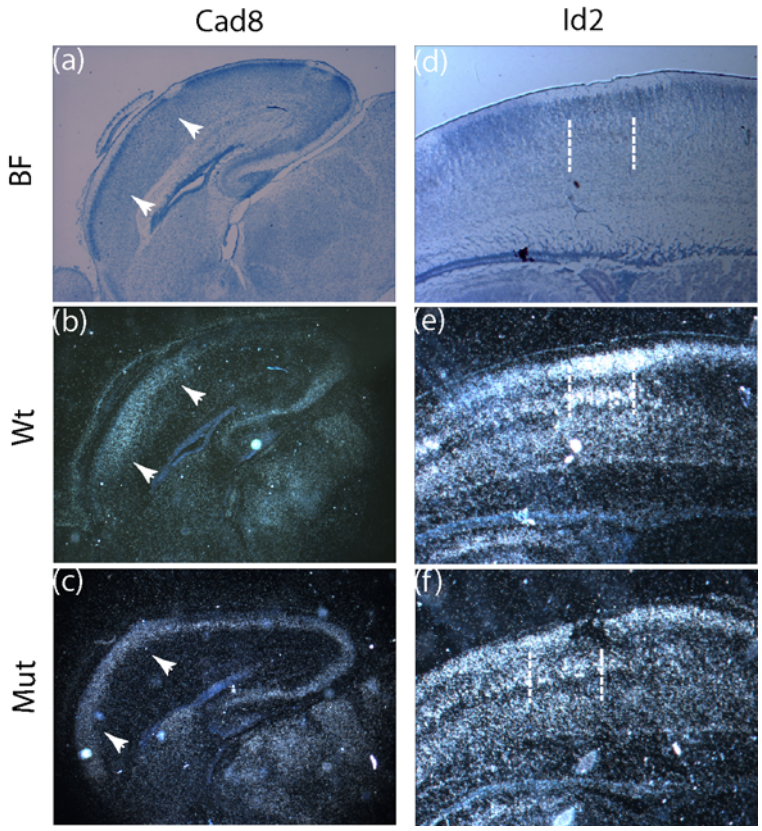

Fig 15: *In situ* hybridization with Cad8 and Id2 probes in AP2γ mutant. The expression of Cad8 (a-c; arrowheads) and Id2 (d-f; dotted lines) in sagittal sections of wt (b and e) and mutant (c and f) P2 cortex is indicated. Borders of Id2 expression is indicated by dotted lines. A rostral shift in Id2 expression can be seen (f). The corresponding bright field images are also shown (a and d). Expression of Cad8 and Id2 are shown at 2.5x and 5x magnification respectively.

4.2.2 Expression of Cux2 and Svet1 does not change in the AP2γ mutants

Cux2 belongs to the family of Cut transcription factors that contain several DNA binding domains. The expression of Cux2 is restricted to the nervous system [Quaggin et al., 1996]. In early embryonic stages Cux2 labels SVZ cells, in adult cortex it is almost exclusively confined to layers 2, 3 and 4 [Nepveu, 2001]. In Pax6 mutant mice Cux2 positive cell number in the SVZ and the upper layers is depleted.

Svet1 (Subventricular tag1) is an embryonic marker of SVZ and specifically marks a subpopulation of cells of the upper layers at postnatal stages [Tarabykin et al., 2001]. In the Pax6/small eye mutant, Svet1 expression was abolished in the SVZ and in the upper cortical layers (layers 2-4) at postnatal stages. The abnormal SVZ cells that lack Svet1 expression do not migrate to the cortical plate; as a result, the SVZ gets overpopulated with cells and expands.

At P2, expression of Cux2 was observed in upper layers of the lateral neocortex but not the medial cortex (Fig: 16b, c). On the other hand, Svet1 was found only in the upper layers of the somatosensory cortex. No difference in expression pattern was found between wt and mutants (Fig: 16e, f).

4.2.3 Expression of Er81 remains unchanged; expression of LMO4 is decreased

ER81 belongs to the ETS family of transcription factors [de Launoit et al., 1997] and is expressed in layer 5 pyramidal cells of wt neocortex [Gray et al., 2004; Hevner et al., 2003]. LIM domain only 4 (LMO4) belongs to LMO family of transcription factors that

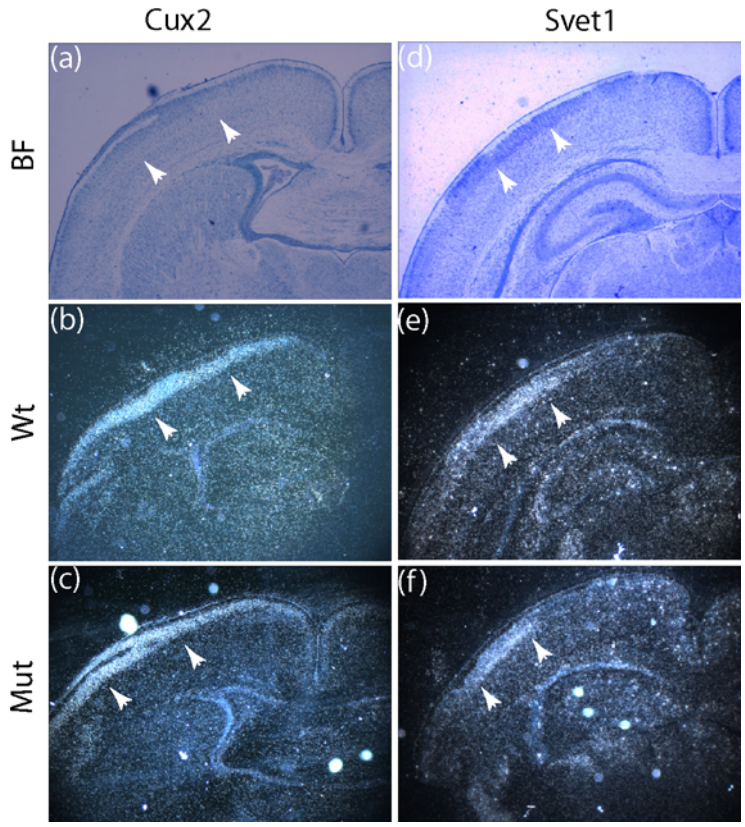

Fig 16: *In situ* hybridization with Cux2 and Svet1 probes in AP2γ mutant. The expression of Cux2 and Svet1 in frontal sections of wt (b and e) and mutant (c and f) P2 cortex is indicated by arrowheads. No changes in expression can be seen. The corresponding bright field images are also shown (a and d). Expression of Cux2 and Svet1 are shown at 2.5x magnification.

play pivotal roles in proliferation and differentiation of blood cells [Boehm et al., 1991]. Lmo4 is expressed in type II layer 5 pyramidal neurons [Arlotta et al., 2005]. It is also expressed throughout layers 2 and 3 of the posterior cortex [Bulchand et al., 2003]. At P2, we found expression of Er81 in layer 5 throughout the neocortex, extending from medial to piriform cortex. A similar pattern of expression was found in both wt and

mutant (Fig: 17b, c). LMO4 was expressed only in layer 5 neurons of the medial cortex. In mutants a lower intensity of expression was observed within the same domain (Fig: 17f).

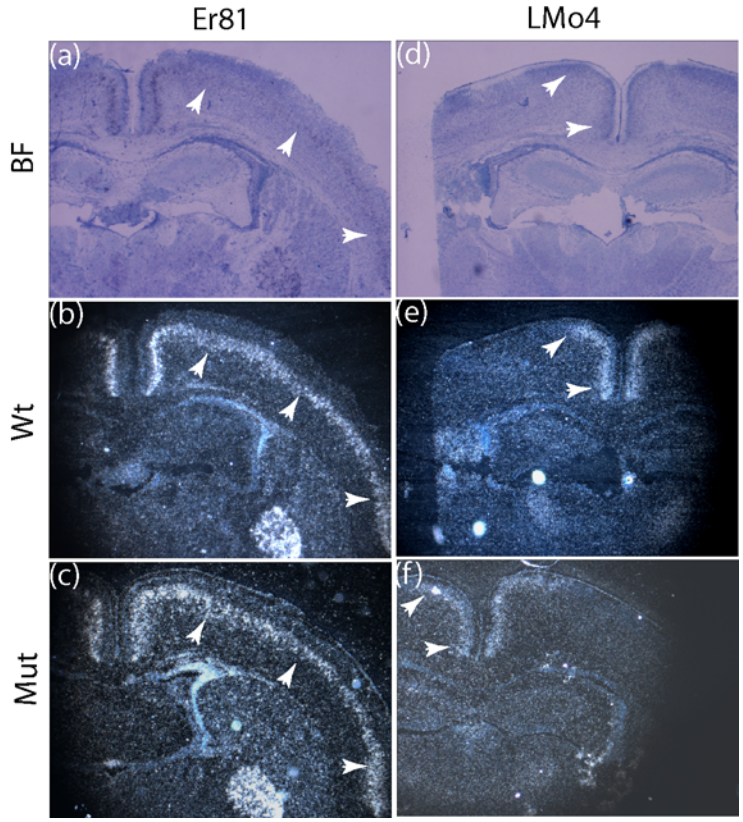

Fig 17: *In situ* hybridization with Er81 and LMO4 probes in AP2γ mutant. The expression of Er81 and LMO4 in frontal sections of wt (b and e) and mutant (c and f) P2 cortex is indicated by arrowheads. A decrease in LMO4 expression can be seen. The corresponding bright field images are also shown (a and d). Expression of Er81 and LMO4 are shown at 2.5x magnification.

4.2.4 Lamination and proliferation in AP2γ mutants is not affected

Since AP2γ is known to repress differentiation [Jager et al., 2003], we wanted to study possible lamination problems in AP2γ mutants. For this, we performed immunohistochemistry with layer specific markers, Satb2 and Ctip2. At E17.5 and P2, we observed expression of Satb2 and Ctip2 in migrating neurons as well as differentiated neurons of layers 2 to 4 and layer 5, respectively. In AP2γ mutants, we did not observe any difference in the pattern of expression of either marker (Fig: 18).

Since AP2γ is known to influence proliferation in breast carcinoma, we wanted to see if it performs a similar role in the cortex. For this, Immunohistochemistry was performed to detect expression of the proliferation marker Ki67 at E17.5. As expected, dividing cells were found in the ventricular zone, subventricular zone, and intermediate zone in wt. This domain of Ki67 expression was unaltered in mutants (Fig: 19).

4.2.5 Pax6 interacts directly with AP2γ promoter

The downregulation of AP2γ in Pax6 mutants prompted us to investigate if Pax6 regulates the expression of *AP2γ* by binding to its putative promoter region. Using E14.5 cortex tissue as the source of DNA, a ChIP assay was performed to see if Pax6 binds to its putative binding sites [Epstein et al., 1994a; Morgan, 2004; Sander et al., 1997] on the promoter of AP2γ. DNA-protein complexes were precipitated with Pax6 antibody. Primers that amplify putative Pax6 binding sites on AP2γ promoter were used to amplify DNA precipitated with and without Pax6 Ab (Fig: 20a). Primers were also designed for a known Pax6 binding site on Pax6 promoter [Morgan, 2004] and used as a positive control

(Fig: 20c). Our results show that Pax6 recognizes two putative sites at 6.8 kb and 3.1 kb upstream of exon1 of AP2γ (Fig: 20b). Thus, AP2γ seems to be a direct downstream target of Pax6.

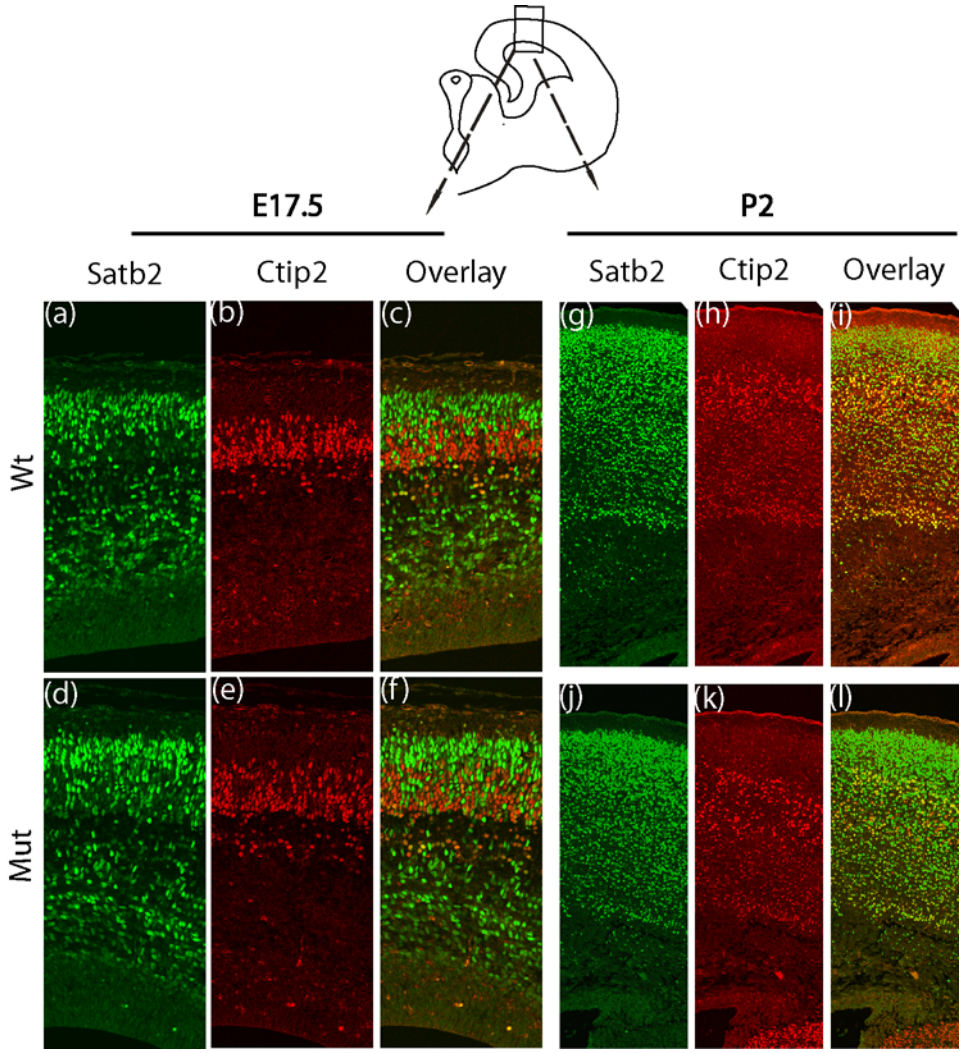

Fig 18: Lamination in wt and AP2γ mutants at E17.5 and P2. To determine possible defects in lamination, immunostaining for Satb2 and Ctip2 was done. No differences were observed between wt (a, b, c; g, h, i) and mutant sections (d, e, f; j, k, l).

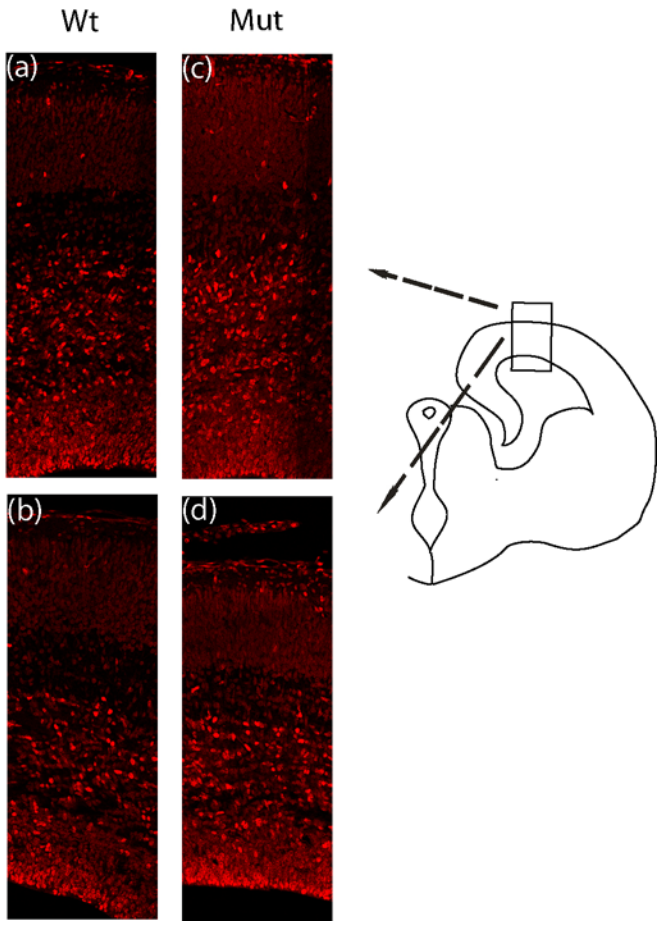

Fig 19: Proliferation in wt and AP2γ mutants at E 17.5. In order to determine possible changes in proliferation, dividing cells were labeled with proliferative marker Ki-67 using immunohistochemistry. No differences were observed between wt (a and b) and mutant sections (c and d).

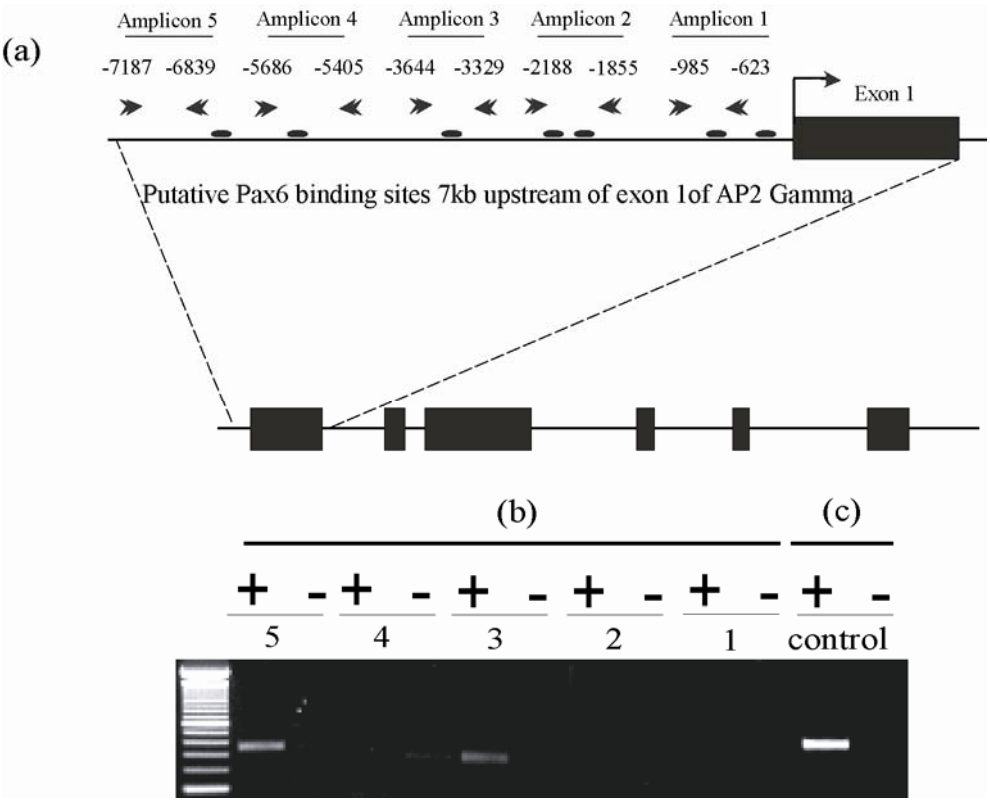

Fig 20: Identification of putative Pax6 binding sites using ChIP assay. Putative Pax6 binding sites (with a few mismatches) and primers to amplify these regions on AP2γ promoter are indicated (a). PCR amplification for putative Pax6 binding sites on AP2γ promoter are shown (b). A known Pax6 binding site on its own promoter was used as a positive control (c). DNA-protein complexes precipitated with Pax6 Ab added (+) and without Ab (-) are indicated. Pax6 seems to bind to AP2γ promoter region.

4.2.6 AP2γ does not influence morphology of radial glial processes expressing Nestin

Loss of Pax6 has been reported to result in a wavy morphology of radial glial processes in the cortex [Gotz et al., 1998]. Since AP2γ is a putative downstream target of Pax6, we wanted to investigate if AP2γ has a role in regulating radial glial morphology. We stained radial glial fibers at E15.5 and E17.5 by immunostaining for Nestin, an

intermediate filament expressed in these processes [Gotz et al., 1998]. No change in Nestin expression was observed in mutants as compared to wt (Fig: 21).

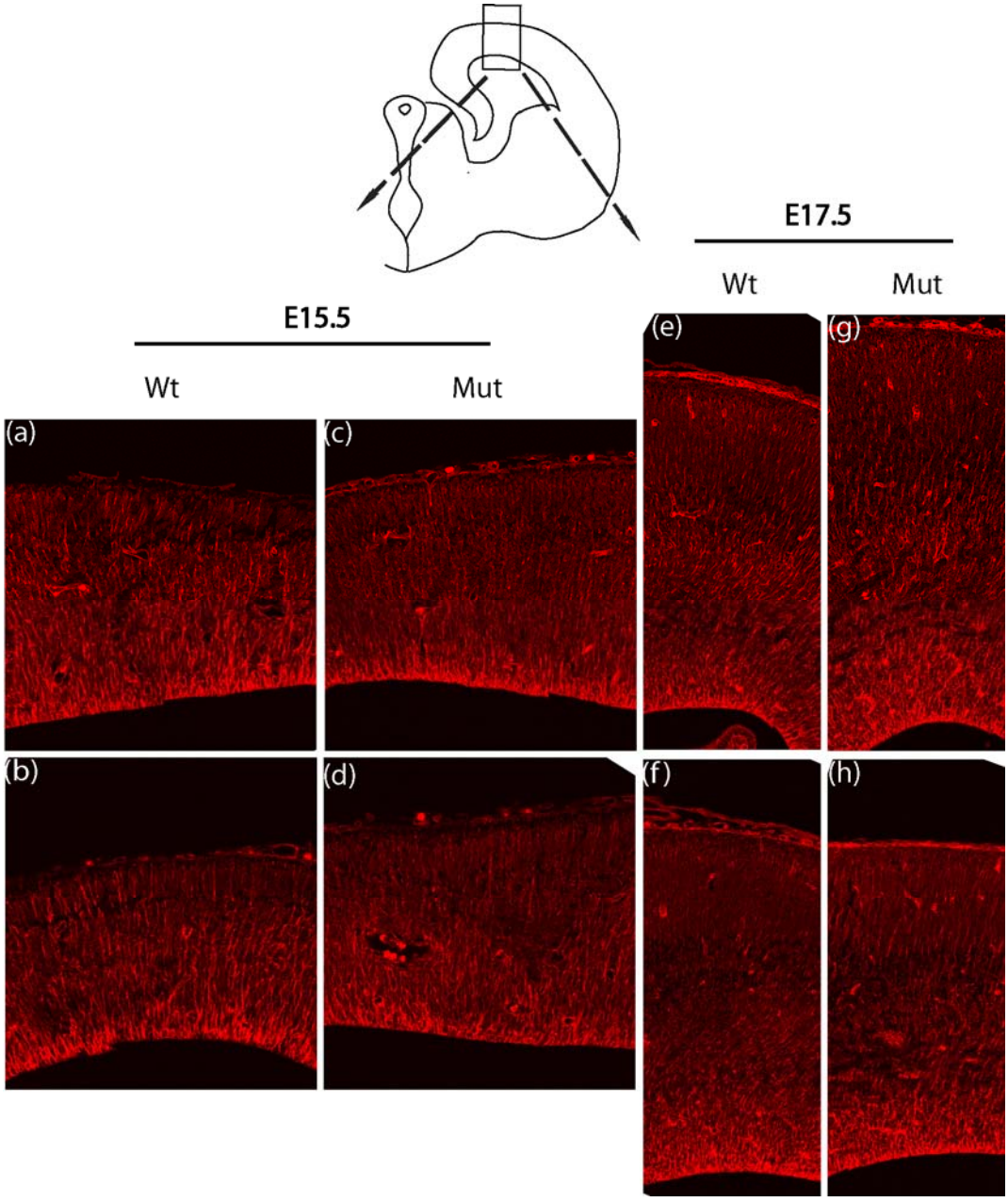

Fig 21: Expression of Nestin in wt and AP2γ mutants. Immunostaining for Nestin, a radial glial marker, at E15.5 and E17.5 indicated no difference in expression in mutant cortex (c, d; g, h) as compared to wt (a, b; e, f).

5. Discussions

In the current study we investigated downstream pathways of the transcription factor Pax6. In the cortex we identified two genes (AP2γ and CRALBP) whose expression was downregulated in Pax6 mutants and investigated the roles of these genes in cortical development.

5.1 CRALBP is a direct downstream target of Pax6 and influences radial glial morphology

Here, we investigate the regulatory activity of Pax6 on CRALBP, a 11- cis retinal binding protein that is mostly expressed in the eye, optic nerve, pineal gland and brain [Saari and Crabb, 2005]. Through microarray experiments we could identify CRALBP to be downregulated in Pax6 mutants. Pax6 is expressed in the ventricular zone (VZ), cortical hem, pallial/subpallial (PSPB) boundary and the thalamus [Manuel and Price, 2005] (Fig: 4b). CRALBP was found to be expressed in all these regions at the mRNA level (Fig: 4c). The borders of CRALBP protein expression corresponded with Pax6 in the cortical hem (Fig: 6a-c), PSPB (Fig: 6d-f), epithalamus (Fig: 7a-c) and hypothalamus (Fig: 7d-f). We did not find regions expressing CRALBP but not Pax6.

CRALBP transcripts were found only in the VZ of the cortex (Fig: 4c), whereas protein expression was observed throughout the cortical plate and the cortical hem (Fig: 6e and 6b). The localization of CRALBP protein in cells of the cortex seemed to be cytoplasmic, as expected. However, we were not able to see radial glial processes through

immunostaining for CRALBP. There are three possible ways to explain why CRALBP protein is detected all over the cortex while the mRNA is seen only in the VZ. One, young neurons derived from cortical progenitors in the VZ express CRALBP under the transcriptional regulation of Pax6. Even though these neurons migrate away from the VZ towards the cortical plate, we continue to observe expression of CRALBP protein possibly due to increased stability and low turnover rate of the protein. Second, synthesis of CRALBP protein takes place in the VZ; this protein is then transported along radial glial processes towards the pial surface. This however does not explain why CRALBP staining cannot delineate individual radial glial processes. The third explanation could be rapid degradation of CRALBP mRNA in the cortical plate.

Pax6 is also expressed in the differentiating field of the amygdala and in the hypothalamus surrounding the third ventricle (Fig: 4b; indicated by double arrow heads). However, we did not find expression of CRALBP in these regions (Fig: 4c). Since the expression of a gene not only depends on the binding of transcription factors but also certain cofactors, we suspect that the cofactors required for CRALBP expression could be lacking in these regions.

Abolishment of CRALBP expression in the spinal cord of Pax6 mutants (Fig: 5d) indicates that CRALBP is a target of Pax6 not only in the cortex but also in other regions of the central nervous system. This also implies that CRALBP might play a role in Pax6 mediated development of the spinal cord.

Using chromatin immunoprecipitation assay, we showed that Pax6 binds to two sites upstream of exon1 of CRALBP gene, at 2kb and 2.9kb (Fig: 9c). Although we could not find any TATA or CAAT boxes between the start site and the first Pax6 binding site, we could confirm the regulatory activity of Pax6 on CRALBP expression through *in vitro* luciferase reporter assay. The increase in reporter activity with increasing concentration of Pax6 (Fig: 10) shows that Pax6 positively regulates the expression of *CRALBP*.

Pax6 is considered a master regulatory gene of the eye [Gehring, 1996]. We found expression of Pax6 in the neuroretina (NR), (Fig: 8a) and that of CRALBP in the retinal pigment epithelium (RPE) that surrounds the neuroretina (Fig: 8b). During eye morphogenesis, Pax6 is expressed in the optic vesicle (OV) and the surface ectoderm (SE) that surrounds the OV. SE thickens to form the lens placoid (LP), whereas the distal OV invaginates to form the optic cup (OC) with the inner layer developing into the NR [Ashery-Padan and Gruss, 2001]. The optic vesicle contains bipotential progenitors which can give rise to both RPE and NR cell types. Separation of progenitors into NR and RPE domains is mediated by external cues [Fuhrmann et al., 2000; Pittack et al., 1997]. We believe that expression of Pax6 in the bipotential progenitor cells of the OV promotes CRALBP expression. After receiving signals from external cues, the bipotential progenitors differentiate into NR and RPE cells. The RPE (expressing CRALBP) then migrate and occupy the periphery surrounding the NR (Fig: 8b). After the optic cup is formed, Pax6 is downregulated in the optic stalk and the RPE, but retained in the neuroretina [Ashery-Padan and Gruss, 2001]. This could possibly explain the expression of CRALBP and Pax6 in distinct non- overlapping regions of the eye (Fig: 8c).

Vitamin A (retinol) and its metabolites (retinaldehyde and retinoic acid) are known for their role in development and maintenance of tissues in many higher animals [Goodman, 1984]. Retinol is metabolized to its bioactive derivative, all-trans-retinoic acid (ATRA) which is synthesized in discrete regions of the brain [Dev et al., 1993]. Retinoic acid (RA) is used for neural induction in embryonic stem cells [Bain et al., 1995; Bain et al., 1996] and has been shown to promote differentiation of excitatory and inhibitory neurons and glia [Finley et al., 1996].

CRALBP belongs to the class of retinoid binding proteins (RBPs) which are carriers of retinol and its derivatives [Vieira et al., 1995]. We found CRALBP transcripts as early as E12.5 in wt cortex (data not shown). By E14.5 expression of CRALBP protein was observed throughout the cortical plate (Fig: 6e) indicating that CRALBP could play a role during early corticogenesis. Early cortical development is associated with proliferation and neuronal migration that requires the aid of radial glial processes, long regarded primarily as a scaffold for migrating neurons [Rakic, 2003]. Immunostaining for BLBP indicated alterations in radial glial morphology in CRALBP mutants (Fig: 13). BLBP belongs to the family of Fatty acid-binding proteins (FABPs) which are a conserved multigene family of intracellular lipid binding proteins (iLBPs). Gene duplications in iLBPs gave rise to four subfamilies [Hanhoff et al., 2002]. Subfamily I comprises proteins that bind vitamin A derivatives like CRABPs and CRBPs [Ross, 1993]. Both CRALBP and BLBP are therefore carrier proteins that are evolutionarily linked. It has been shown that BLBP is a downstream target of Pax6 in rats [Arai et al., 2005] and

knock-down of BLBP using small interfering RNA (siRNAs) results in rounded neuroepithelial cells. BLBP expressing radial glial cells in the cortex of wt mice have straight processes that run from the ventricular surface towards the pial surface (Fig: 12 and 13c). However, in CRALBP mutants these processes have a “wavy” morphology (Fig: 13d). This phenotype recapitulates the radial glial morphology observed in Pax6 mutants [Gotz et al., 1998]. It is however subtle in comparison. We believe that both CRALBP and BLBP contribute to specification of radial glial morphology. In addition to BLBP, we also investigated the expression of Nestin (Fig: 14). Here we did not find the “wavy” phenotype that we observed with BLBP staining in CRALBP mutants. It has been shown that in Pax6 mutants, morphological alterations of radial glial cells can be observed with radial cell 2 (RC2) immunostaining [Gotz et al., 1998]. The authors report that while almost all Pax6-positive cells express RC2, a high proportion of Pax6-negative cells express Nestin [Gotz et al., 1998]. Since Nestin is not expressed in a high majority of Pax6- positive radial glial cells, we assume that the morphological effects mediated by deletion of Pax6, cannot be recapitulated in Nestin-positive cells via CRALBP.

CRALBP is the carrier of 11-cis-retinol in Muller glial cells of the eye. Muller glial cells of the retina have recently been shown to form neurospheres and express CRALBP [Monnin et al., 2007]. Retinol and its metabolites are known for their role in development [Goodman, 1984]. The impairment of 11-cis-retinol transport in CRALBP mutants could lead to decreased activity of RA signaling. Retinoic acid has previously been shown to induce Pax6 expression efficiently in embryoid bodies [Gajovic et al., 1997]. Low activity of RA signaling could result in decreased induction of Pax6 expression, thereby

partly contributing to the "wavy" phenotype of radial glial cell processes in CRALBP mutants.

We did not observe any defects in either layer-specification or migration of neurons in CRALBP mutants (Figs: 11). However, it is probable that other RBP's expressed in the brain compensate for the loss of CRALBP to some extent thereby leading to a relatively mild phenotype of "wavy" radial glial processes. Our data suggest that CRALBP acts downstream of Pax6 in regulating radial glial morphology.

5.2 AP2γ does not regulate lamination and proliferation but might influence arealization of the cortex

The AP2 family of transcription factors consists of five members that are [Zhao et al., 2001] mainly involved in cell proliferation and differentiation. AP2γ has previously been demonstrated to be essential for early embryogenesis [Werling and Schorle, 2002a]. Through microarray experiments we found that expression of AP2γ was downregulated in Pax6 mutants. We then investigated the phenotype of AP2γ mutants in the cerebral cortex. We did not observe any differences in arealization or regionalization using markers like Cad8, Cux2, Svet1 or Er-81 (Figs: 15, 16 and 17 respectively). However, we could find some differences in the expression of Id2 (Fig: 15f) and LMO4 (Fig: 17f).

We found weaker expression of LMO4 in layer 5 in mutants as compared to wt (Fig: 17e-f). It has previously been suggested that LMO4 could be a downstream target of fibroblast growth factor receptor 1 (FGFR1) [Saga et al., 1997]. Several putative binding

sites of AP2 transcription factors have been located on FGFR3 [Perez-Castro et al., 1997]. This might explain the regulatory role of AP2 transcription factors on FGFR genes. AP2γ could also regulate the expression of LMO4 by regulating the expression of FGFR1. However, we have not attempted to look for putative binding sites of AP2γ in the promoter region of FGFR1. Since putative binding sites of AP2 genes are known [Hilger-Eversheim et al., 2000], it would be interesting to investigate this possibility. In vivo binding assays could provide better insight on transcriptional regulation by AP2γ.

It has been reported that overexpression of AP2γ promotes proliferation and impairs differentiation [Jager et al., 2003]. Therefore, in AP2γ mutants, impaired proliferation and premature differentiation can be expected. Premature differentiation can often lead to problems in lamination. To verify this, layer specific populations of neurons were labeled with markers like Satb2 (layers 2-4) and Ctip2 (layer5). We did not find any difference in the size of these populations or their position within the cortex (Fig: 18). This indicates that patterning and layer specification are not affected in AP2γ mutant cortex. At physiological levels, AP2γ probably does not influence differentiation of neurons.

Expression of Id2 marks not only the boundary between motor and somatosensory cortex, but also the lateral boundary of the somatosensory cortex. In AP2γ mutants, this boundary of expression and probably the different cortical areas are shifted rostrally (Fig: 15f). Hence, we believe that AP2γ might be involved in arealization of the cortex.

AP2γ has been shown to promote proliferation in breast carcinoma [Jager et al., 2005]. However, we did not find any changes in the expression of the cell cycle marker Ki67 in the germinal zones of the neocortex (Fig: 19). Hence, we conclude that AP2γ does not seem to regulate proliferation in this region.

Nestin, a radial glial specific intermediate filament, did not show any differences in expression at E15.5 and E17.5 (Fig: 21) between wt and mutants. Since the possibility for the existence of Nestin positive and Pax6 negative radial glial cells has been shown [Gotz et al., 1998], one could expect to find Nestin positive radial glial processes that do not show any morphological changes on deletion of Pax6.

Since AP2γ is strongly downregulated in Pax6 mutants we looked for Pax6 binding sites on AP2γ promoter. Although we could not find consensus sequences that would indicate putative Pax6 binding sites [Epstein et al., 1994a; Morgan, 2004; Sander et al., 1997], we did find possible Pax6 binding sites with one or two nucleotide mismatches. As Pax6 recognizes an array of different consensus sequences, we began investigating the binding capabilities of Pax6 to these sequences using Chromatin immunoprecipitation assay (ChIP) in combination with semi-quantitative PCR. Our results show that Pax6 binds *AP2γ* promoter (Fig: 20b) and possibly regulates its expression. This indicates that *AP2γ* could be a direct downstream target of Pax6, which in turn implies that the expression of AP2γ should be completely abolished in Pax6 mutants. However, we did observe a weak expression of AP2γ in these mutants. We therefore believe that cortical progenitors possess alternate pathways to regulate AP2γ expression. We are yet to perform luciferase

reporter assay with *AP2γ* promoter constructs and constructs carrying Pax6 coding sequence, to test whether binding of Pax6 can regulate the expression of *AP2γ* gene. In conclusion, it seems that AP2γ does not play a role in lamination or proliferation in the cortex. However, the shift in Id2 expression suggests that it might have a role in arealization. Decrease in LMO4 expression also suggests that AP2γ might be involved in specification of deep layer neurons in the medial cortex. These data indicate the possibility of Id2 and LMO4 as direct or indirect targets of AP2γ.

6. Summary

Transcription factor Pax6 plays an essential role in the development of diverse tissues in vertebrates and invertebrates. It regulates the expression of other transcription factors and cell adhesion molecules, and is crucial for neurogenesis in the developing forebrain. Analysis of gene expression profiles through microarray experiments in Pax6 mutants allowed us to focus on two (AP2γ and CRALBP) of the many genes that were downregulated. We chose these genes since they were expressed in the VZ of the cortex, a proliferative zone known for Pax6 expression. The aim of the project was to analyze if Pax6 mediates some of its functions through these genes.

CRALBP binds 11-cis-retinol with high affinity and acts as a substrate carrier protein. It is expressed in retinal pigment epithelium (RPE) of the eye and is known to have a role in the visual cycle. CRALBP mRNA was expressed in Pax6 expression domains in wt cortex and spinal cord and completely abolished in Pax6 mutants. Protein expression in the brain showed that Pax6 and CRALBP had coinciding boundaries of expression in different regions of the cortex and thalamus. However, in the eye, Pax6 and CRALBP do not share an expression domain. *CRALBP* promoter was found to contain several putative Pax6 binding sites. We confirmed that Pax6 directly binds *CRALBP* promoter and positively regulates its expression. Therefore it can be concluded that *CRALBP* is a direct downstream target of Pax6.

BLBP expressing radial glial processes in the cortex of CRALBP knockout mouse acquire a wavy morphology, partly recapitulating the phenotype seen in Pax6 mutants.

This suggests that the morphology of radial glial processes is regulated by Pax6 via CRALBP.

Transcription factor AP2γ positively regulates proliferation and is important for early murine development. Expression analysis (mRNA) in AP2γ mutants using cortical area-specific and layer-specific markers showed differences in expression of Id2 and LMO4. While Id2 showed a shift in expression boundaries, expression of LMO4 was decreased. However, there are no apparent lamination or proliferation defects in AP2γ mutant cortex. We also found that Pax6 binds the putative promoter region of AP2γ gene. This suggests that AP2γ might also be a direct downstream target of Pax6.

7. Conclusions

In the current study we investigated the role of CRALBP and AP2γ, putative downstream targets of Pax6, in cortical development.

RNA and protein expression data show that CRALBP expression was completely lacking in Pax6 mutants. We also show that Pax6 binds the promoter of CRALBP and activates its expression. Therefore, we conclude that CRALBP is a direct downstream target of Pax6. Also, in CRALBP mutants, radial glial processes acquire a wavy morphology that partly recapitulates the phenotype seen in Pax6 mutants. Thus, it can be concluded that Pax6 regulates radial glial morphology via CRALBP.

We also investigated the role of AP2γ in the cerebral cortex by conditional gene inactivation. We did not find lamination problems or changes in proliferation in AP2γ mutants. We found that Pax6 directly interacts with AP2γ promoter.

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……..Chance favours the prepared mind

Louis Pasteur