Transcription factor Pax6 regulates cell cycle progression and cell fate determination: the modular logic of complex transcriptional control

Dissertation der Graduate School of Systemic Neurosciences
der Ludwig-Maximilians-Universität München

Graduate School of
Systemic Neurosciences
LMU Munich

Submitted by
Stefania Petricca
München, 2nd of July 2015

Supervisor: Dr. Jovica Ninkovic

Second reviewer: Prof. Dr. Magdalena Götz

Third reviewer: Prof. Dr. Federico Calegari

Date of submission: 02.07.2015

Date of oral defense: 29.09.2015

Abstract

The development of the central nervous system relies on the tight regulation of the neural progenitor proliferation and differentiation in order to generate new neurons. The transcription factor Pax6 coordinates these functions during the development of the mammalian forebrain, using the paired DNA binding domain. This is a bipartite DNA-binding domain constituted by two subdomains, the PAI and the RED, binding the DNA in a cooperative or independent manner in order to control specific targets.

Focusing on the activity of Pax6 as regulator of cell proliferation and of cell fate determination, I aimed to understanding how these functions are regulated at the molecular level, using the developing ventral forebrain as a model.

In this work the role of the RED domain of Pax6 as a regulator of cell cycle progression is investigated. The mutation of the RED subdomain leads to an increase of progenitors in active mitosis (phospho-histone3 positive cells) in the ventral telencephalon at midneurogenesis. Similar result was obtained in the progenitors of the dorsal telencephalon. The increment in the phospho-histone3 positive cells is followed by the increase in cell death in both dorsal and ventral forebrain. These evidences suggest impairments in the cell cycle progression of the progenitor cells in the RED domain mutant.

The importance of full activity of the RED domain of Pax6 for the proper progression of the cell cytokinesis is shown via ex-vivo live imaging, performed on ventral developing forebrain of $Pax6^{Leca2}$ mutant animals (RED domain mutant).

To elucidate the molecular mechanisms underlying the observed phenotype, transcriptome of the $Pax6^{Leca2}$ mutants and their age matching siblings is analyzed, identifying a potential candidate gene: the Holliday junction recognition protein (HJURP). The overexpression of the HJURP protein in the wild type progenitors in vitro resembles the impairment of cytokinesis observed in $Pax6^{Leca2}$ mutant.

In summary, my data suggest new mechanisms for the regulation of cytokinesis in progenitors mediated by the RED domain of Pax6 and indicate that the full functionality of the paired domain is a prerequisite for Pax6 to function as fate determinant.

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List of abbreviations

%	Percent
°C	Degree Celsius
µg	Microgram(s)
µl	Microlitre(s)
µM	Micromolar
AP	Apical Progenitor
bHLH	Basic helix-loop-helix
BP	Basal Progenitor
Bp	Base pairs
cDNA	Complementary Deoxyribonucleic Acid
CNS	Central Nervous System
CO_2	Carbon Dioxide
Ctx	Cortex
DAPI	4',6-diamidino-2-phenylindole
dATP	DeoxyAdenosine TriPhosphate
dCTP	DeoxyCytidine TriPhosphate
Dcx	Doublecortin
dGTP	DeoxyGuanosine TriPhosphate
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
dNTP	DeoxyNucleoside TriPhosphate
dNTP	DeoxyNucleoside TriPhosphate(s)
dTTP	DeoxyThymidine TriPhosphate
E	Embryonic Day
e.g.	For example (Latin ‘exempli gratia’)
EDTA	Ethylene-Diamine-tetra-Acetic-Acid
EGF	Epidermal Growth Factor
ENU	*N*-ethyl-*N*-nitrosourea

FCS	Fetal Calf Serum
FGF	Fibroblast growth factor
G	Gram
GAPDH	GlycerAldehyde 3-Phosphate DeHydrogenase
GE	Ganglionic Eminence
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
H	Hour(s)
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HJURP	Holliday Junction recognition protein
Kb	Kilobase
kDa	Kilodalton
Kg	Kilogram(s)
L	Litre
LB	Luria-Bertani medium
Leca	Lens Corneal Adhesion
LGE	Lateral Ganglionic Eminence
MGE	Medial Ganglionic Eminence
Min	Minute
miRNA	Micro Ribonucleic Acid
mM	Millimolar
mRNA	Messenger Ribonuicleic Acid
NaCl	Sodium Chloride
Ng	Nanogram(s)
OB	Olfactory Bulb
Olig2	Oligodendrocyte transcription factor2
P	Postnatal day
Pax6	Paired Box 6

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDL	Poly-D-Lysine
PFA	Paraformaldehyde
PSB	Pallial-subpallial boundary
qPCR	Quatitative Polymerase Chain Reaction
RG	Radial Glia
RMS	Rostral Migratory Stream
RNA	Ribonucleic Acid
rpm	Revolutions per minute
RT	Room Temperature
Sec	Seconds
SEM	Standard Error of the Mean
SEZ	Subependymal Zone
SP	Subapical Progenitor
Str	Striatum
SVZ	Subventricular Zone
Taq	DNA polymerase of bacterium Thermus aquaticus
U	Enzyme unit
VZ	Ventricular Zone
w/v	Weight/Volume
WT	Wild Type

1 Introduction

The mammalian brain comprises a variety of cell types. The two major populations are the neurons, specialized in conducting signals to other cells, and the glia cells comprising various cells important to preserve homeostasis and provide support and protection to the neurons.

During brain development the neural stem cells represent the source of all the neurons in the mammalian brain as well as of the astrocytes and oligodendrocytes (Gotz and Huttner, 2005). The commitment of a cell to a specific fate can be determined by the environment surrounding the cells providing different cues (non-cell autonomous) and by a gene expression program pre-specified in the cells (cell autonomous). The proper development of the brain requires a fine regulation of the combination of these extrinsic and intrinsic fate determinants in order to define the correct number of each cell type at the right time and place (Guillemot et al., 2006; Johansson et al., 2010; Lui et al., 2011; Tiberi et al., 2012).

A cell intrinsic factor, that is regulating the commitment of the neural stem cells to the neuronal fate, is Pax6. In the developing cortex in fact the transcription factor Pax6 is expressed in neural stem cells and controls their proliferation as well as the time they exit the cell cycle in order to proceed to differentiation (Warren et al., 1999). Pax6 is important for the generation of superficial cortical neurons (Schuurmans et al., 2004; Georgala et al., 2011b). In addition Pax6 is also important for the generation of olfactory bulb interneurons originating from the ventral part of the telencephalon and are produced lifelong in the adult lateral wall of the subependymal zone (Merkle et al., 2004; Hack et al., 2005; Merkle and Alvarez-Buylla, 2006). More insights on the functions of this transcription factor will be described further in the chapter.

1.1 Early brain development

After the neurulation process and prior to the onset of neurogenesis (embryonic days E7-9 in mice), the neural plate and the neural tube are composed by a single layer of neuroepithelial cells (NE).

The NE cells, characterized by apical- basal polarity, form a pseudostratified layer of cells. The appearance of the neuroepithelium in layers is given by the nuclei of the cells that are performing the so called interkinetic nuclear migration (INM). During this process the nucleus of every NE cell moves in the direction of the basal lamina (a thin layer of extracellular matrix at the pia surface) during G1 and S phase of the cells cycle and moves back to the apical side (at the ventricular surface) in G2 phase undergoing to mitosis directly at the apical side (Sauer, 1935; Takahashi et al., 1993).

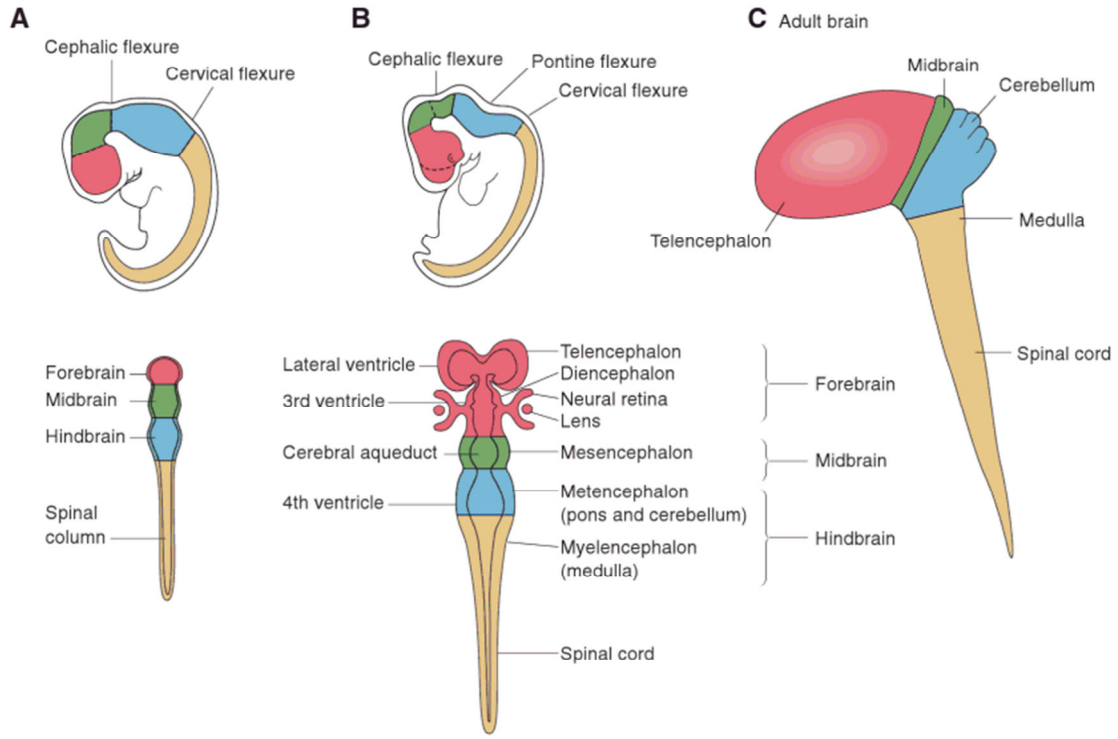


Figure 1: Vertebrate brain and spinal cord development
Schemes taken from Sanes, Reh and Harris; Development of the Nervous System.
(A) The primary structures appearing are the forebrain (prosencephalon), midbrain (mesencephalon), and hindbrain (rhombencephalon). These structures further differentiate and the forebrain generates the telencephalic vesicles and the diencephalon, while the rhombencephalon divides into the metencephalon and the myelencephalon (B). The basic brain divisions are then maintained in the adult one (C).

The rostral region of the neural tube differentiates in three primary vesicles: forebrain (prosencephalon), midbrain (mesencephalon) and hindbrain (rhombencephalon) (Figure 1 A). These three regions further subdivide in five secondary brain vesicles. The forebrain gives rise to the anterior telencephalon and the more caudal diencephalon. The hindbrain further differentiates into an anterior metencephalon, forming the pons and the cerebellum, and a posterior myelencephalon giving rise to the medulla

oblongata (Figure 1 B) (Melton et al., 2004). The anterior-posterior regionalization finally ends in the caudal part of the neural tube where it specifies the spinal cord.

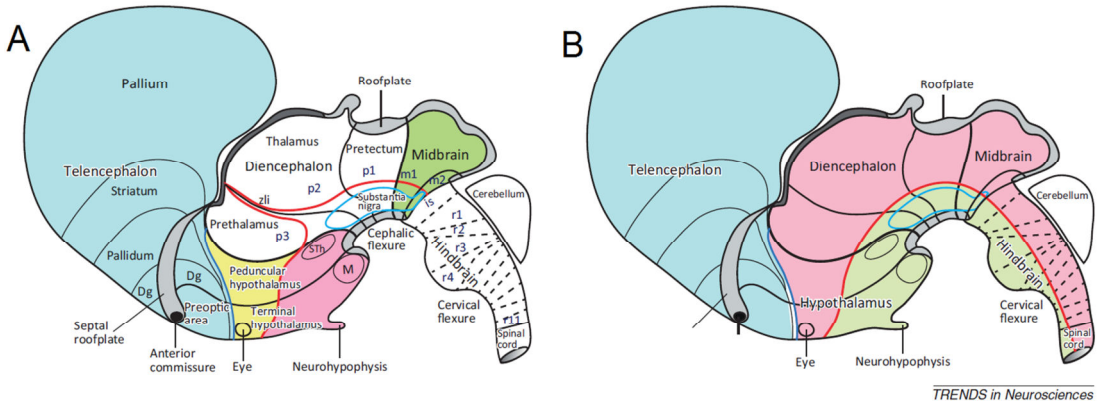


Figure 2: Organization of the telencephalon
Scheme taken from (Puelles et al., 2013) summarizing the subdivision of the telencephalon area of interest in this work. (A-B) Diagrams of a lateral view of developing mouse brain. The forebrain is subdivided in the telencephalon and diencephalon. The telencephalon subdivides into the pallium, in the dorsal side, and subpallial regions ventrally.

The most anterior vesicle, the telencephalon, gives rise to the cerebral cortex or pallium dorsally whereas the ventral region generates the lateral and the medial ganglionic eminences (LGE and MGE) which will result in the striatum and the pallidum respectively (Figure 2).

At the cellular level, while the neurogenic process proceeds, the NE cell populating the telencephalic area are transformed in the radial glia cells (RG).

The RG cells similarly to the NE have apical- basal polarity and undergo INM. These cells differentiate from the NE because of the expression of some astroglial markers like vimentin, the glial glutamate/ aspartate transporter GLAST, the brain lipid binding protein (BLBP) which are all lacking in the NE (Gotz and Huttner, 2005; Sild and Ruthazer, 2011).

In the dorsal telencephalon, the RG cells either directly differentiate to neurons or give rise to basal progenitors (BP), also known as intermediate progenitors, that are located in the subventricular zone (abventricular region)(Figure 3). These progenitors are distinguished from the RG cells by the expression of distinct transcription factors (Englund et al., 2005). Aside the expression of different factors, the BP cells differentiate morphologically from the RG. BPs are lacking the apical-basal polarity and do not undergo INM. In the dorsal telencephalon, these progenitors divide in basal

subventricular area and undergo symmetric cell division which leads to the increase of the numbers of neurons generated per round of division (reviewed in (Gotz and Huttner, 2005).

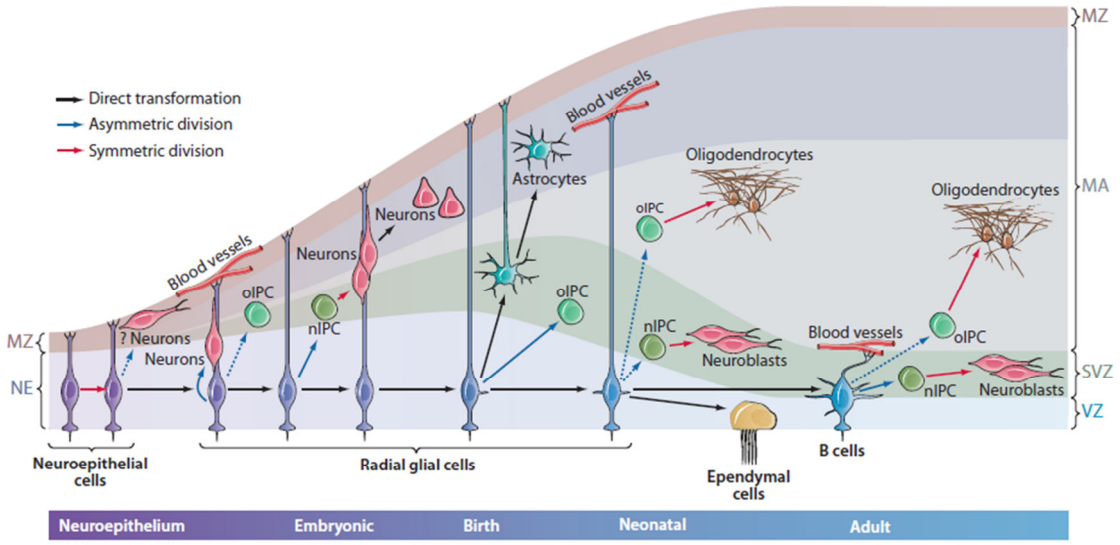


Figure 3: Glial nature of embryonic and adult neural stem cells
Scheme from (Kriegstein and Alvarez-Buylla, 2009) summarizing the different progenitors in the embryonic and adult brain and their mode of division. Neuroepithelial cells in early development divide symmetrically to amplify their pool. As the brain development proceeds neuroepithelial cells convert into radial glial (RG) cells. RG divide asymmetrically to generate neurons directly or indirectly through intermediate progenitor cells (nIPCs). A subpopulation of RG retain apical contact and continue functioning as adult NSCs in the neonate in specific regions where neurogenesis continues lifelong. Colors depict symmetric, asymmetric, or direct transformation. IPC, intermediate progenitor cell; MA, mantle; MZ, marginal zone; NE, neuroepithelium; nIPC, neurogenic progenitor cell; oIPC, oligodendrocytic progenitor cell; RG, radial glia; SVZ, subventricular zone; VZ, ventricular zone.

1.2 Ventral telencephalon

Dorso-ventral patterning starts, in the telencephalon, early during development after the closure of the anterior neuropore with the appearance of three primitive ventral structures composing the subpallium. These structures are all protruding into the ventricular lumen in a specific temporal order: first the medial ganglionic eminence (MGE) at embryonic day 9 (E9), then the lateral ganglionic eminence (LGE) at E10 and finally the caudal ganglionic eminence (CGE) at E11 (Kohtz et al., 1998; Rallu et al., 2002).

The subpallial area gives rise to the multiple neuronal types that form the basal ganglia, parts of the amygdala and the septum. In particular the LGE is giving rise to

the caudate and the putamen and the MGE is forming amygdala and globus pallidus (Reiner et al., 1998).

From the subpallial region is also originating a variety of cortical interneurons.

Differently from the cerebral cortex where the projection neurons build up a cortical plate of 6 layers (Molyneaux et al., 2007), the striatum is organized in a mosaic pattern of patches and surrounding matrix (Gerfen, 1992). Patch neurons are born in two waves; the first wave is between E10.5-E11, the second wave of patch neurons is between E12.5-E13.5. Matrix neurons are born later (Fishell and van der Kooy, 1987; Mason et al., 2005; Liao et al., 2008).

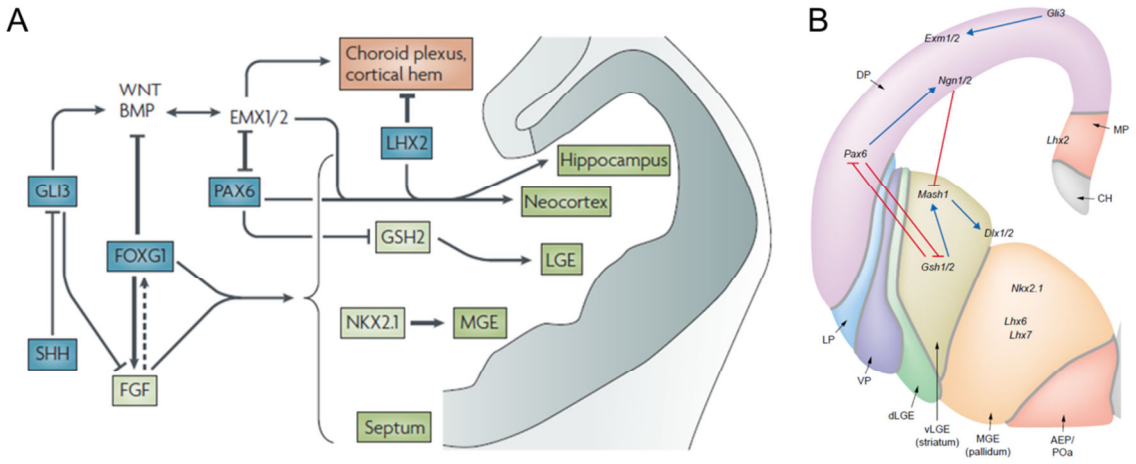


Figure 4: Dorsal and ventral telencephalon specification
(A) Scheme taken from (Hebert and Fishell, 2008); (B) scheme taken from(Schuurmans and Guillemot, 2002). (A). The factors that act early to establish broad telencephalic regions are in blue. Sonic hedgehog (SHH) ventralizes the telencephalon antagonizing the effect of GLI3 (dorsalizing factor). FoxG1 and FGF signaling are necessary to form all regions of the telencephalon (shown in green), except for the dorsomedial region (shown in orange). Downstream transcription factors, such as GSH2 and NKx2.1, then form specific subdivisions. (B) The dorsal telencephalon specification directed by the expression of Pax6 and Ngn1/2 antagonizing with the ventralizing factors Gsh1/2 and Ascl1/ Mash1.

The development and the specification of the ventral telencephalon identity are governed by a variety of morphogens and transcription factors like Gsx1/2, Ascl1 and Dlx1/2 (Schuurmans and Guillemot, 2002; Hebert and Fishell, 2008) (Figure4).

1.2.1 Lateral Ganglionic Eminence specification and neurogenesis

The LGE is anatomically defined as the bulge that is protruding into the ventricle in the area located between the cortex and the MGE. From the molecular point of view, the LGE is characterized by the expression of Dlx1/2/5 (Bulfone et al., 1993) as well as the

expression of Isl1, Gsx2, ER81 and Pax6 (Stoykova et al., 1996; Szucsik et al., 1997; Sussel et al., 1999; Stenman et al., 2003; Flames et al., 2007). The dorsal border of the LGE with the cortex is defined as ventral pallium (Puelles et al., 2000). In the ventral pallium Tbr2, Ngn2, and Dbx1 are expressed whereas Dlx2 is absent (Puelles et al., 2000; Yun et al., 2001). The border between the LGE and the MGE is identified by a high expression of Nkx2.1 (Puelles et al., 2000; Garcia-Lopez et al., 2008) (Figure 4).

The progenitors in the LGE are distinct in four domains, all extending (except the ventral pLGE4) along the rostro-caudal extent of the subpallium (Flames et al., 2007). Two domains of the dorsal LGE are discriminated by the differential expression of Pax6 and ER81 (Stenman et al., 2003; Carney et al., 2006; Waclaw et al., 2006; Flames et al., 2007). The dorsal LGE is thought to give rise to interneurons migrating to the olfactory bulb (Toresson et al., 2000; Yun et al., 2001; Stenman et al., 2003; Waclaw et al., 2006) and to contribute to distinct populations of postmitotic neurons forming the striatum proper and the central amygdala (Moreno et al., 2009).

The ventral LGE is the most prominent part of the LGE and is divided in two further domains. These two domains express Nkx6.2 (Flames et al., 2007) and give rise to Isl1 expressing cells in the SVZ (Stenman et al., 2003).

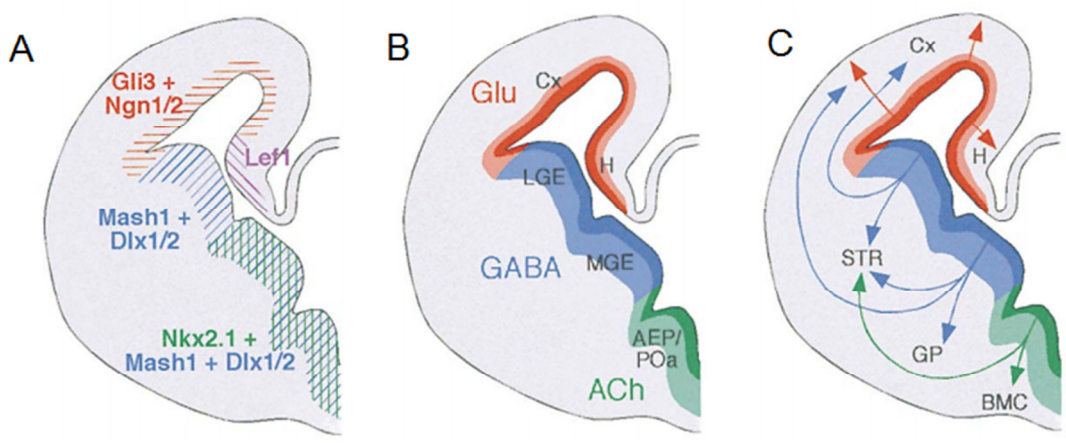


Figure 5: Production and migration of different neurons in the ventral telencephalon
Scheme representing the telencephalic hemispheres from E14 mouse embryos (Wilson and Rubenstein, 2000). (A) Expression patterns in the progenitor zones of the transcription factors implicated in the regulation of telencephalic patterning and differentiation. (B) Subdivisions of the telencephalic proliferative zone and location of precursor cells producing neurons expressing the neurotransmitters, glutamate, GABA, and acetylcholine. (C) Migration pathways from different progenitors zones.

The progression of neurogenesis in the ventral telencephalon is under the control of the proneuronal factor Ascl1 (Mash1) (Casarosa et al., 1999). In this context Ascl1 is

not only acting as neural determinant but it is also conferring ventral neuronal identity due to the regulation of Dlx and GAD67, the enzyme synthetizing GABA (Casarosa et al., 1999; Fode et al., 2000; Parras et al., 2002; Poitras et al., 2007; Castro et al., 2011). Ascl1 is also responsible for the generation of an early population of subpallial oligodendrocytes cooperating with the transcription factor Olig2 (Parras et al., 2007).
Differently from the pallium, where the projection neurons (glutamatergic neurons) reach their final position via radial migration from their ventricular progenitor zone, in the subpallial region the majority of telencephalic interneurons migrate following tangential routes to integrate into their final destination (Marin and Rubenstein, 2001; Marin and Rubenstein, 2003) (Figure 5). The streams of migration of the interneurons are classified according to the timing of migration, the subpallial domain of origin and the target region (Corbin et al., 2001; Nadarajah and Parnavelas, 2002; Flames and Marin, 2005; Metin et al., 2006). Hence, cortical GABA interneurons arise through two main tangential migrations, a superficial pathway, which originates in the MGE (Anderson et al., 1999; Lavdas et al., 1999; Sussel et al., 1999; Wichterle et al., 1999; Pleasure et al., 2000; Anderson et al., 2001) and a deep pathway originating from the LGE (de Carlos et al., 1996; Tamamaki et al., 1997; Pleasure et al., 2000; Anderson et al., 2001) (Figure 5).

1.2.1.1 Progenitor cells diversity in the LGE

Differently from the cortex where the vast majority of progenitor cells are within the ventricular zone (VZ), in the LGE the most prominent number of proliferating progenitors is located abventricularly (Pilz et al., 2013).
The apical progenitors (AP) are the cells undergoing mitosis at the luminal surface of the VZ. The AP cell population in the ganglionic eminence, is composed by two cells types that undergo interkinetic nuclear migration: the bipolar radial glia cells (RG) and the short neural precursors (SNP) (Pilz et al., 2013). The SNPs are unipolar with a unique apical process. The apically dividing RG cells asymmetrically divide and, besides self-renewing, they produce either SNPs or a subapically dividing progenitors (SP) (Pilz et al., 2013) (Figure 6). The SNPs undergo further steps of amplification. The SNPs amplification represents a peculiar characteristic of the ventral telencephalon (Pilz et

al., 2013); in the cerebral cortex, in fact, the SNPs directly generate postmitotic neurons (Gal et al., 2006; Stancik et al., 2010; Tyler and Haydar, 2013).

Both RGs and SNPs are giving rise to SP (Figure 6). This population of cells has been recently identified in the LGE (Pilz et al., 2013). The SP progenitors undergo mitosis in a region comprised within 10 rows of cells from the apical surface. Beside the common proliferative zone, the SP cells are very heterogeneous in terms of morphology.

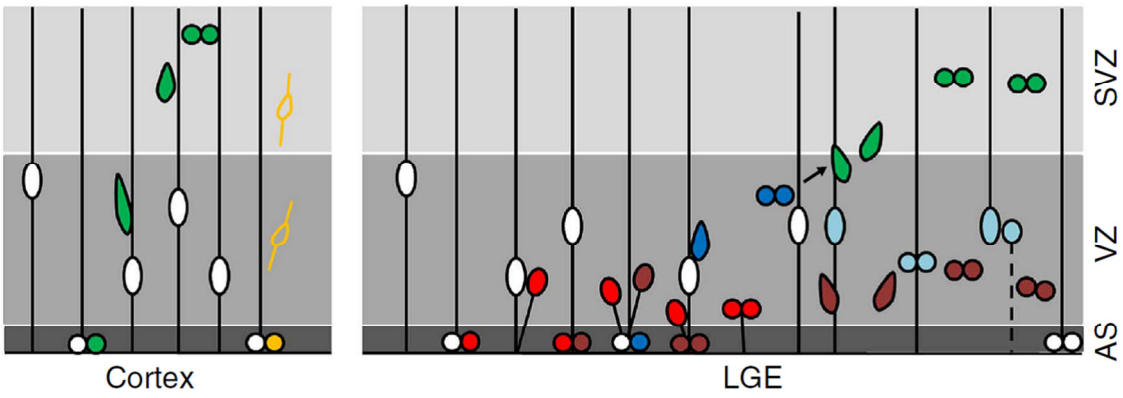


Figure 6: Progenitor cells in the cortex vs LGE

Scheme illustrating the progenitors present in the LGE and in the cortex.(Pilz et al., 2013). RG are in white, short neural precursors (SNP) are in light red; they both divide apically but the SNPs present only one process at the apical side. In light blue the SP dividing within 10cells rows from the ventricle. BPs are depicted in green and divide in the SVZ. In the cortex RG cells divide at the ventricle and give rise to neurons in yellow directly or to BP in green dividing in the SVZ. Abbreviations: AS (apical side); VZ (ventricular zone); SVZ (subventricular zone).

In the LGE SPs are positive for radial glia markers like RC2 and GLAST and also express Sox2, Ascl1, Dlx2 and Pax6 (Pilz et al., 2013). The SPs amplify the pool of proliferating progenitors in the LGE and they also give rise to basal progenitors (BP) (Pilz et al., 2013).

In term of cell cycle progression, live imaging experiments in the LGE area revealed that the RGs are the cells with the longest cell cycle (25h in average). The cell cycle length of SNPs and SPs is about 17h and the second generation of SPs from SNPs or SPs themselves is significantly shorter (12h) (Pilz et al., 2013). These observations suggest that besides a series of division that fasten the cell cycle of the progeny, these fast divisions are necessary to amplify the number of progeny in a given time; those are all hallmarks of the adult neural stem cells lineages emerging from this region (Pilz et al., 2013).

1.3 Adult brain from the LGE

In mammals, radial glial cells populating the brain during development disappear after birth even if some RG-like cells persist in few areas during adulthood (Figure 7). The adult NSCs (aNSC) and the radial glia cells belong to the same lineage (Merkle et al., 2004). In particular, radial glial cells of the neonatal lateral ventricular wall (Figure 7 C) occupy the same region as the astrocytic stem cells of the adult subedendymal zone (SEZ) (Figure 7 D). This led to the conclusion that the primary progenitors for the postnatal generation of new neurons in the adult SEZ zone are regionally specified and the organization of the aNSCs in different domains is defined before birth (Figure 7) (reviewed in(Merkle and Alvarez-Buylla, 2006)).

The adult SEZ is the largest germinal zone in the adult mammalian brain and is located at the lateral wall of the lateral ventricle. In this region are generated the new neurons that migrate through the OB via the rostral migratory stream (RMS) (Luskin, 1993; Lois and Alvarez-Buylla, 1994) and glial cells destined to the corpus callosum (CC) (Hack et al., 2005; Menn et al., 2006).

The SEZ niche consists of slow proliferating cells, the stem cells (also called Type B cells), that, via multistep process of fate restriction, switch from multipotent cells towards more fate-restricted precursors the transit amplifying progenitors (type C) and neuroblasts (type A), which further differentiate resulting in new neurons that functionally integrate into the OB system.

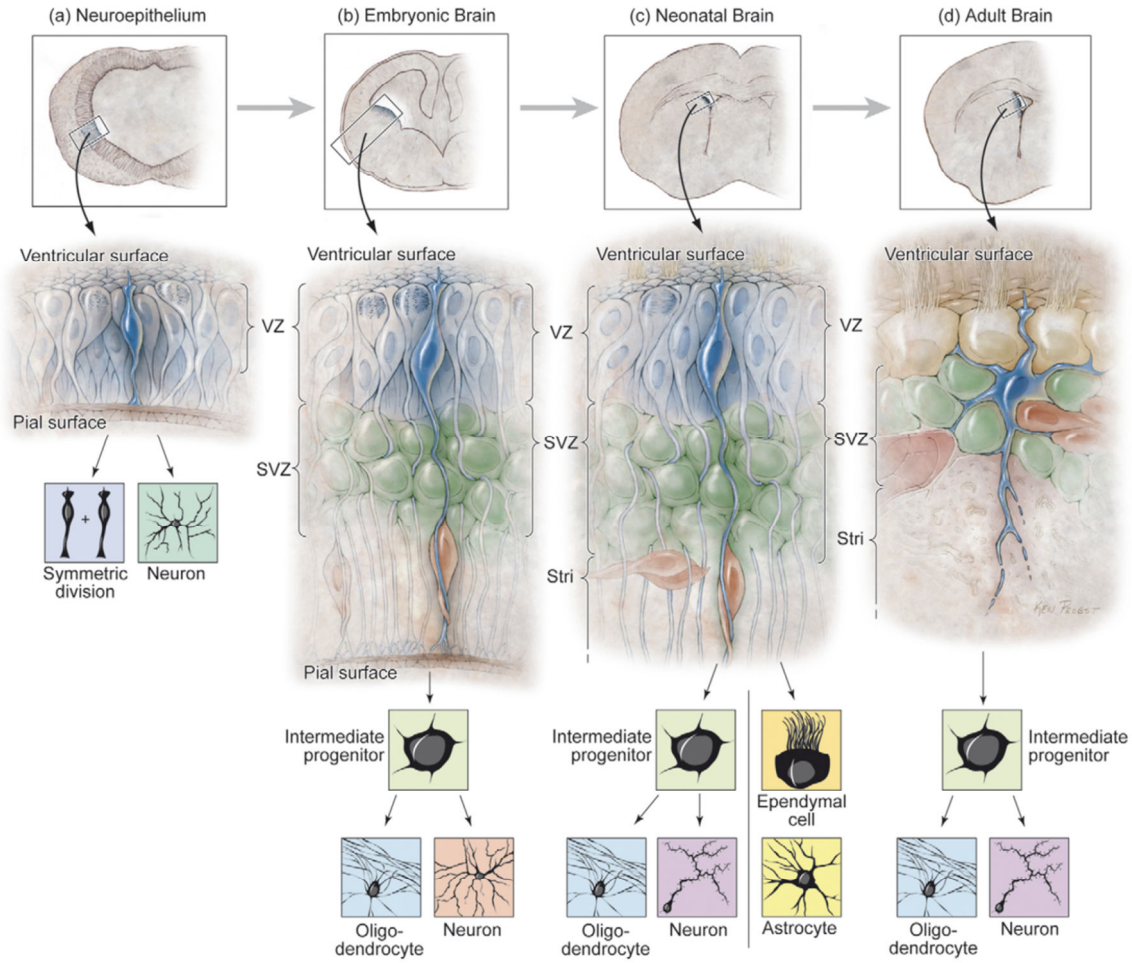


Figure 7: Neural stem cells and their progeny in adult brain
Schematic drawing from (Merkle and Alvarez-Buylla, 2006).
The NSCs of the lateral ventricule, in blue, change their shape and produce different progeny as the brain develops. They begin as neuroepithelial cells and transform into radial glial cells having NSCs characteristics. RGs contact with the ventricle, into which they project a primary cilium and produce progeny either directly or via an intermediate progenitors, in green. (A) At early developmental stages the CNS is a tubular structure, composed of neuroepithelial cells, which divide symmetrically to expand the stem cell pool. (B) Neuroepithelial cells differentiate into embryonic radial glial cells, which divide to generate striatal neurons and oligodendrocytes, either directly or via an intermediate progenitor. The radial processes of radial glial cells support the migration of neuroblasts, in red. (C) Radial glial cells persist in the neonatal brain, where they generate oligodendrocytes, olfactory bulb interneurons, and ependymal cells. They also generate astrocytes, some of which remain stem cells in the adult. (D) In the adult brain, adult NSCs retain a radial process and contact both the ventricle and the basal lamina of blood vessels. They generate oligodendrocytes and olfactory bulb interneurons. Abbreviations: Stri, striatum; SVZ, subventricular zone; VZ, ventricular zone.

1.3.1 Transit amplifying progenitors

The transit-amplifying progenitors (TAPs or Type C) represent a class of fast cycling cells within the SVZ. They originate directly from the slow dividing aNSCs.

Clonal analysis highlighted that TAPs undergo 3-4 rounds of symmetric proliferative divisions (Ponti et al., 2013; Calzolari et al., 2015) and seem to divide in close proximity to blood vessels (Tavazoie et al., 2008).

At the molecular level, this cell type can be isolated from the other populations by the expression the EGF receptor (Morshead et al., 1994; Doetsch et al., 2002).

At least two different lineages of the fast proliferating intermediate progenitors have been identified within the SVZ: TAPs that generate oligodendrocytes migrating towards the white matter (Hack et al., 2005; Marshall et al., 2005; Menn et al., 2006; Colak et al., 2008) and TAPs that give rise to immature neuroblasts migrating via the rostral migratory stream (RMS) into the OB (Doetsch et al., 2002; Parras et al., 2004; Hack et al., 2005; Menn et al., 2006; Colak et al., 2008). This difference in TAPs fate is generated at the level of the aNSCs where they either generate neuronal or oligodendroglial lineage (Ortega et al., 2013).

1.3.2 Neuroblasts

Neuroblasts or Type A cells originate from the TAPs. These cells are characterized by their capability to tangentially migrate to the OB. Once the neuroblasts reach the OB, they radially migrate to reach their final location.

These young neurons are characterized by the expression of factors like polysialic acid (PSA-NCAM), doublecortine (Dcx), and CD24. They show properties of already differentiated neurons but continue to divide in the SVZ and on their way to the OB. For this reason, they appear to be BrdU positive upon short pulses of this compound similarly to the TAPs (Luskin, 1993).

The main feature of these cells is that they migrate covering long distances from the lateral wall of the ventricle to reach the OB. The migration along the rostral migratory stream is mediated by molecules like chemoattractants and chomorepellents, as well as proteins of extracellular matrix and proteins present on the cell surface (Hagg, 2005). In addition their migration is regulated by the blood vessels delimiting the route of migration (Snapyan et al., 2009) and the astrocytic tube that avoids the dispersion of cells in the surrounding tissue (Ghashghaei et al., 2007).

In the SEZ-RMS system, both neuroblasts and some TAPs are characterized by the expression of the transcription factor Pax6 (Hack et al., 2005; Brill et al., 2008) and this feature will be relevant in the following chapters.

1.4 The transcription factor Pax6

Pax6 is a member of the Pax family of transcriptional regulators. This gene is located on the chromosome 2 in mice (chromosome 11 in humans) and encodes for a highly conserved protein of 422 amino acids.

Pax6 protein is involved in the development of the eyes, brain, spinal cord, and pancreas. Pax6 is additionally expressed in the pituitary, the gut and the olfactory epithelium (Walther and Gruss, 1991; Callaerts et al., 1997).

In mice, losing the functionality of this protein leads to the death of the animal shortly after birth (Hogan et al., 1986).

In the developing CNS Pax6 is widely expressed including the forebrain, hindbrain, cerebellum and spinal cord (Walther and Gruss, 1991; Grindley et al., 1995).

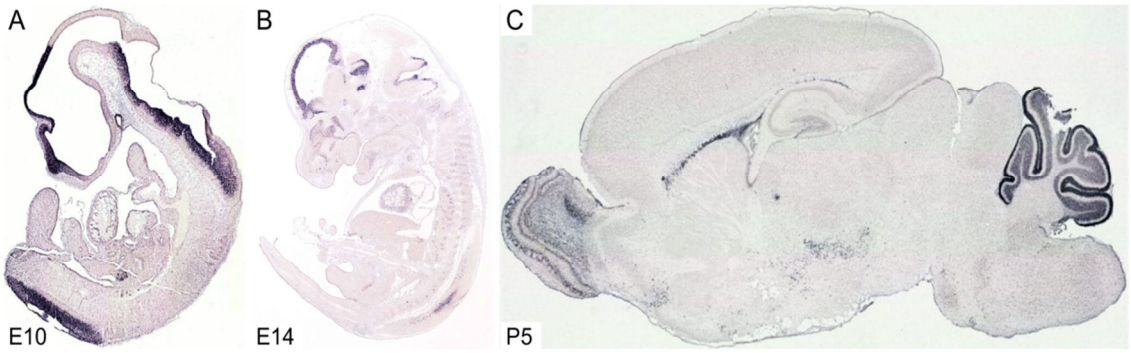


Figure 8: Pax6 expression in embryonic and adult brain
(A-C) Pax6 in situ hybridization from the GenePaint.org the digital atlas of gene expression patterns in the mouse. (A) Pax6 expression at E10, (B) at E14 and (C) at postnatal stages.

In mice (Figure 8) Pax6 starts to be expressed as early as embryonic day 8.5 (E8.5) when the neural tube is closing. In the dorsal telencephalon the peak of expression is reached at E14 (peak of neurogenesis) and declines as gliogenesis starts (E18) (Gotz et al., 1998; Englund et al., 2005).

In the adult CNS, Pax6 is detected in the neurons of various regions including the cerebellum, the thalamus, the olfactory bulb and the amygdala (Stoykova and Gruss, 1994; Engelkamp et al., 1999; Kawano et al., 1999; Yamasaki et al., 2001; Tole et al., 2005).

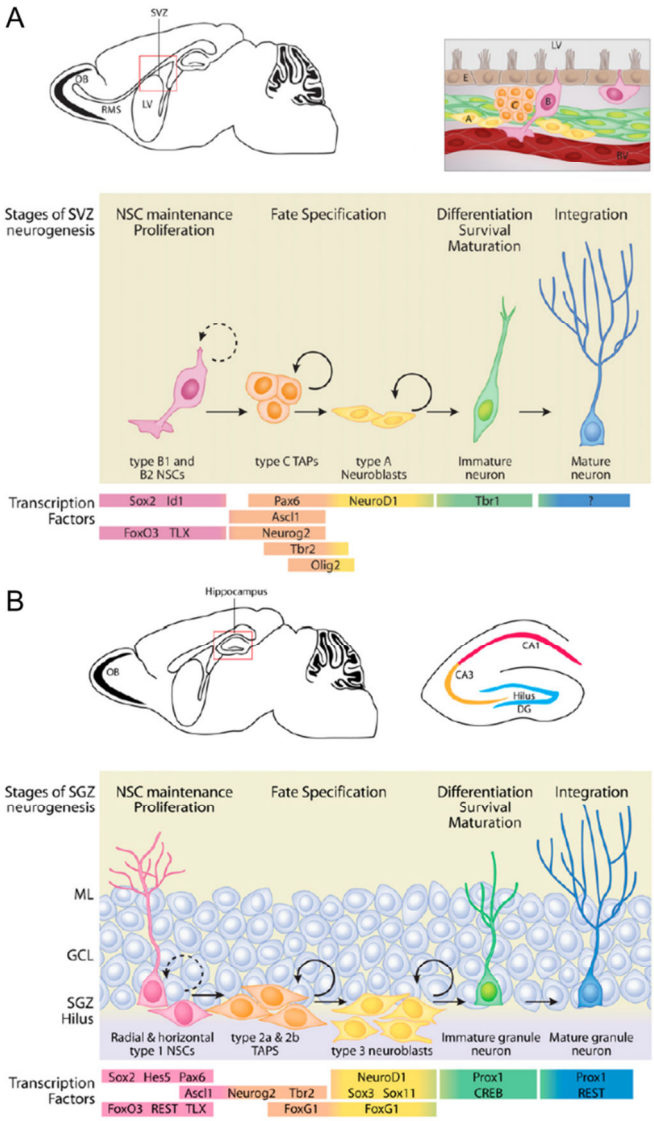


Figure 9: Pax6 expression in the SEZ and SGZ population
(A-B) Schemes taken from (Hsieh, 2012). The scheme is depicting the SEZ (A) and SGZ (B) niches respectively and highlights the expression of Pax6 in certain cells population along the lineage.

Pax6 is expressed in the two most known neurogenic regions of the postnatal brain: the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) and the subependymal zone (SEZ) in the ventricular zone (Hsieh, 2012)(Figure 9).

In the SGZ, the transcription factor is expressed in a subpopulation of neural stem cells, in early progenitor cells presenting a radial glia like morphology and in a small population of late progenitors (Maekawa et al., 2005; Nacher et al., 2005) (Figure 9).

In the SEZ, Pax6 is expressed in a subpopulation of TAPs and immature neurons that migrate via the rostral migratory stream (RMS) to the olfactory bulbs (OB) (Figure 9). In

the OB Pax6 is relevant for the survival of the dopaminergic neurons (Hack et al., 2005; Kohwi et al., 2005; Ninkovic et al., 2010).

1.4.1 Pax6 in development

Most of the knowledge concerning the functions of Pax6 derives from the analysis of the phenotype of mutants animals where the Pax6 function is lost. One of these is the small eye mutant ($Pax6^{Sey}$) which is carrying a non-functional Pax6 protein.
The $Pax6^{Sey}$ is characterized by a natural occurring point mutation that generates a truncated protein lacking the transactivation domain at the C-terminus and considered, for this reason, non-functional (Hill et al., 1991).
From the analysis of these mutants was possible to define the necessity of the Pax6 protein for the eye and brain development. In the heterozygotes mice the eye is smaller, as suggested by the name, but is completely missing when the mutation is in both alleles of the gene (Hogan et al., 1986; Osumi et al., 2008). Pax6 is also essential for the development of the lens and the retina, (Ashery-Padan et al., 2000; Ashery-Padan and Gruss, 2001; Kozmik, 2005; Graw, 2010; Shaham et al., 2012; Cvekl and Ashery-Padan, 2014).
In the brain, losing Pax6 functionality leads to a reduction of the brain size due to a smaller cerebral cortex composed by a thinner cortical plate (Schmahl et al., 1993; Heins et al., 2002; Asami et al., 2011). In the developing telencephalon the transcription factor regulates essential processes like the proliferation of the cells, the neurogenic fate determination and the regionalization of the telencephalon defining the borders between the dorsal and the ventral area (Haubst et al., 2004; Georgala et al., 2011a).

1.4.1.1 Patterning

The establishment of the dorso ventral border delimited by the so called pallial-subpallial boundary (PSB), a physical boundary composes by RG cells highly expressing Pax6, is under the control of the transcription factor Pax6. When this protein is non-functional (e.g.$Pax6^{Sey}$), the PSB is severely disrupted (Stoykova et al., 1997) leading to

an increase of the cell migrating from the ventral telencephalon to the cerebral cortex (Chapouton et al., 1999). In addition, the expression of dorsal factors like Ngn2 and Tbr2 is lost (Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001; Haubst et al., 2004) and ventral factors like Gsx1/2, Ascl1, Dlx1/2 and Olig2 take over spreading in the dorsal area (Stoykova and Gruss, 1994; Stoykova et al., 1996; Stoykova et al., 1997; Toresson et al., 2000; Yun et al., 2001; Heins et al., 2002).

It is not only the specification of dorsal versus ventral regions in the telencephalon that depends on Pax6, but also arealisation of the cerebral cortex (Bishop et al., 2000; Bishop et al., 2002; Muzio et al., 2002a; Muzio and Mallamaci, 2003).

Pax6 is expressed in a gradient with high expression rostro-lateral and low expression caudal-medial, in an opposing manner if compared with the expression of Emx1 and Emx2 (other two dorsally expressed transcription factors). Losing the functionality of Pax6 leads to the expansion of Emx2 and in turn to the anterior expansion of the primary visual area. The loss of a functional Emx2 leads to the caudal expansion of Pax6 and the consequent expansion of domains containing somatosensory and motor neurons (Bishop et al., 2002; Muzio et al., 2002a; Muzio et al., 2002b; Muzio and Mallamaci, 2003).

Changes in regionalization due to loss of functional Pax6 have also been observed in other regions of the CNS, as in the ventral diencephalon, hindbrain and spinal cord (Briscoe et al., 1999) (Stoykova et al., 1996; Grindley et al., 1997; Osumi, 2001).

1.4.1.2 Fate determinant

Pax6 is involved in the regulation of the neurogenic process.

In the dorsal telencephalon of Pax6 deficient mice, neurogenesis is strongly impaired (Schmahl et al., 1993; Heins et al., 2002), whereas the ventral telencephalon is not affected. This impairment is due to the fact that at the peak of neurogenesis the RG cells express the Pax6 protein (Gotz et al., 1998; Hartfuss et al., 2001). The loss of Pax6 functionality in the $Pax6^{Sey}$ mice leads to a decrease in the RG cells neurogenic potential (Heins et al., 2002; Haubst et al., 2004) whereas the basal progenitors in the SVZ are not affected (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). Conversely Pax6 overexpression enhances *in vitro* neural production from RG (Heins et

al., 2002; Haubst et al., 2004). This neurogenic potential is not restricted in time and region since the overexpression of Pax6 results in a fate conversion of already committed glia cells in postnatal (Heins et al., 2002) and fully adult brain derived cells (Hack et al., 2004).

1.4.1.3 Proliferation

The neurogenic function of Pax6 and the capability of regulating the cell cycle are very much linked. The fact that it drives cells to the neuronal fate means that, when overexpressed, Pax6 forces cells to exit the cell cycle and start differentiating.
In the developing cortex, the deletion of Pax6 leads to an increase of proliferation in the progenitors located in non-apical positions (Gotz et al., 1998; Estivill-Torrus et al., 2002; Haubst et al., 2004; Quinn et al., 2007; Tuoc et al., 2009). This increase in proliferation was explained also by the loss of the dorsal area specification, as described before. In addition, when Pax6 is not functional, progenitors located apically delaminate prematurely and contribute to the non-apical increase of proliferating progenitors (Asami et al., 2011).
Differently from the developing cortex, where loss of Pax6 contribute to increase of proliferation, in other regions like the diencephalon, the eye and in different type of cells like the postnatal glia progenitors, when Pax6 is lost proliferation decreases (Warren and Price, 1997; Marquardt et al., 2001; Sakurai and Osumi, 2008). In the cerebellum of $Pax6^{Sey}$ animals, the proliferation of progenitors is not affected (Engelkamp et al., 1999).
These various responses upon lacking of Pax6 functionality underline the fact that the capability of Pax6 to regulate proliferation of cells is not only cell type specific but also region related.

1.4.2 Pax6 protein structure

The Pax6 protein contains different DNA binding domains: the paired domain (PD) at the amino terminal, shared by all the members of the Pax family (Chalepakis et al., 1992); a homeodomain (HD) and a transactivation domain (TA) at the carboxy terminal

(Glaser et al., 1992). The PD is further differentiated in two DNA binding subdomains: the N-terminal subdomain or PAI and the C-terminal subdomain or RED (Walther et al., 1991; Czerny et al., 1993; Epstein et al., 1994b; Epstein et al., 1994a; Jun and Desplan, 1996; Xu et al., 1999). The sequence that is recognized by the PAI is named P6CON whereas the one recognized by the RED is the 5aCON (Epstein et al., 1994b; Chauhan et al., 2004; Xie et al., 2013; Xie et al., 2014).

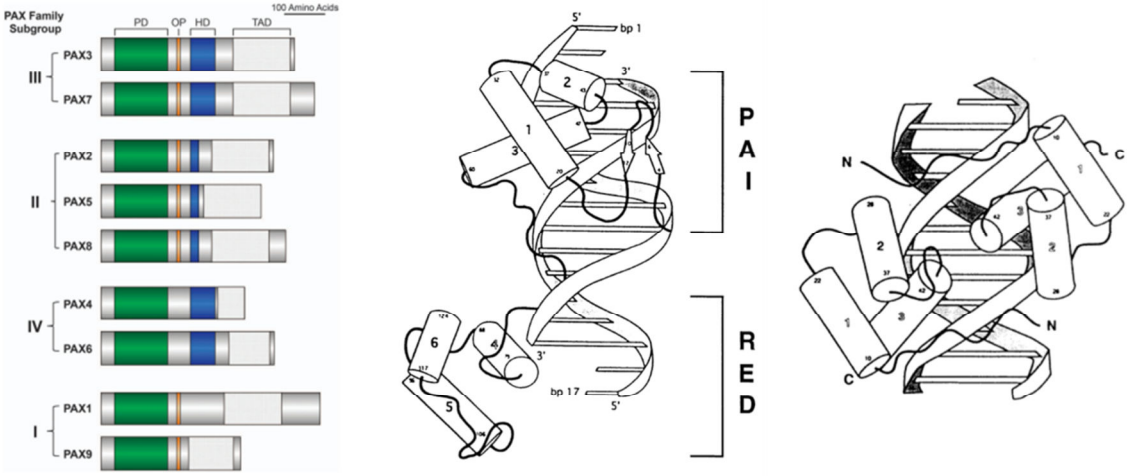


Figure 10: Pax family and Pax6 domains
Graph depicting all the members of the Pax family from (Mahajan et al., 2014) and the structure of the PD and HD of the human Pax6 gene from crystallographic analysis (Callaerts et al., 1997).

Crystallographic analyses of the human PD DNA complex of Pax6 revealed that the PAI domain contains two extended β-sheets and three α-helices between 20 to 60 amino acids residues. The RED subdomain is constituted by three α helical regions (Figure 10). The HD is predicted to have three α-helices where the third of them is contacting the major groove of the DNA and is necessary for sequence recognition (Bruun et al., 2005). Differently from other sequence specific DNA binding proteins that use only one DNA binding domain, Pax6 can use all its DNA binding parts or a range of their combinations to bind to the DNA (Xie and Cvekl, 2011).

The Pax6 gene generates different transcripts variants either by alternative splicing or by the usage of an alternative promoter. One of the most commonly expressed Pax6 variants is the Pax6(5a). This splice variant has an insertion of 14 amino acids in the PAI subdomain (Walther and Gruss, 1991; Glaser et al., 1992; Puschel et al., 1992) leading to abolishment of the binding activity of the subdomain with the retention of the RED domain functionality (Epstein et al., 1994b; Kozmik et al., 1997; Anderson et al., 2002).

The canonical form of Pax6 together with the splice variant Pax6(5a) are expressed in the developing brain, eye, spinal cord and in the olfactory epithelium (Walther and Gruss, 1991; Glaser et al., 1992) even if the Pax6(5a) is expressed at lower levels.
The second major isoforms is missing the PD domain and its activity is restricted to the functional HD and TA. The PD-less form (Mishra et al., 2002) is present in the adult brain, pancreas and eye (Walther et al., 1991; Turque et al., 1994; Grindley et al., 1995; St-Onge et al., 1997) and is found also in the invertebrates differently from the Pax6(5a) which is specific for vertebrates (Miles et al., 1998).
As mentioned in the previous sections, Pax6 is involved in the regulation of many developmental processes, such as the regulation of cell proliferation, neurogenesis and brain patterning. Recently many genes regulating these functions have been identified (Sansom et al., 2009; Wolf et al., 2009; Coutinho et al., 2011; Xie and Cvekl, 2011) and it was also shown how Pax6 positively regulates them (Sansom et al., 2009). However, in order to understand how Pax6 coordinates the regulation of these multiple functions at the molecular level, the role of the different DNA-binding domains has been analyzed in loss- and gain- of function approaches in different regions of the brain (Haubst et al., 2004).
From this analysis was evident that the homeodomain (HD) of Pax6 is not relevant for any of the above mentioned functions of Pax6 during brain development. In the mutant line $Pax6^{4NEU}$, where the DNA-binding properties of the HD are disrupted by a point mutation leading to an amino acid substitution, no defect was evident in the brain region specification, in cell proliferation or differentiation (Haubst et al., 2004). Moreover no defect was detectable also at later stages (Ninkovic et al., 2010). These observations imply that most of the roles exert by Pax6 during development might be mediated by the bipartite PD domain.

1.4.2.1 Pax6 PD mutants: $Pax6^{Leca2}$ and $Pax6^{Leca4}$

In order to discriminate the role of the two subdomain of the PD in regulating neurogenesis, brain patterning and cells cycle regulation, two mouse line $Pax6^{Leca2}$ and $Pax6^{Leca4}$ were analyzed during their brain development (Walcher et al., 2013).

Each of these two mutants is carrying a single point mutation in one of the two subdomains (Thaung et al., 2002) resulting in a substitution of lysine for asparagine (N50K) in the PAI domain ($Pax6^{Leca4}$) or of cysteine for arginine (R128C) in the RED domain ($Pax6^{Leca2}$). The RED domain mutation has been observed also in patients (Azuma et al., 1996).

Both these mutations are predicted to affect the binding capacity of the domains to their consensus site and the milder eye phenotype, compared with the $Pax6^{Sey}$ (full KO for Pax6), would be consistent with a selective disruption of only one of the subdomain at the time (Thaung et al., 2002).

According to the eye phenotype, the craniofacial defects, and the reduction of the OB sizes both the homozygote mice for the two mutants line show similarities with the $Pax6^{Sey}$ (Hill et al., 1991; Tzoulaki et al., 2005). However, the $Pax6^{Leca2}$, in contrast with $Pax6^{Sey}$ and the PAI domain mutants ($Pax6^{Leca4}$), survive to adulthood.

The $Pax6^{Leca4}$ mutants are more similar to the $Pax6^{Sey}$ than the $Pax6^{Leca2}$. While the $Pax6^{Leca4}$ have reduction in brain size due to a reduction in the cortical thickness and the misspecification of the dorso-ventral borders, the RED domain mutants do not presents these aberrations. In the $Pax6^{Leca2}$ the cortical size is comparable with the wildtype (WT) control and as well the borders between dorsal and ventral area are maintained. Those are all considered insights that neither the neurogenic capacity of Pax6 nor its regulation of the brain regionalization is under the control of the RED domain (Walcher et al., 2013) (Figure 11).

In terms of cell proliferation both the domains (PAI and RED) are important to regulate this function of Pax6 but in an opposite manner. In the $Pax6^{Leca2}$ the cells in active mitosis, both the apical and basal progenitors, are almost doubled in their number when compared to the WT. Conversely when the same analysis is carried in the $Pax6^{Leca4}$, both progenitors were significantly decreased. Apical and basal progenitors in both the mutants were still Pax6 and Tbr2/Eomes positive as expected and the RG cells were normal in their morphology. Hence only the number of cells in mitosis but not their identity is affected by the mutation of the PAI and the RED domains (Walcher et al., 2013). Notably this is in contrast with the $Pax6^{Sey}$ where the apical progenitors proliferation is unaffected and the increase in non-apical proliferating cells is resulting

from the migration of cells from the ventral area (Gotz et al., 1998; Haubst et al., 2004; Tuoc et al., 2009) (Figure 11).

The analysis of the RED and PAI mutants showed that Pax6 can exert opposing functions on the same cortical progenitor population, having the intact RED domain the capability of inhibiting proliferation and the PAI domain the capability of promoting proliferation.

Interesting goals would be unraveling the molecular network controlled by the RED domain involved in the cell cycle control and, since this mutants survive to postnatal stages, investigate the repercussion of this mutation also on the adult neurogenesis. These achievements are partially reached with this work.

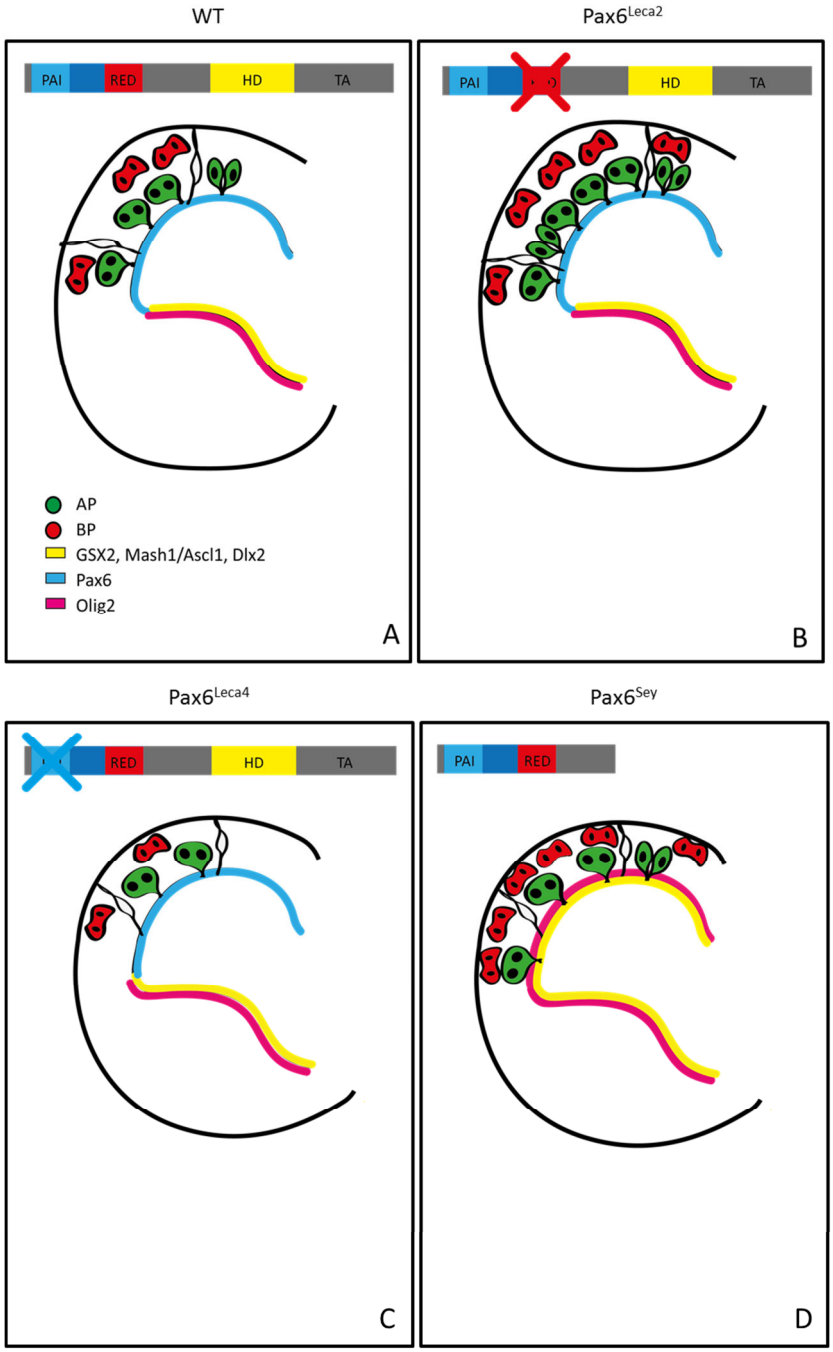


Figure 11: PD domain mutants Pax6^{Leca2} and Pax6^{Leca4}

(A-D) Summary of the phenotype observed in the developing cortex of Pax6^{Leca2} and Pax6^{Leca4} mice in comparison to the WT and the Pax6^{Sey} in terms of regulations of progenitors proliferation, patterning and neurogenic functions of Pax6. (A) WT, (B) Pax6^{Leca2} that have no defects in patterning and neurogenic potential but have an increase in the mitotically active cells in SVZ and VZ. Opposite situation in Pax6^{Leca4} (C) where mitotic figures are decreased, there are patterning defects and the brain size is reduced with a thinner cortical plate. (D) Pax6^{Sey}. Abbreviations: AP, apical progenitors; BP basal progenitors; PD, paired domain, HD, homeodomain; TA, transactivation domain.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Chemical	Company
4',6-diamidino-2-phenylindole (DAPI)	AppliChem
5'-bromo-2'-deoxyuridine (BrdU)	Sigma-Aldrich
Acrylamide 30%	Roth
Agarose	Biozym
Ampicillin	Roth
Aqua ad iniectabilia	Braun
Bovine Serum Albumin (BSA)	Sigma-Aldrich
Difco LB-Agar	Hartenstein Laborversand
DNA Ladder (Generuler 1kb)	Fermentas
D-Saccarose	Roth
EDTA	Merck
Ethanol	Merck
Glycerol	Sigma-Aldrich
Glycine	AppliChem
HEPES	Roth
Hydrochloric acid	Merck
Isopropanol	Merck
Kanamycin sulfate	Roth
Low melting Agarose	Biozym
Mercaptoethanol	Merck
Methanol	Merck
Milk powder	Roth
Normal goat serum (NGS)	Vector Laboratories

N-P40 (Nonidet-P40)	ICN Biomedicals
Paraformaldehyde (PFA)	Merck
PCR dNTP Mix (25mM each)	Fermentas
Protein ladder Page ruler plus	Thermo Scientific
Proteinase K	Roth
Sodium dodecyl sulphate (SDS)	Roth
Sodium phosphate ($Na_2HPO_4 \bullet 7H_2O$)	Merck
SYBR green	Qiagen
SYBR Safe	Life Technologies
TEMED	Sigma-Aldrich
Tissue Tek	Hartenstein Laborversand
Tris Base	Sigma-Aldrich
Tris-HCl	Roth
Triton-X100	Roth
Tween-20	Sigma-Aldrich

2.1.2 Kits

Kit	Company
BigDye Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems
DC Biorad protein Assay Kit	Biorad
Dye Ex 2.0 Spin Kit	Qiagen
ECL Western Blotting Detection Reagents(Amersham)	GE Healthcare
EndoFree Plasmid Maxi Kit	Qiagen
Maxima First Strand cDNA Synthesis Kit	Thermo Scientific
QIAquick Gel Extraction Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
RNase Free DNase set	Qiagen

RNeasy Mini Kit	Qiagen
Taq DNA Polymerase	Qiagen

2.1.3 Tissue culture reagents

A summary of the media and reagents, needed to cultivate cells used in this work, follows in this section.

Reagent	Company
B-27 Serum-Free Supplement	Gibco (Life technologies)
BD Matrigel Matrix Growth Factor Reduced	BD Biosciences
Bovine serum albumin (BSA)	Sigma-Aldrich
D-Glucose (Stock 45%)	Sigma-Aldrich
DMEM (Dulbecco's Modified Eagel Medium)+4.5g/ml glucose and GlutaMAX	Gibco (Life technologies)
DMEM:F12 (Dulbecco's Modified Eagel Medium:Nutrient Mixture F12)+ GlutaMAX	Gibco (Life technologies)
DMSO	Sigma-Aldrich
EGF (Epidermal Growth Factor)	Roche
FCS (Fetal Calf Serum)	Pan Biotech
FGF2 (Fibroblast Growth Factor-2)	Roche
HBSS 1X (Hank's balanced salt solution)	Gibco (Life technologies)
HEPES (1M)	Gibco (Life technologies)
Horse serum (HS)	Pan Biotech
Hyaluronidase	Sigma-Aldrich
Laminin	Roche
L-Glutamine (200mM)	Gibco (Life technologies)
Lipofectamine 2000	Life technologies
MEM Non-Essential Amino Acids Solution (100X)	Gibco (Life technologies)
N2 supplement	Gibco (Life technologies)

Neurobasal	Gibco (Life technologies)
Opti-MEM +GlutaMAX	Gibco (Life technologies)
Penicillin/Streptomycin (Pen/Strep)	Gibco (Life technologies)
Poly-D-Lysine (PDL)	Sigma-Aldrich
Poly-L-ornithine	Sigma-Aldrich
Trypan Blue	Gibco (Life technologies)
Trypsin	Sigma-Aldrich
Trypsin (EDTA), 0.05%	Gibco (Life technologies)

2.1.4 Standard solution and media

2.1.4.1 Phosphate Buffer Saline (PBS) (0.15M)

1.4M NaCl

27mM KCl

83mM Na_2HPO_4

15mM KH_2PO_4

5L of 10X stock solution of PBS are prepared by dissolving 400g NaCl; 10g KCl; 10g KH_2PO_4 and 73.66g Na_2HPO_4 x 2 H_2O in ultrapure water followed by adjustments of the pH to 6.8. After autoclaving, the stock solution is diluted 1:10 with autoclaved water to have 0.15M 1X PBS with a pH 7.4.

2.1.4.2 Paraformaldehyde (PFA) 4%

4% diluted PFA is prepared dissolving 40g of Paraformaldehyde in 800ml ultrapure water by gentle heating during stirring with the addition of 3NaOH pellets to enable the PFA powder to dissolve. The solution is cooled down to room temperature and the volume adjusted to 1L with 1X PBS The pH is then adjusted to 7.2 -7.3. The solution is then filtered through a paper filter and stored at 4°C.

2.1.4.3 50X Tris Acetate EDTA (TAE) Buffer

2M Tris acetate

50mM EDTA

To prepare 1L of 50X TAE-buffer 242g Tris Base; 57.1ml Acetic Acid and 100ml 0.5M EDTA are dissolved in 800ml autoclaved H_2O. The pH is then adjusted to 8.0 with HCl and the solution filled up to 1L with autoclaved H_2O.

2.1.4.4 Lysis Buffer for tail DNA extraction

100mM Tris, pH 8.0-8.5

200mM NaCl

5mM EDTA, pH8.0

2% SDS

1L of Lysis buffer is prepared by mixing 100ml 1M Tris HCl; 10ml 0.5M EDTA; 20ml 10% SDS; 200ml 1M NaCl and 660ml autoclaved H_2O. To proceed to the lysis, 10µl/ml of Proteinase K (10mg/ml) is added freshly to the Lysis solution.

2.1.4.5 LB medium (Luria-Bertani)

0.5% (w/v) NaCl

1% (w/v) Bacto-Tryptone

0.5% (w/v) yeast extract

20mM Tris/HCl pH 7.5

The pH value was adjusted to 7.0 with NaOH.

2.1.5 Solutions and media

2.1.5.1 Ex vivo live imaging

2.1.5.1.1 Dissection Medium

7.8g DMEM/F12 powder

0.6g NaOH

1.45g Glucose

5ml Penicillin/Streptomycin

495ml qH_2O

2.1.5.1.2 Reconstitution Medium

10ml HEPES 1M

1.25ml 2M NaOH

1.09g $NaHCO_3$

40ml qH_2O

2.1.5.1.3 Collagen Matrix

320µl cellmatrix Type I-A (Nitta Gelatin)

120µl 5xDMEM

60µl Reconstitution medium

2.1.5.1.4 Slice culture Medium

17.4ml Dissection Medium

1ml HS

1ml FCS

400µl B27

200µl N2

2.1.5.2 Ex vivo SEZ explant

2.1.5.2.1 Explants culture Medium

Neurobasal (Gibco-Life Technology)

1ml B27 supplement

400µl HEPES

500µl P/S

500µl D-glucose

2.1.5.3 Cell culture Media

2.1.5.3.1 Neurosphere preparation and maintenance

2.1.5.3.1.1 Solution1

10% HBSS

1.8% Glucose

Diluted in ddH_2O with final pH 7.5

2.1.5.3.1.2 Solution2

30% sucrose dissolved in 0.5x HBSS with pH7.5.

2.1.5.3.1.3 Trypsin inactivating solution (Solution3)

4% BSA

20mM HEPES

Diluted in EBSS with a final pH 7.5

2.1.5.3.1.4 Dissection medium

1.33mg/ml trypsin

0.7mg/ml hyaluronic acid

Trypsin and hyaluronic acid are dilute in a Solution1

2.1.5.3.1.5 Adult neurosphere Medium

8mM HEPES

1% penicillin/streptomycin

2% B27

10ng/ml EGF/FGF2

Diluted in DMEM:F12 (GlutaMAX)

2.1.5.3.1.6 Embryonic neurosphere Medium

1% D-glucose

1% penicillin/streptomycin

2% B27

0.2% EGF andFGF2

Diluted in DMEM: F12 (GlutaMAX)

2.1.6 Cell lines

In this work are used different cell lines for various purposes. These are recorded below.

Bacterial cell lines:

- DH5α Competent E.Coli (Invitrogen- Life Technologies)
- DB 3.1, ccdB resistant, Competent E. Coli (Invitrogen- Life Technologies)

Eukaryotic cell lines:

- P19 carcinoma cells (kindly provided by Dr. Francois Guillemot)

2.1.7 Cloning and expression plasmids

Plasmid	Origin
164-pENTR1A	Kindly provided by Dr. Alexandra Lepier
311-pCAGGS-Dest	Kindly provided by Dr. Alexandra Lepier
374-pLV(CAG)-Dest-GFP	Kindly provided by Dr. Alexandra Lepier
pCAGCre	Shitamukai et al. 2011
pCAG-Floxp-mKO2-F	Shitamukai et al. 2011
pCAG-GFP-DEST	Kindly provided by Dr. Paolo Malatesta
pCAG-HJURP-GFP	Stefania Petricca
pCAG-Leca2-GFP	Tessa Walcher
pCAG-Leca4-GFP	Tessa Walcher
pCAG-Pax6-GFP	Kindly provided by Dr. Robert Blum
pENTR6-GW-grandestuffer	Kindly provided by Dr. Paolo Malatesta
pmiRNA-1202	Stefania Petricca
pmiRNA-946	Stefania Petricca

2.1.8 Primary antibodies

Following is the list of primary antibodies used for immunostaining.

Antigen	Species-Isotype	Company	Catalog #	Dilution	Treatment
Anti-5-bromo-2'-deoxyuridine (BrdU)	Rat	Abcam	AB6326	1:200	HCl treatment
Anti-Acetylated Tubulin	mouse IgG2b	Sigma Aldrich	6-11B-1	1:500	
Anti-Activated Caspase3	rabbit	Millipore	AB3640	1:200	
Anti-Doublecortin (Dcx)	guinea pig	Millipore	AB2253	1:1000	

Anti-Glial fibrillary acidic protein (GFAP)	rabbit	Dako	Z0334	1:500	
Anti-Glial fibrillary acidic protein (GFAP)	mouse IgG1	Sigma Aldrich	G3893	1:1000	
Anti-Green Fluorescent Protein (GFP)	Chick	Aves Labs	GFP-1020	1:1000	
Anti-Holliday Junction Recognition Protein (HJURP)	rabbit	Sigma Aldrich	HPA008436	1:250	
Anti-Paired box protein6 (Pax6)	rabbit	Millipore	AB2237	1:500	
Anti-Paired box protein6 (Pax6)	mouse IgG1	Abcam	ab78545	1:200	
Anti-Phospho Histone H3 (Ser10)	rabbit	Millipore	06-570	1:200	
Anti-Phosphorilated Vimentin (Ser55)	mouse IgG2b	MBL	D076-3	1:100	
Anti-T-box brain protein 2 (Tbr2)	Chick	Millipore	AB15894	1:500	
Anti-β-III-Tubulin	mouse IgG2b	Sigma Aldrich	T8660	1:500	

2.1.9 Primers used for qPCR analysis

Below are catalogued the primers used to evaluate the levels of specific genes via qPCR.

Primer	Sequence
Paired forward	CAGCTTGGTGGTGTCTTTGT
Paired reverse	GCAGAATTCGGGAAATGTCG
Splice forward	GCAGATGCAAAAGTCCAGGT
Splice reverse	CTCGTAATACCTGCCCAGAA
Homeo forward	TCTAATCGAAAGGGCCAAATG
Homeo reverse	AGGAGGAGACAGGTGTGGTG
Bub1 forward	AGCATGAGCAGTGGGTTAGT
Bub1 reverse	TCCTGCTGGGAGCAAGTATT
Bub1 forward_2	GCATGAGCAGTGGGTTAGTG
Bub1 reverse_2	TTCCTGCTGGGAGCAAGTAT
HJURP Forward	GCCCCAGGGGTTGAGAGTTT
HJURP Reverse	TAAGCTCGTATCCGCACCGC

2.2 Methods

2.2.1 In vivo methods

2.2.1.1 Mouse lines

All the animals used in this work were kept in the animal facility of the Helmholtz Zentrum München. All the experimental procedures were performed in accordance with German and European Union guidelines. Animals are maintained on a 12h light-dark cycle. The day of the vaginal plug is considered as embryonic day 0 (E0) and the day of birth as postnatal day 0 (P0). In this study, except when specifically expressed, the $Pax6^{Leca2}$ mouse line (Thaung et al., 2002) is used.

This line was originally obtained from the GlaxoSmithKlein research.

Animals were crossed two generation with the CD1 wild type strain and afterwards maintained by interbreeding (original background: 129S6). Currently animals are backcrossed to C3HeB/Fej background (3^{rd} generation).

Characteristic of the mouse line used in this work:

Name	Symbol	Mutation description	Mouse strain
Paired box 6; lens corneal adhesion 2	$Pax6^{Leca2}$	Chemically induced (ENU) Single point mutation (C586T RED domain)	Original Background:129S6 crossed 2 generations with CD1WT and maintained by interbreeding

2.2.1.2 Anesthesia

To perform in utero operations, mice are anaesthetized by intraperitoneal injection of a “Sleep” solution containing: Fentanyl (0.05 mg/kg), Midazolam (5 mg/kg) and Medetomidine (0.5 mg/kg). The anaesthesia is terminated with a subcutaneous injection of an “Awake” solution composed of: Buprenorphine (0.1 mg/kg), Atipamezol (2.5 mg/kg) and Flumazenil (0.5 mg/kg).

To perform transcardial perfusion, juvenile and adult animals are anesthetized with an intraperitoneal injection of Ketamine (Ketamine hydrochloride 100µg per gram of bodyweight) and Rompun (Xylazinhydrochloride, 20µg per gram of bodyweight) prepared in 0.9% NaCl solution (Braun).

2.2.1.3 In utero electroporation

Surgeries were performed on animals in accordance with the guidelines of Government of Upper Bavaria under licence number 55.2-1-54-2532-79/11.
E13 pregnant females are anesthetized as described in the section above and operated as previously described in (Saito, 2006). In brief, the shaved abdomen is opened by caesarian section in order to expose the uterine horns. These are kept wet and warm by continuous application of pre-warmed saline. Endotoxin free plasmids -diluted to 1µg/µl- are mixed to Fast green (2.5 mg/µl, Sigma). 1µl of mix is injected into the ventricle with the aid of glass capillaries (self-made with a micropipette puller).
DNA is electroporated into the telencephalon (dorsal or ventral according to the orientation of the electrodes) with four pulses of 38mV and 100ms. At the end of the electroporation, the uterine horns are repositioned into the abdominal cavity, which is then filled with pre-warmed saline. The abdominal wall is then sewn closed by surgical sutures with medical sewing equipment. Anaesthesia is terminated as described above and animals are monitored while they wake up and recover from anaesthesia.
The day after (E14), operated animals are anesthetized with CO_2 and sacrificed by cervical dislocation. Embryos are placed into HBSS (Hank's Balanced Salt Solution -Gibco, Life Technologies-) supplemented with 10mM Hepes (Gibco, Life Technologies). Embryos are dissected and brains are fixed; used for FACS sorting or used for *ex vivo* life imaging.

2.2.1.4 Fixation of embryonic mouse brain and cryosectioning

Embryonic brains are fixed in 4% Paraformaldehyde (PFA) with gentle rotation at 4°C for 1,5-6h (E10/E12: 1,5h; E14: 2h; E16: 4h; E18: 6h). Brains are then washed with PBS and cryoprotected in 30% sucrose (diluted in PBS) overnight followed by embedding in

Tissue-Tek and frozen on dry ice. Tissue is then stored at -20 °C until it is cryosectioned in 20-30µm slices with a Cryostat (Leica).

2.2.1.5 Ex vivo live imaging

To perform *ex vivo* live imaging Pax6^{Leca2} embryos are electroporated in utero as described before (section 2.2.1.3) and the embryos processed the day after (E14).
Briefly to the procedure: the pregnant mother is sacrificed by cervical dislocation at day E14 and the uterus with the embryos is placed in the ice cold dissection medium.
After dissection of the brains, with consequent removal of the meninges, the brains are transferred into a plate with low melting agarose at 42°C.
The brains are then oriented with the olfactory bulb facing the operator and the plate left in ice to solidify.
The blocks of agarose containing the brains are then fixed on the vibrotome support (Leica) using the Roti Coll I (Carl Roth) and cut in 300µm tick sections.
The sections are then cleaned of the excess of agarose and embedded in collagen matrix.
The slices embedded in the matrix are finally placed onto the filter (cell culture insert; Millicell PICMORG50) and the plate is put in the incubator (37°C, 5%CO_2) for 10 min to allow the collagen to solidify and only at that point the culture medium is added.
The slices are then incubated for at least 30min in order to stabilize the whole culture system and minimize movements during the movie acquisition.
The movie is performed using a confocal microscope Olympus FV1000 cLSM system (Olympus), using the FW10-ASW 4.0 software (Olympus).
Images are acquired every 15min for about 24h.
Data processing and analysis are performed with Fiji software, an open source image processing package based on ImageJ (Schindelin et al., 2012).

2.2.1.6 BrdU administration

In adult mouse, 100mg/kg bodyweight of DNA base analogue 5 bromodeoxyuridine (BrdU) is injected intraperitoneally 1h before sacrifice to label fast proliferating cells.

BrdU is dissolved at a concentration of 10mg/ml in sterile 0.9 % NaCl solution (Braun) by continuous stirring at 37°C for 2h.

2.2.1.7 Fixation of adult mouse brain and sectioning

Adult animals are anesthetized by intraperitoneal injection as previously described (section 1.2.1.2). They are transcardially perfused first with a PBS in order to clean up the circulation from the blood and afterwards with 4% PFA as fixative. The fixed brains are then removed and post fixed in 4% PFA overnight at 4°C in continuous rotation.
After post fixation, brains are washed in PBS for a few hours and embedded in 3% agarose diluted in PBS. After the agarose hardened, brains are sliced with a vibrotome (Zeiss) at thickness of 70-100µm and collected in PBS.

2.2.1.8 Ex vivo SEZ explant preparation

To assess the migratory ability of neuroblasts (cells located in the lateral wall of the SEZ characterized by the capacity to migrate via the RMS to the OB) of postnatal animals, explant of the SEZ were taken out from P15 animals and cultured for 7div.
The procedure implies that P15 animals are sacrificed by cervical dislocation and the brain dissected.
The SEZ is isolated as described before in (Fischer et al., 2011) and the explants are made according to the guidelines proposed in (Gierdalski et al., 2011)
Briefly, explants of the diameter of about 0.5mm (using Harris Uni-Core) are moved from ice cold dissection medium (1xHBSS supplemtend with 10mM HEPES), into a drop (0.25-0.5ml) of ice cold Matrigel. Using a 20µl pipette tip (kept on ice to avoid that the Matrigel sticks on it) the explants are posed on a glass coverslip and let seed for 5 min at room temperature. The explants are then covered with additional 30µl of Matrigel and let it set in the incubator at 37°C for 15min.
Finally 2ml of explants culture medium is added to the explants.
The explant are then incubate at 37°C in 5%CO_2, 95%O_2 for 3 days, time in which the migratory behavior is analyzed.

2.2.2 Methods in cell biology

2.2.2.1 Cell culture

2.2.2.1.1 Preparation of coated coverslips

Glass cover slips are washed with acetone and boiled in ethanol containing 0.7% HCl for 30min. After two washing steps in 100% ethanol, these are dried at RT and autoclaved for 2h at 180°C. To each well of a 24-well plate one sterilized cover slip is added and pre-wetted with PBS. The PBS was replaced by 1% poly-D-lysine (PDL) dissolved in PBS or 1% Poly-dL-Ornithine (PORN) dissolved in water and plates are incubated for at least 2h at 37°C.
The wells are washed three times using autoclaved ultrapure water. The PDL coated plates are left drying for 2h in the laminar flow before being transferred to the fridge and stored at 4°C until needed; on the PORN coated plate, Laminin (dissolved in water) is directly put on them and left for about 3h before use.

2.2.2.1.2 Adult neurosphere preparation

Adult animals are scarified by cervical dislocation and decapitated. The skull is opened; the brain dissected and collected in ice-cold HBSS containing 10mM HEPES. The adult subependymal zone (SEZ) is dissected out as described before in (Fischer et al., 2011). Afterwards the tissue is incubated in the dissociation solution at 37°C for 15min and after gentle trituration, incubated for another 15min. In order to inactivate trypsin Solution 3 is added and cells are passed through a 70µm cell strainer. Next cells are centrifuged at 1300rpm for 5min and suspended in Solution2. Another centrifugation step follows at 2000rpm for 10 min. The cell pellet is suspended in 2ml of Solution3, added on top of 10ml of cold Solution3 and centrifuged at 1500rpm for 7min. Finally, cells are suspended in adult neurosphere medium. The cell concentration is determined by Trypan Blue dye exclusion and primary sphere forming cells are seeded at a cell density of 5-10cells/µl.

Flasks containing the floating cells are incubated at 37°C with 95%O_2 and 5% CO_2. Every second day, fresh EGF and FGF2 are added.

Cells are passaged three times before differentiation. The passaging takes place once a week by centrifugation at 1200rpm for 5min, followed by enzymatic dissociation (0.05% trypsin [EDTA]) and re-plating in 50% neurosphere conditioned medium and 50% fresh adult neurosphere medium. From the second passage on, cells are seeded at a clonal density of 1cell/µl.

To differentiate the neurosphere forming cells after the third dissociation step (third passage) 200.000 cells per well are plated in a 24 well plate (PDL coated) in 1ml of adult neurosphere medium. After 2h from plating, cells are virally transduced and left in their medium for 7days before analysis.

2.2.2.1.3 Embryonic neurosphere

E14 embryos are removed from the mother and collected in ice-cold HBSS containing 0.01M HEPES. The brains are dissected out with careful removal of the meninges. Dorsal telencephalon is cut as well as the ganglionic eminence but separated from the olfactory bulb and the hippocampal anlage. Cortices and GEs are kept in separate tubes filled with ice cold HBSS. The tissue is then allowed to settle; the balanced salt solution is replaced by 1ml 0.05% trypsin (EDTA) and incubated for 15min at 37°C. Afterwards 2ml of DMEM (4.5g glucose, GlutaMAX) supplemented with 10% FCS and 1% penicillin/streptomycin, is added in order to block the enzymatic activity. The tissues are then mechanically dissociated with a fire polished Pasteur pipette coated with serum and centrifuged for 5min at 1200rpm.

The pellet cells are then cultivated in flask in embryonic neurosphere medium.

The neurosphere are passaged 3 times for three consecutive weeks. After the third passage, the cells are allowed to seed, on PDL coated dishes and let them differentiate in neurophere medium and normally 2h after plating the cells were transduce with different viral constructs.

2.2.2.1.4 P19 cells cultivation

P19 cells (embryonic carcinoma cell line derived from an embryo-derived teratocarcinoma in mice) are cultivated as described next.

Medium

- DMEM (Dulbecco's Modified Eagles Medium)
- 10% FCS
- 1% Penicillin/Streptomycin
- 1% MEM Non-Essential Amino Acids Solution (Life Technology)

The cells are cultivated at 37°C and 5% CO_2, 95% O_2. They are passaged when they reach the confluence of 80%.

For passaging, cells are washed with sterile 1xPBS once, and let detach with 2ml of Trypsin-EDTA for 1-2min at 37°C. The detached cells are re-suspended in 2ml fresh growth medium until a single cell suspension is present. Aliquots (1/20-1/3) of the re-suspended cells are plated on Petri dish containing 10ml fresh medium and equally distributed by gentle shaking.

2.2.2.1.5 Cell time lapse imaging

Live imaging of dissociated cultures (2.2.2.1.2,2.2.2.1.3) or from P19 cells (2.2.2.1.4) is performed with a cell observer (Zeiss) at a constant temperature of 37°C and 5% CO_2.

Phase contrast images are acquired every 10min for 2-7 days, according to the type of culture, using a 20x phase contrast objective (Zeiss) and an AxioCamHRm camera with a VBA module remote controlling Zeiss AxioVision 4.7 software (Rieger and Schroeder, 2009). Single-cell tracking is performed using a custom made computer program (TTT)(Rieger and Schroeder, 2009). Movies are analyzed using the TAT player from the TTT software and assembled using the open source imaging software Fiji (Schindelin et al., 2012).

2.2.2.2 Immunostaining

Immunohistochemistry (immunostaining performed on fixed tissue) and immunocytochemistry (immunostaining performed on cell culture sample) are performed with the primary antibodies listed in paragraph 2.1.8 diluted in a solution containing 0.5% TritonX in PBS and 10% NGS (Normal Goat Serum).

In general, primary antibodies are applied overnight at 4°C in humidified chambers to avoid evaporation of the staining solution. Afterwards the cells coverslips or the tissue slices are washed with PBS prior the application of the secondary antibody staining solution for 2h at room temperature. To visualize cell nuclei, DAPI (4', 6-diamidino-2-phenylindole) is included in the solution with a concentration of 0.1µg/ml.

The secondary antibodies used are isotype specific antibodies conjugated to Alexa-488, Alexa-543, Alexa-633 (dilution 1:1000 in staining solution –Invitrogen, Life Technologies-) or Cy2, Cy3, Cy5 (dilution 1:500 in staining solution -Jackson Immuno Research-).

As last step, cells and sections are again washed in PBS and mounted with Aqua Poly/Mount, a mounting medium that enhances and retains fluorescent stains.

The mounted samples are kept for long storage at room temperature.

Some antibodies required a specific pre-treatment to retrieve the antigen.

In particular for the anti- BrdU antibody the HCl pretreatment is required. This consists of a 30min incubation of the samples at room temperature with 2N HCl and two consequent washing for 15min each with 0.1M sodium-tetraborate.

All the antibodies used, together with the working concentration and the treatment conditions are listed in the section 2.1.8.

2.2.2.3 Image acquisition and processing

Immunohistochemistry images are acquired with an Olympus FV1000 cLSM system (Olympus), using the FW10-ASW 4.0 software (Olympus). The system is equipped with laser diodes 405nm, 559nm and 635nm. Colour adjustments are performed in the acquisition software, or using the open source imaging software Fiji (Schindelin et al., 2012).

2.2.2.4 Transfection of cells

The Lipofection, also called liposome transfection, is used to be able to introduce specific genetic materials into cells. To do so cells are plated on 6 well dishes (when need form RNA or protein extraction) or 24 well dishes (in case of transfection prior to 2D-imaging) at 30-40% confluency the day before transfection in 2ml total volume. Before transfection, growth medium is collected and replaced with OptiMEM. Cells are transfected with Lipofectamine2000 according to the protocol of the manufacturer. 2µg of plasmid DNA is used per well in a 6 well dish format and 1µg is used per well of a 24 well dish. 2-4 hours after transfection, OptiMEM is replaced by growth medium. Reporter expression was generally visible 12-16 h later.

2.2.3 Methods in molecular biology

2.2.3.1 Mouse tail DNA extraction

Parental animals are maintained in a heterozygote allelic state and crossed to obtain all three possible genotypes (WT/WT; WT/-; -/-) from the same litter.

To sort out the different genotypes in the $Pax6^{Leca2}$ colony, the homozygote mice are distinguished by eye phenotype whereas wild type and heterozygote mice are genotyped by PCR followed by sequencing on tail DNA.

In details, 1-3mm long tails from ear marked mice (4-5 weeks old) are incubated overnight at 55°C in 300µl of Lysis Buffer supplemented with 2µl of Proteinase K (1mg/mL) in order to digest contaminating proteins and inactivate nucleases in the nucleic acid preparation.

The following day, the samples are cooled down to room temperature with the addition 100µl of protein precipitation solution. Samples are then vortexed and centrifuged at 13000rpm to remove hairs and tissue residues. The supernatant is poured in 300µl of Isoporapanol and mixed by inverting. The precipitate is then washed with 300µl of 70% ethanol and dissolved in 50µl of DNA hydration solution (Qiagen) and purified DNA is dissolved at 65°C for 1h.

2.2.3.2 PCR genotyping

$Pax6^{Leca2}$ colony is genotyped with a standard PCR mix (Qiagen kit) containing 1nM of each required primer (Pax6Leca2 forward primer: TCCAGGATGGCTGGGAGCTT; Pax6Leca2 reverse primer: TTGCGTGGGTTGCCCTGGTA); 1nM of dNTPs (dATP, dCTP, dGTP, dTTP); 0.5µl of $MgCl_2$; 1.5U of Taq DNA polymerase; 2.5µl of 10X PCR buffer and 2µl of genomic DNA (dilution1:10 in H_2O) with a final volume of 25µl per reaction. The DNA is amplified under these cycling conditions:

	94°C	2min
30x	94°C	50sec
	57°C	50sec
	72°C	50sec
	72°C	5min
	16°C	∞

2.2.3.3 Sequencing

To be able to genotype the colony, characterized by the presence of a single point mutation, it is necessary to sequence the Pax6 RED domain to localize the mutation. To do so, consequently to the first PCR, the products need to be cleaned. The 295bp amplicone is purified using the QIAquick Gel Extraction Kit (Qiagen) according to the instructions of the manufacturer. A second PCR is then run using 0.5µl of Big Dye Terminator (containing the polymerase); 2µl of Big Dye Buffer; 0.5µl of Primer sense or antisense (from 100µM stock) and 2µl of DNA template with a total of 5µl PCR reaction volume.

The sequencing PCR is then performed according to the following cycling conditions:

	96°C	1min
35x	96°C	10sec
	50°C	5sec
	60°C	4min
	16°C	∞

The sequencing PCR is then purified using Dye Ex 2.0 Spin Kit (Qiagen) according to the instructions of the manufacturer and filled in a sequencing plate.

The sequencing is performed at the Sequencing Core Facility of the Helmholtz Zentrum München and the sequence analyzed with the use of the free available software ApE (A plasmid Editor by Wayne Davis).

2.2.3.4 RNA extraction

The total RNA for qPCR analysis was extracted from cortices and lateral ganglionic eminences of WT and $Pax6^{Leca2-/-}$ littermates in different developmental stages in order to evaluate the expression of the two isoforms of Pax6 and confirm Microarray data from previous study (Walcher et al., 2013).
The RNA derived from P19 cell lines was also used in order to evaluate gene expression changes upon overexpression of different forms of Pax6 and HJURP at different time points after overexpression.
In all the cases the RNA was extracted using RNeasy Mini Kit (Qiagen) according to the protocol suggested by the manufacturer.

2.2.3.5 Determination of the concentration and quality of nucleic acids

The concentrations of nucleic acids are determined using the Nanodrop (NanoDrop 1000 Thermo Scientific. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio around 1.8 is generally accepted as “pure” for DNA; a ratio around 2.0 is generally accepted as “pure” for RNA.
The quality of RNA was further assessed by using the Agilent Bioanalyzer (Agilent 2100 Bioanalyser). Agilent Bioanalyzer creates electrophorograms measuring the abundance of ribosomal 5S, 18S, and 28S RNAs. This also assigns RNA Integrity Number (RIN) that ranges from 1 (totally degraded RNA) to 10 (completely intact RNA). For Real Time PCR were used high quality RNA samples (RIN > 7).

2.2.3.6 cDNA synthesis and quantitative PCR analysis

The cDNA is synthetized from the total RNA extracted (500ng/µl) using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) according to the instructions of the manufacturer.

The qPCR is performed using a QuantStudio™ 6 Flex Real-Time PCR System (Life Technology), gene specific primers listed above in the section 2.1.9, and the SYBR green master mix (Qiagen). GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) is used as reference gene for normalization of the expression of the target gene. The relative expression of each mRNA is calculated using the delta Ct method between the gene of interest and the reference gene:

($R = \frac{1}{2^{-(-\Delta Ct)}}$).

Following the cycling conditions:

Hold stage		
	95°C	5min
PCR stage		
40x	95°C	10sec
	60°C	30sec
Melt curve stage		
	95°C	15sec
	60°C	1min
	95°C	15sec

2.2.3.7 Cloning

In order to overexpress the HJURP, its cDNA (Source BioScience) (first confirmed for the correct sequence upon sequencing) was cloned into the pENTR1A vector by sequential digestion with the restriction enzymes SalI and NotI. Finally the HJURP cDNA was shuttled into the pLVCAG-GFP destination vector by LR reaction (Gatway system). The resulting plasmid was checked by sequencing.

To achieve the knock down of the HJURP gene, the RNAi technology was used by designing miRNA against the gene of interest using the BLOCK-iT™ RNAi Express tool (Life Technology).

The double stranded oligos were generated according to the protocol of the manufacturer. The two miRNAs (HJURP946 and HJURP1202, according to the designing coordinates) were cloned into the pENTR6_GW_grandestuffer and finally shuttled into the pDest-311 vector by LR reaction (Gateway system).

2.2.3.7.1 Transformation into chemically competent *E.Coli*

The amplification of a specific plasmid is achieved inserting it into chemically competent cells. The insertion of exogenous genetic material into a bacterial cell happens by a process called transformation.
To perform a transformation the following materials are needed:

- LB-medium
- Chemo-competent E.coli
- Bacterial agar plates
- Antibiotic:
    - Ampicillin stock (100 mg/ml): use 1:1.000
    - Kanamycin stock (50 mg/ml): use 1:1.000

An aliquot of 50µl containing chemo-competent cells are thawed on ice. DNA (5-200 ng) is added to it and incubated for 20min on ice. Cells are heat-shocked for 45s at 42°C, and cooled on ice for 2min. 200µl LB medium is added and cells are incubated for 45min at 37 °C on a roller shaker. Afterwards, different aliquots are plated on bacterial agar plates (50-500µl) and incubated overnight at 37°C.

2.2.3.7.2 Bacterial liquid cultures and DNA purification

For large scale (200ml) and small scale (5ml) liquid bacterial cultures, a single colony from the agar plate is inoculate into LB buffer, supplement with the appropriate antibiotic and incubated overnight at 37°C under vigorous shaking.
Plasmid DNA is then isolated with Qiagen Maxi or Mini kits according to the instruction of the manufacturer.

2.2.3.7.3 Restriction digestion of DNA

A restriction reaction is required to be able to cut DNA in specific region. This is prepared as described below:

- 1µg DNA plasmid
- 2µl 10xreaction buffer (NEB)
- 5-10U restriction enzyme (NEB)
- Up to 20µl of H_2O

The reaction is incubated at the appropriate temperature according to the restriction enzyme used and for a time that ranges from 2h to overnight. The DNA digestion is assessed by gel electrophoresis.

2.2.3.8 Gel electrophoresis

To perform gel electrophoresis, an appropriate amount of Agarose to prepare 1-2% (w/v) gel, depending on the size of the nucleic acid of interest, is dissolved in 1X TAE buffer let to melt in a microwave. The solution is then cooled down and SYBR® Safe DNA Gel Stain (Life Technology) is added in 1:10000 dilution before pouring the gel in an electrophoretic chamber. After the gel solidified, DNA samples mixed with 6X loading dye and a DNA ladder of appropriate size is loaded and electrophoresis performed at 100-200V. Then the band patterns of the samples are documented under UV light. In case of further usage of the DNA fragments for downstream applications; the DNA fragments with the right size are cut under UV light (254nm), transferred into an Eppendorf tube and purified using the QIAquick gel extraction kit (Qiagen) according to the protocol of the manufacturer.

2.2.3.8.1 Ligation

In order to allow ligation, the insert of DNA is used in a 5-10 fold excess from the DNA vector. The ligation reaction is let at 16°C overnight and transformed the following day. Colonies are picked, DNA purified and the final vector analyzed by sequencing.

The sequencing is performed at the Sequencing Core Facility of the Helmholtz Zentrum München and the sequence analyzed with the use of the free available software ApE (A plasmid Editor by Wayne Davis).

2.2.4 Data analysis

Quantifications of cells in mitosis (PH3+ cells) were performed at E14 and E16 across the entire LGE from lateral to medial using the Olympus Flow View software. All quantifications were done on images from ventral telencephalon using level matched sections of at least three stage matched embryos of each genotype.
Quantifications of BrdU+ and Dcx+ cells in the adult brain were performed in P56 sections and total numbers of the different categories defined per field of view. Fields of view are pictures taken with a 40x magnification 800*800 pixels. 2-3 sections per animals were measured of at least three staged matched animals of each genotype.

2.2.4.1 Statistics

Calculation of the arithmetic average:

$$\bar{x} = \frac{1}{n}\sum_{i=1}^{N} x_i$$

Standard deviation (SD):

$$\sigma = \sqrt{\frac{\sum_{i=1}^{N}(x_i - \bar{x})^2}{N-1}}$$

Standard error of the mean (SEM):

$$\sigma_M = \frac{\sigma}{\sqrt{n}}$$

were performed with GraphPad Prism5 and the same software used to test statistical significance. Data were considered as significant with P≤0.05 (*), very significant with P≤0.01 (**) and highly significant with P≤0.001 (***).

3 Aim of the thesis

Neural stem cells proliferation, specification and differentiation are three processes that need to be tightly regulate in order to allow the production of specific neuronal subtype at the right time and place in the brain during development and in the neurogenic regions at postnatal stages.

The transcription factor Pax6 is one of the factors able to orchestrate all these functions. It is defined as master regulator, being implicated in the control of fate determination and cell proliferation (Osumi et al., 2008; Georgala et al., 2011a). To control these aspects of the neurogenic process, Pax6 utilizes the paired (PD) DNA binding domain (Haubst et al., 2004; Ninkovic et al., 2010).

The PD domain is structured in a modular bipartite manner with two subdomains PAI and RED that bind cooperatively and independently to different binding sites. Both these subdomains exert effect on neurogenesis and on the control of cell proliferation (Walcher et al., 2013).

Given these evidences this work is aiming to dissect the molecular network controlled by the PD domain which is involved in the regulation of the cell cycle progression. Particular attention is given to the RED subdomain and how it affects the progenitors of the ventral telencephalon in the lateral ganglionic eminence (LGE).

In this work will be also explored the influence of the RED subdomain of Pax6 on the neurogenic lineage progression in the subependymal zone (SEZ), the postnatal neurogenic region laying at the ventricular surface, which is originating from the developing LGE.

4 Results

The transcription factor Pax6 is a pivotal gene in the central nervous system compelling, among other functions, for the proper patterning of the brain during development (Stoykova et al., 1996; Stoykova et al., 2000; Toresson et al., 2000).
Pax6 is highly expressed in the developing cortex (Walther and Gruss, 1991; Gotz et al., 1998; Heins et al., 2002), delineating the major border between the dorsal and the ventral forebrain with the pallial-subpallial boundary (PSPB).
In the ventral telencephalon, although at lower level if compared with the dorsal forebrain, the Pax6 protein is expressed in the lateral ganglion eminence (LGE) (Hallonet et al., 1998). Additionally in the ventral most side of the forebrain it is expressed in the amygdaloid complex (Tole et al., 2005) and in the latero-cortical stream (Carney et al., 2006), but these two areas will not be further discussed.
To address the contribution of the RED domain of the Pax6 protein in the regulation of the proliferation mode of the progenitors cells located at the ventricular surface, we took advantage of the $Pax6^{Leca2}$ mutants harboring a single point mutation in the RED DNA binding domain which is predicted to abolish its binding activity.
In this chapter will be underlined the role of the RED domain in regulating cell division of progenitor cells and the repercussion of such regulation on the postnatal brain.

4.1 Pax6 in the lateral ganglionic eminence

4.1.1 Pax6 isoforms expression in the developing telencephalon

In vertebrate the Pax6 locus encodes for three different protein isoforms: the canonical form, the Pax6(5a) and the PD-less form. The Pax6 PD-less form is expressed higher than the other two isoforms early in development (E10-E12), but it decreases at the onset of neurogenesis when the PD-containing forms play the major roles (Haubst et al., 2004).
The levels of expression of the two PD-containing forms of Pax6 are different between the dorsal and the ventral region. Pax6 and Pax6(5a), consisting of an insertion of 14aa

in the paired (PD) binding domain (Figure 12A-B), are expressed higher in the cortex than in the LGE and this difference in expression in maintained during development at E14 and E16 (Figure 12 C-D). Moreover, the levels of expression of the two forms of Pax6 tent to decrease as neurogenesis declines in both cortex and LGE (Figure 12D).

During early development the canonical Pax6 and the isoform Pax6(5a) are expressed in a ratio 8:1 in favor of the canonical Pax6 (Kozmik et al., 1997; Pinson et al., 2006). In the cerebral cortex, the ratio between the two transcripts is around 3:1 at the midneurogenesis (E14) and 2:1 at later time points (Figure 12C-D) always in favor of the canonical form of Pax6. In the LGE of the ventral telencephalon the two transcripts are equally expressed at midneurogenesis but this ratio increases at the later time points during brain development, in favor to the canonical Pax6 (Figure 12 C-D).

The proportion of the two PD-containing forms of Pax6 is maintained in favor of the Pax6 canonical form also in mutant conditions (Figure 12 E).

In the cortex of the $Pax6^{Leca2}$ mutants which harbor a mutation in the RED subdomain of the PD (Figure 12 E), the mRNA expression levels of Pax6 and its isoform Pax6(5a) have been measured in the mutant cortex and compared to the wild type (WT). In this case, even if the levels of Pax6 and Pax6(5a) in the mutant cortices are increased if compared with the WT levels of the same mRNAs, probably due to the fact that the inactivity of the RED subdomain disrupts any auto-regulatory loop of Pax6, the proportion between the two forms of Pax6 is preserved. This suggests that if the expression of the two isoforms of Pax6 is regulated in a region specific manner, this is independent on target genes recognized by the RED domain of Pax6.

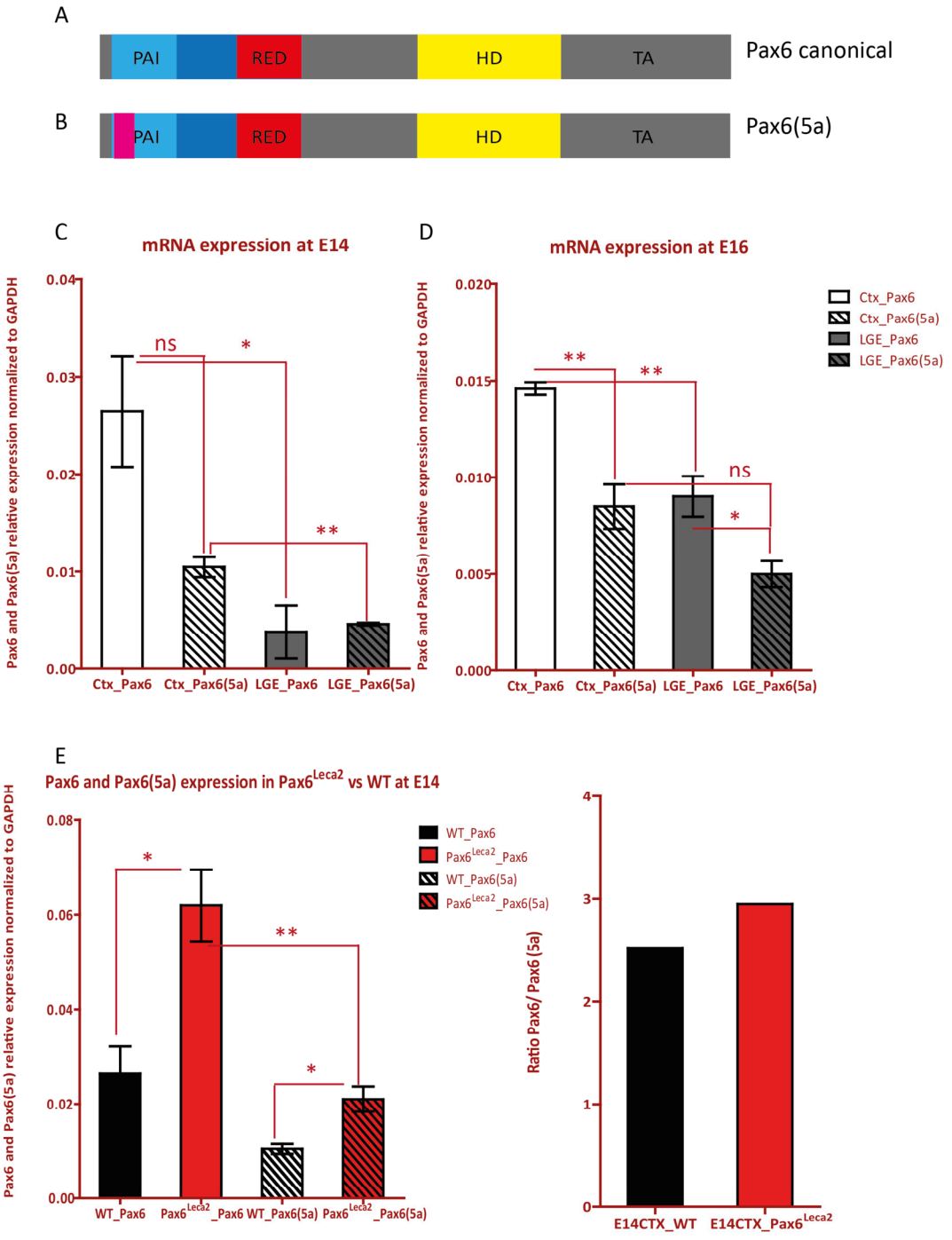


Figure 12: Pax6 isoforms expression in the cortex and in the LGE

(A-B) Scheme depicting two isoforms of the Pax6 protein present in the developing brain. (A) Canonical form prevalent in the developing brain and the (5a) isoform (B) consisting of an insertion of 14aa in the PD DNA binding domain impairing the binding capacity of the domain itself.

(C-D) mRNA expression level measured via qRT-PCR of the two isoforms in the dorsal and ventral telencephalon at E14 in (C) and at E16 in (D). (E)Histogram depicting the expression levels of the canonical form of Pax6 and the Pax6(5a) isoform in the cortex of WT and Pax6Leca2 The ratio of expression of the Pax6 vs Pax6(5a) in Ctx of E14 WT and Pax6Leca2. Data are shown as mean ± SEM of the relative expression of Pax6 and Pax6(5a) normalized to GAPDH (R=1/2-(ΔCt) ΔCt values have been measured for 3 independent samples of E14 and E16 brains). Significance defined via Unpaired TTest two-tailed pvalues. ns: non-significant; **P<0.01; *P<0.05.

4.1.2 Proliferation of progenitors in Pax6 RED domain mutants

Pax6 controls the proliferation of progenitors during the development of the cortex and the PD domain at the N-terminus is determinant for such function (Warren et al., 1999; Estivill-Torrus et al., 2002; Haubst et al., 2004).

As previously mentioned, Pax6 expression is not only constrained in the cortical area but also expressed in the LGE of the ventral telencephalon. To assess the role of Pax6 in controlling the proliferation of the progenitors in the LGE, I analyzed the number of phospho-histone 3 (PH3)-positive cells in $Pax6^{Leca2}$ mutants compared to their age matching siblings.

The antibody PH3 labels cells in the G2/M phase of the cell cycle, allowing the localization of actively dividing cells (Hendzel et al., 1997). While in the cortex PH3-positive cells are mostly located in apical positions (the basal division represent around 25% of the total PH3 positive cells (Pilz et al., 2013)), in the LGE around 60% of progenitors divide in non-apical position (Pilz et al., 2013). These non-surface mitoses are located within 10 rows of cells from the ventricular side (subapical progenitors SP) and in more basal positions (basal progenitors BP) (Figure 13).

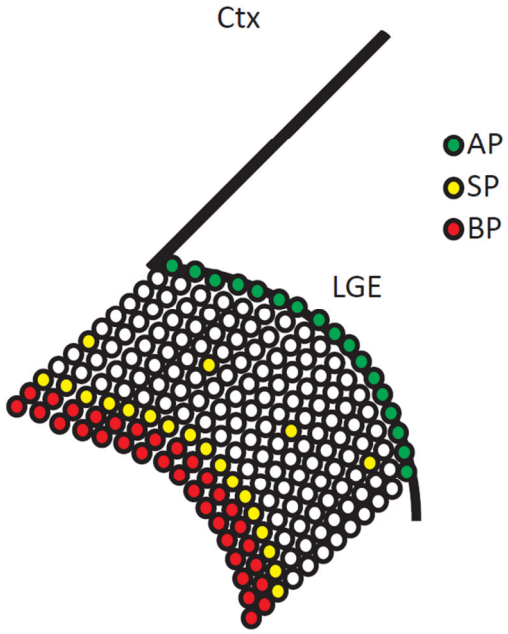


Figure 13: Proliferating cells in LGE
Scheme depicting the position of dividing progenitors in the lateral ganglionic eminence of the ventral telencephalon classified as apical progenitors (AP) dividing at the ventriclular surface; subapical progenitors (SP) dividing within 10 rows of cells from the ventricle and basal progenitors (BP) dividing more than 10 rows of cell away from the ventricle.

In the mutant LGE the PH3-positive cells distribute similarly to the WT littermate and all the progenitors populations are represented (Figure 14 A-B). The mutation of the RED domain induces to the increase of mitoses in the population of the apical progenitors, the cells that divide at the ventricular surface (Figure 14 C-D).

The increased number of PH3-positive AP cells is evident at E14 and then maintained at later times (E16), suggesting a constitutive effect due to the defective binding of the RED domain and not only a transient phenomenon.

The increase in apically dividing progenitors is a common feature between the progenitors in the LGE and the progenitors in the developing cerebral cortex (Walcher et al., 2013). This suggests a common role for the RED domain in the regulation of the cell cycle independently from the region of origin of the cells.

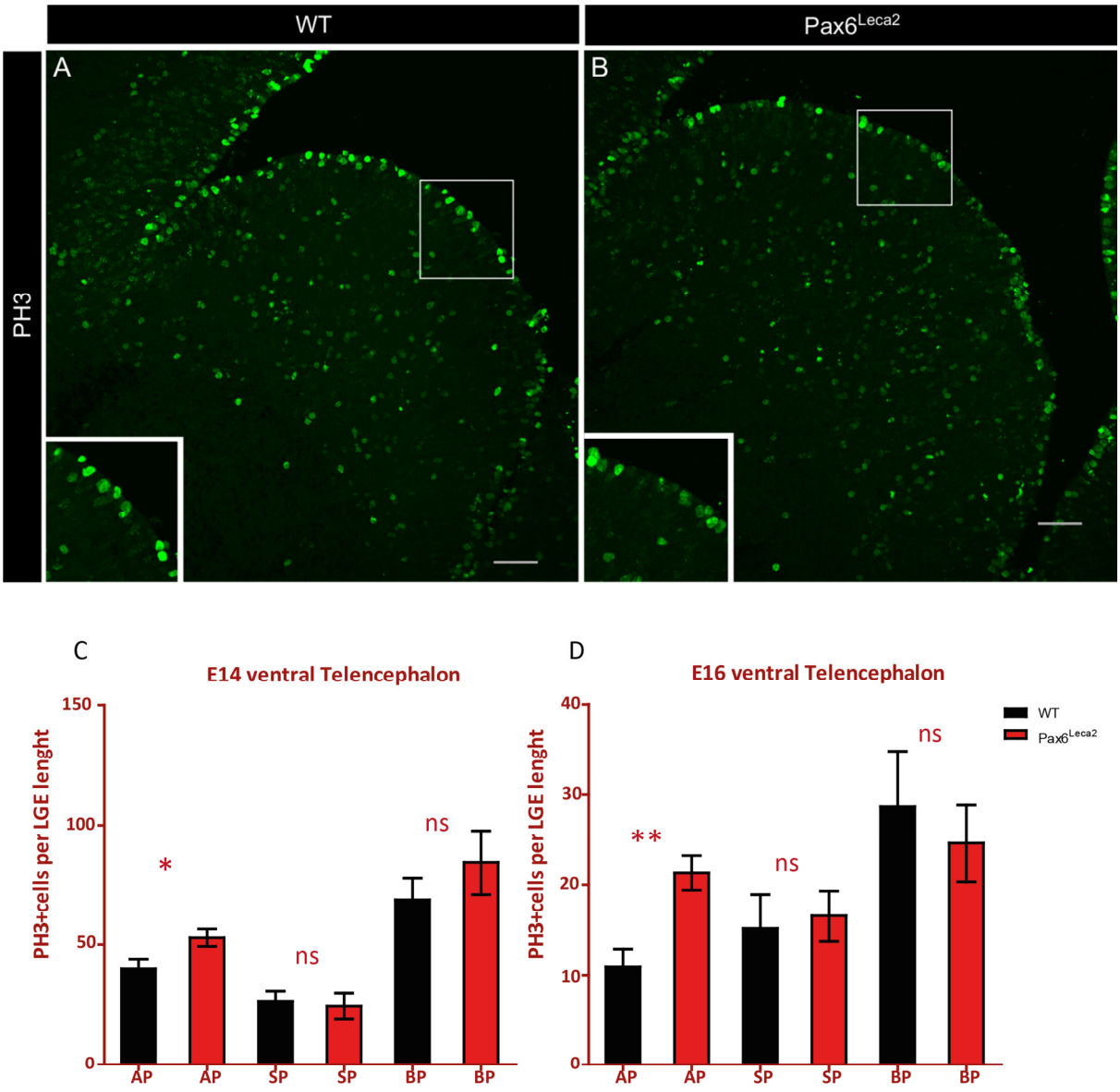


Figure 14: Progenitor proliferation in Pax6^{Leca2} LGE

(A-B) Micrographs of E14 ventral telencephalic sections depicting cells immunoreactive for phospho-histone H3 (PH3) in Pax6^{Leca2} brains and their WT littermates. The insert shows a zoom of the ventricular area where the cells are mostly affected. Scale bars 50µm. (C-D) Histogram depicting the distribution of PH3+ cells in the LGE of E14 and E16 Pax6^{Leca2}. Data are shown as mean ± SEM of the PH3+ cells per LGE length. Significance defined via Mann Whitney test. ns: non-significant; **P<0.01; *P<0.05. E14 AP WT vs AP Pax6^{Leca2}pvalue=0.019. E16 AP WT vs AP Pax6^{Leca2} pvalue=0.0031. n≥4 LGE sections per genotype, at least three different animals per genotype.

The exclusive affection of the AP population in the Pax6^{Leca2} mutants resides on the fact that all the cells proliferating at the ventricular side express Pax6 (Figure 15).

In the ventricular area (Figure 15) the phospho- vimentin (pVim) positive cells (marker used to label cells in the M-phase of the cell cycle homologous of PH3) are all expressing Pax6. This is different from the SP and BP where only some cells express the Pax6 protein.

In the populations of the SP and the BP the effect of the RED domain mutation might be diluted and masked in the analysis of the all non-apically proliferating progenitors.

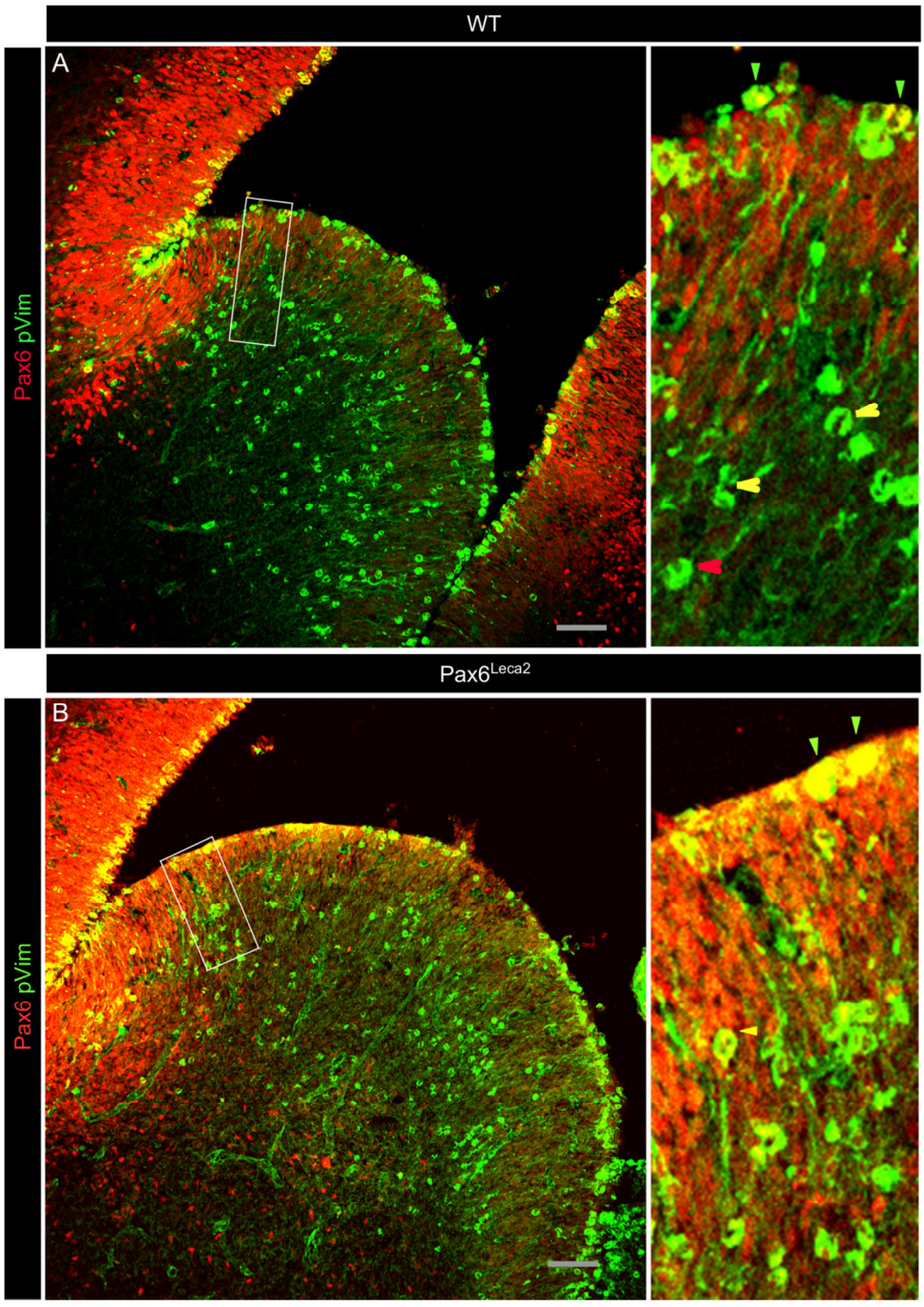


Figure 15: Pax6 expression in the progenitors of the LGE.
(A-B) Micrographs depicting the expression of Pax6, in red, and pVim, in green, in WT (A) and in the mutant (B). in the high magnification examples of different progenitor co-immunostained with Pax6 and pVim (filled arrow heads) or expressing only pVim (outlined arrow heads). In green AP, in yellow SP and red BP. Scale bars 50µm.

4.1.3 Cell death analysis in RED domain mutants

The increment of mitotic progenitors in the $Pax6^{Leca2}$ is a general effect registered in the dorsal and ventral telencephalon.

In the cortex the increase in PH3-positive apical progenitors upon the RED mutation has been explained as the effect of the Pax6 RED domain in controlling cells survival (Walcher et al., 2013). Pax6 is implicated to play a role in this context (Nikoletopoulou et al., 2007; Ninkovic et al., 2010).

Supposedly this is a general effect exerted by Pax6 independently from the region of expression. In order to validate whether Pax6 RED domain is relevant for preserving progenitors survival, has been analyzed the level of apoptosis in the progenitors of LGE of the Pax6 mutants and their WT siblings. The immunostaining against activated-Caspase3 was performed in the ventral telencephalon of E14 animals (Figure 16).

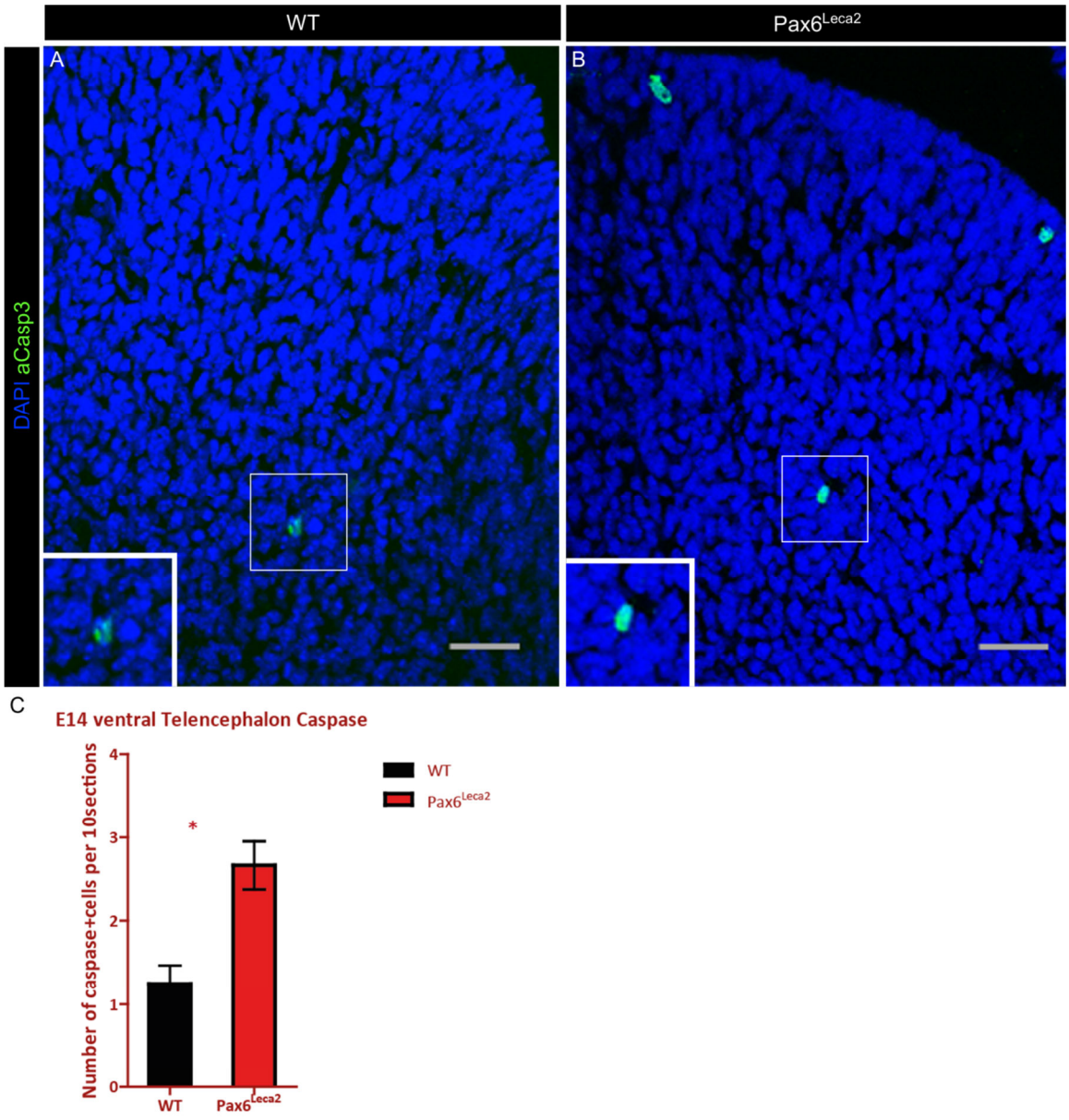


Figure 16: Survival of progenitors in Pax6^{Leca2}

(A-B) Micrographs of E14 LGE depicting cells immunoreactive for activated-Caspase3 (green) in WT and mutants siblings. Scale bar 20µm. (C) Histogram depicting the number of activated-Caspase3 positive cells per total LGE. Data are shown as total number per 10 sections ±S.E.M. n (number of brains)≥3. *p<0.05; Mann-Whitney test.

This analysis revealed that the mutation of the RED domain of Pax6 leads to a decrease of the cell survival in the LGE of the ventral telencephalon, similarly to the effect that it exerts on the progenitors of the developing cortex.

This evidence, together with the increment of cells in mitosis (Figure 16 and Figure 14), suggested a direct involvement of the RED subdomain on the regulations of the mitotic progression that, if impaired, leads to cells aberrations and eventually cell death.

4.1.4 Cell cycle progression in Pax6^{Leca2} ventral telencephalon: loss and gain of function experiments

In LGE of the Pax6^{Leca2} mutant animals, the increase in apically dividing progenitors and the reduction of cell survival could be explained by the AP cells failing in completing the cell cycle and the division of the two daughter cells during the last step of the cytokinesis. Failure in the cytokinesis would lead to the visualization of more nuclei, as in the analysis of fixed tissue where there is an increment of about 30% in PH3-positive cells (Figure 14), but those could be predicted as divided nuclei of daughter cells that did not completely cleaved their cytoplasm.

To prove this hypothesis, we performed ex vivo live imaging of progenitor cells in the LGE. This technique offers the ability to follow single cells overtime during their cell cycle and allows answering if the abundance of mitotic figure in Pax6^{Leca2} is due to impairment in cytokinesis.

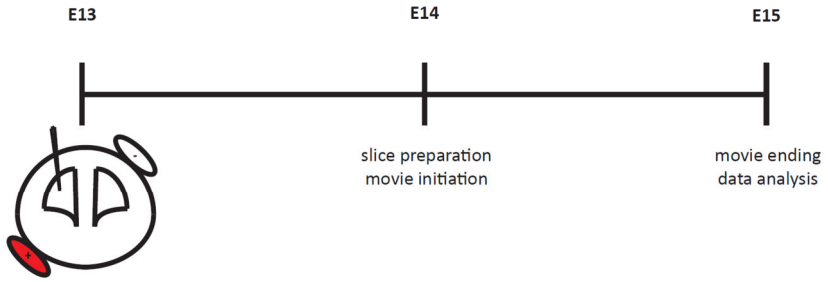


Figure 17: Ex vivo life imaging:
Scheme depicting the ex vivo live imaging paradigm as described in the method paragraph 2.2.1.5

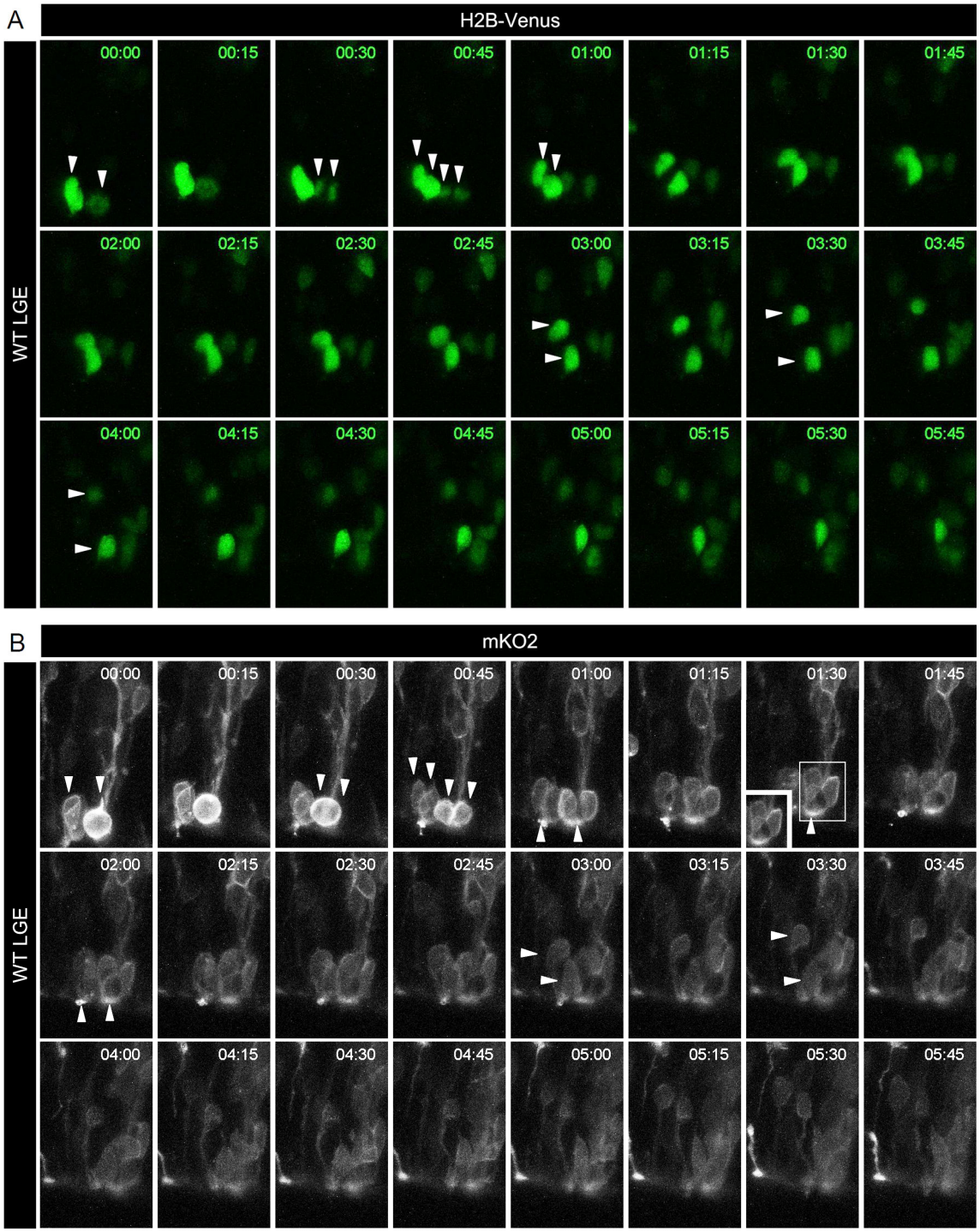
As described in the method paragraphs 2.2.1.3, 2.2.1.5, progenitor cells in the LGE are labelled via in utero electroporation (Figure 17) of embryos at E13. Three plasmids are introduced into the cells of the LGE of WT and mutant animals:

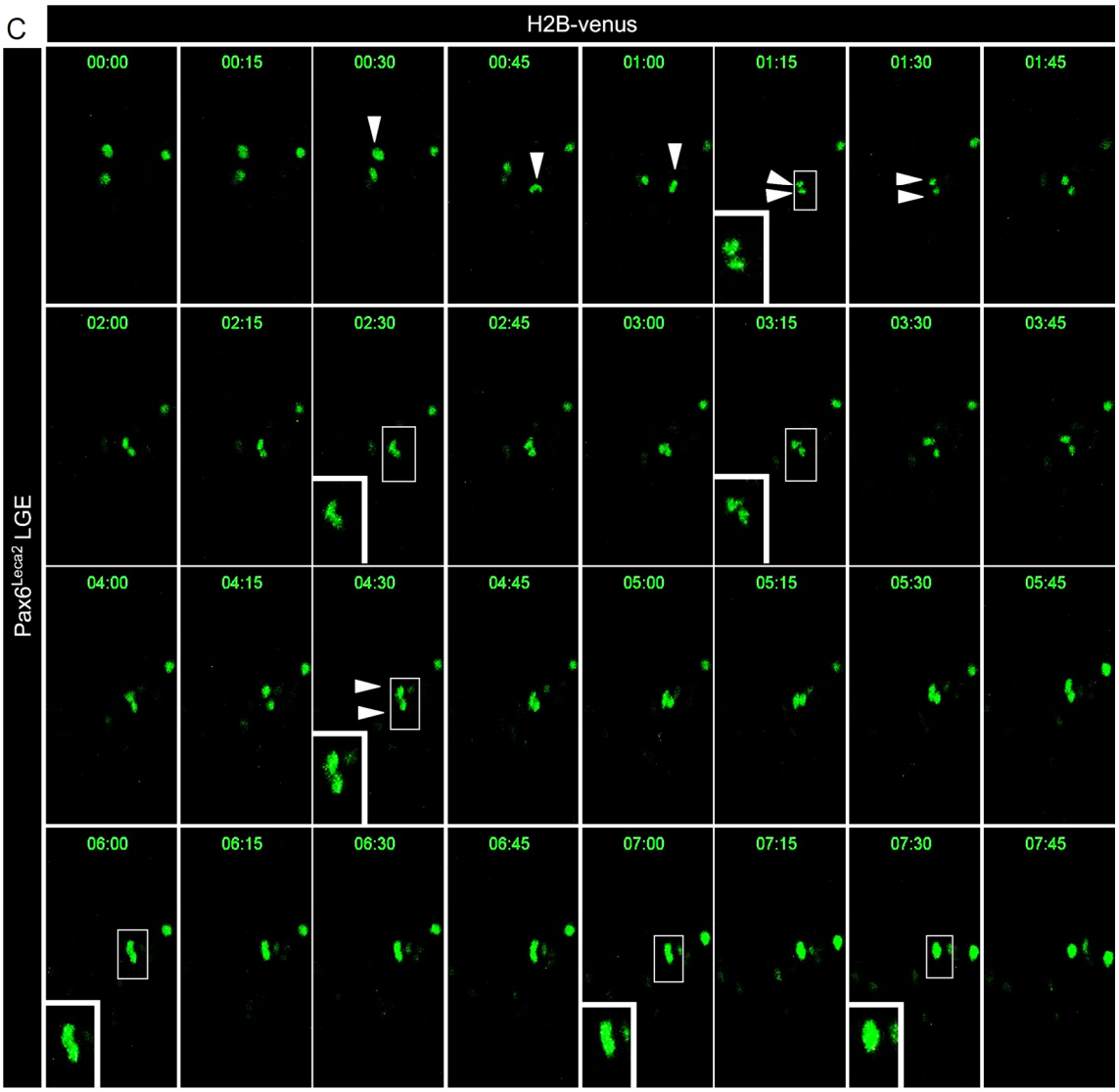
- H2B-Venus is used to identify the nuclear movements since H2B is a member of the core histones that package DNA in the nucleus.
- pCAG-Floxp-mKO2-F (Shitamukai et al., 2011) combined with a Cre expressing plasmid are used to highlight the cell membrane and to visualize the phase of the cytoplasmic scission.

The day after the operation, embryos at E14 are sacrificed, sliced and imaged (Figure 17). The imaging session is running for about 24 hours to be able to visualize at least

one cell division. The cell cycle of an apical progenitor cells is in fact lasting about 25h (Pilz et al., 2013).

During this time was possible to monitor the progenitor cells during their division and estimate the time required for their nuclear and cytoplasmic division (example of time series in Figure 18).

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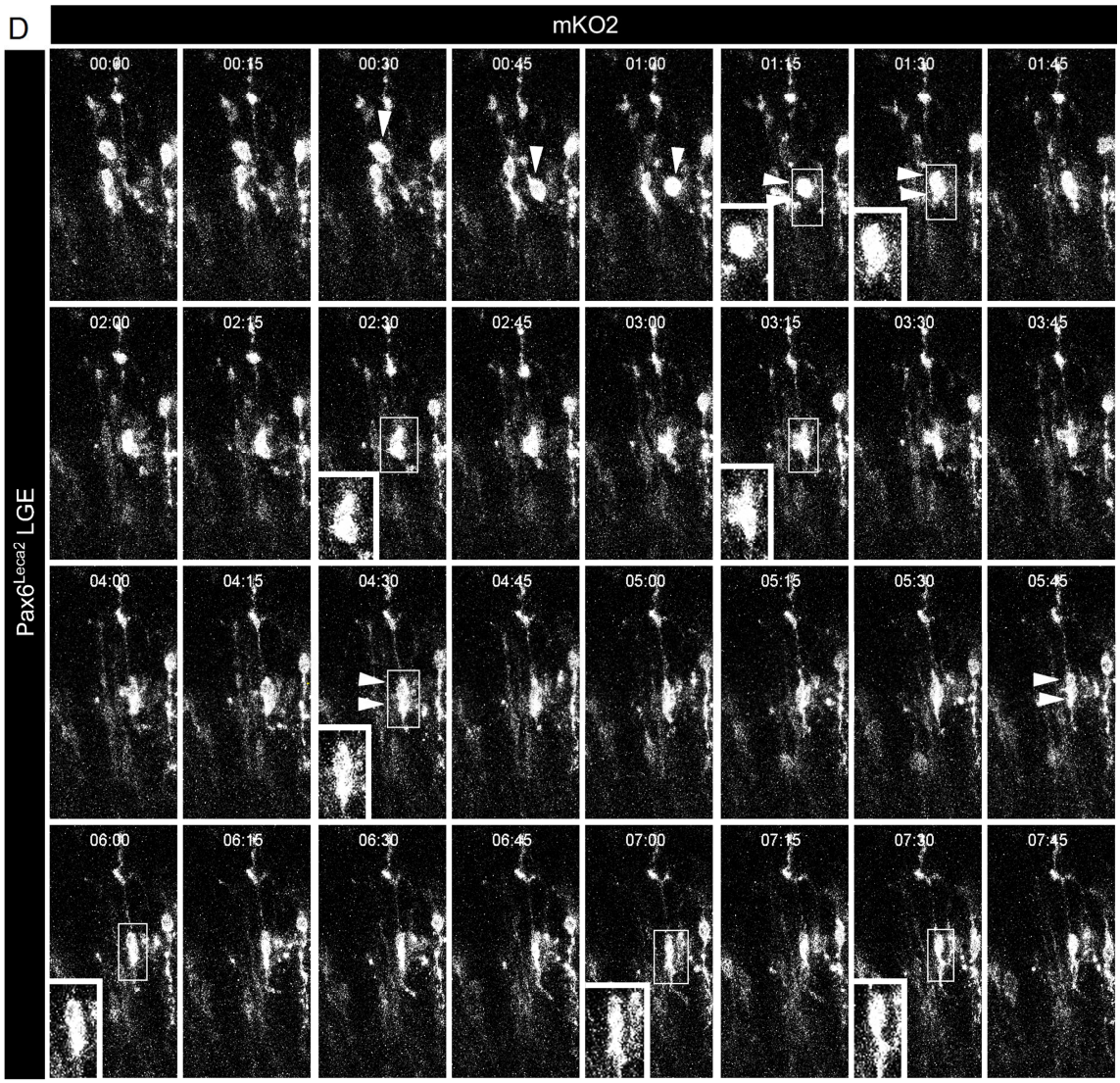


Figure 18: LGE ex vivo live imaging

(A-D) Micrographs depicting examples of cells in the WT (panel A-B, curtesy of Dr Sven Falk) and Pax6^{Leca2} (panel C-D). Images are acquired with an interval of 15 minutes with a confocal microscope Olympus FV1000 cLSM system, 20 x magnification ((A-B) 2x zoom as acquisition setting). (A-C) H2B-Venus used to visualize nuclear movements. (B-D) mkO2 used to visualize the cytoplasm and cell membrane movements. Arrow heads indicate examples of the cells analyzed.

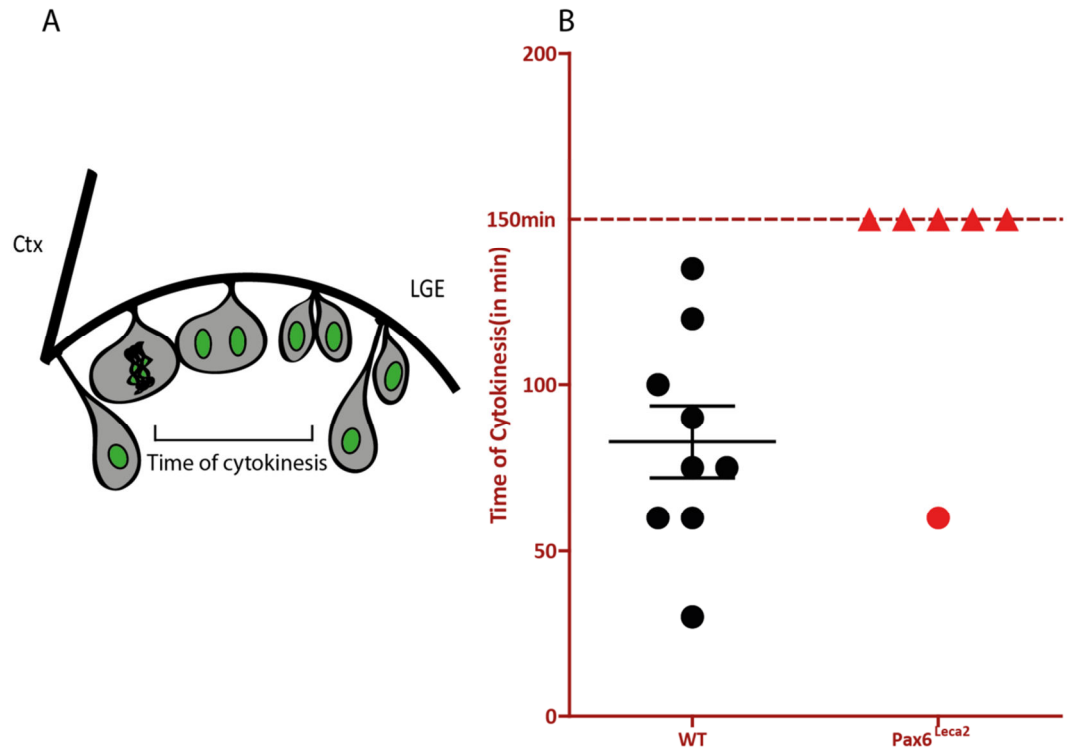


Figure 19: Time of cytokinesis definition and measuring
(A) Scheme depicting the definition of "time of cytokinesis" measured as the time between the division of the nucleus and the full scission of the cytoplasm in two different daughter cells. (B) Dot blot representing the time that the cells analyzed need to divide into two daughter cells. Dots in black represent the WT cells measured (9 cells). The red dot represents the Pax6^{Leca2} cells that divided in the 150min of observation whereas the red triangles represent the cells that did not fulfill the requirement in the given time (cell1=510min: cell2=315min: cell3=150min; cell4=180min; cell5=255min).
For the WT the horizontal bar represents the mean ±SEM.

To prove the idea of a prolonged cytokinesis, we measured the "time of cytokinesis". This is defined (Figure 19 A) as the time between the division of the nuclei, right after the condensation of the heterochromatin that is a synonym of the nuclear division initiation, and the full division at the membrane level of the two daughter cells. This analysis, (Figure 19 B) revealed that the time of cytokinesis in Pax6^{Leca2} mutants is longer than in the WT. Given an observation time of 150 minutes all the WT cells managed to complete division (average time: 82.8min) whereas only one Pax6^{Leca2} completed the cytokinesis in the same time.

These measurements could confirm that cells upon mutation of the RED domain have a prolongation of the time the cells share their cytoplasm after their nuclear division.

This evidence was further consolidate when E14 brains, derived from Pax6^{Leca2} background, were analyzed and fixed after the electroporation of the same plasmids listed above (Figure 20).

Also via fixed analysis we could detect that the mutation of the RED domain is impairing the cell cycle progression of the apical progenitors of the LGE. The progenitors in the Pax6^{Leca2}, even if composed by two nuclei (Figure 20), are still surrounded by a unique membrane. The situation in the WT is different (Figure 20 A), the cells are mostly in G phase with one nucleus ready to divide or differentiate.

These data underline the importance of the RED domain of Pax6 in controlling the final step of the cell cytokinesis.

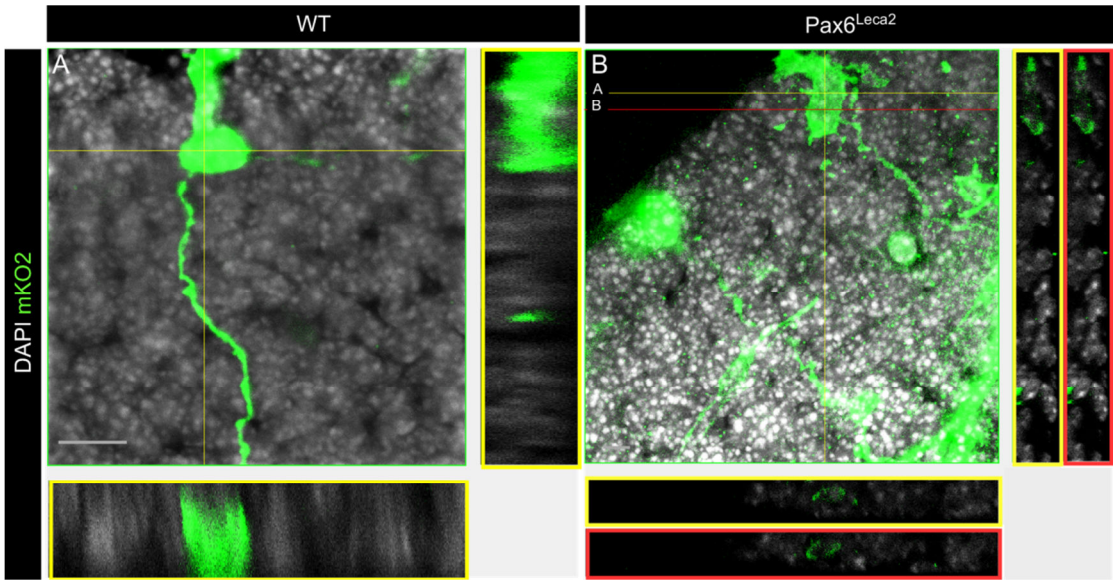


Figure 20: Cell division in vivo
Orthogonal projections depicting examples of radial glia cells after in utero electroporation of membrane protein in the mutant (B) and WT littermate (A) LGE. Scale bars A 20µm, B 10µm.

The implication of the RED domain mutation affecting cell cycle progression and compromising the capacity of the cells to complete their mitosis was further supported by in vivo experiments. In these experiments we performed loss of the RED domain functions in the WT background. Cells in the WT brains were transduced with retroviral vectors co-expressing the Pax6^{Leca2} form and the GFP protein. The viruses were injected at the E14 stage and the brains analyzed 3 days later (E17). The injection was directed in the ventricle and labelled cells were found in the cortex.

The cells found, even if they were only few probably due to the low titer of the virus, were all in duplets (Figure 21).

This is considered an additional evidence that the mutation of the RED domain of Pax6 would lead to impairments in the progression of the mitotic phase.

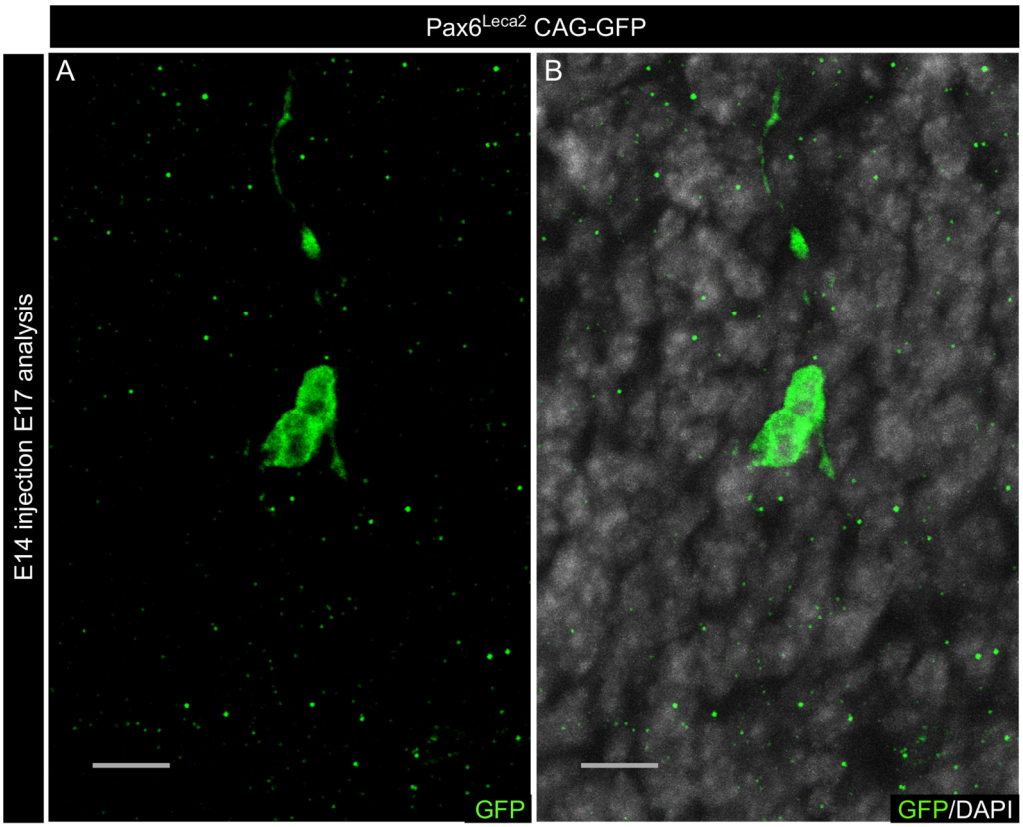


Figure 21: In vivo overexpression of the RED domain mutation of Pax6
(A-B) Micrographs depicting duplets of cells in the cortical area upon in vivo transduction with retro viral vector Pax6^{Leca2} CAG-GFP in WT background. (A) GFP immunostaining, (B) GFP immunolabeling merged with the DAPI. Scale bars 10µm.

Pax6 is known as a regulator of cell division (Asami et al., 2011), but its involvements in the control of the progression of cytokinesis is a novel role which opens as new question whether the RED domain controls specific targets sufficient to explain the observed phenotype.

To have new insights on the molecular partners regulated by the RED domain that could explain the phenotype in the developing brain, the microarray data derived from the developing cortex of the Pax6^{Leca2} (Walcher et al., 2013) were analyzed. From this analysis the Holliday junction recognition protein (HJURP) emerged as an interesting candidate.

4.1.5 Candidate gene: HJURP

The effect of the RED subdomain mutation of Pax6 on the proliferation of progenitors observed via live imaging, motivates to analyse genome–wide expression changes previously collected (Walcher et al., 2013), in order to identify possible candidates suitable to explain the phenotype.

Microarray analysis was carried out using rostral developing cortices from mutants and wild type animals at the developmental stage E14 (Figure 22). The rostral part of the cortex was selected based on the knowledge that Pax6 is expressed in a rostral high - caudal low gradient (Stoykova et al., 1997; Muzio et al., 2002b). In the microarray analysis, among the most upregulated genes (35 genes out of the 94 differentially expressed probe sets detected), two seemed particularly promising in explaining the prolongation of the cell mitosis: Bub1 (Budding uninhibited by benzimidazoles 1) and HJURP (Holliday junction recognition protein).

The Bub1 gene encodes for a serine/threonine-protein kinase that plays a central role in mitosis: it is essential for spindle-assembly checkpoint signaling and for correct chromosome alignment (Elowe, 2011).

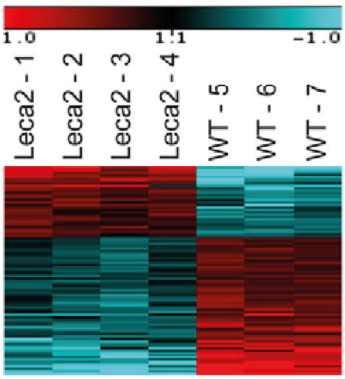
Since the upregulation of Bub1 could not be confirmed via quantitative RT-PCR with all the sets of primers in independent samples (data not shown), the attention was focused on the second candidate: HJURP.

HJURP is a centromeric protein playing a central role in the incorporation and maintenance of histone H3-like variant CENPA at centromeres. HJURP is a chaperone for CENPA and is required for the incorporation of newly synthesized CENPA molecules into nucleosomes at replicated centromeres. (Stellfox et al., 2013; Muller and Almouzni, 2014). The proper organization of the centromere is fundamental for the assembly of the centromere-kinetochore structure and the consequent proper segregation of the chromatids during mitosis.

The HJURP gene is upregulated in the $Pax6^{Leca2}$ cortices and such upregulation is confirmed via quantitative RT-PCR in independent samples derived from the cortices of mutants and wild types (Figure 22 C).

A

Heat map Pax6^{Leca2} vs WT

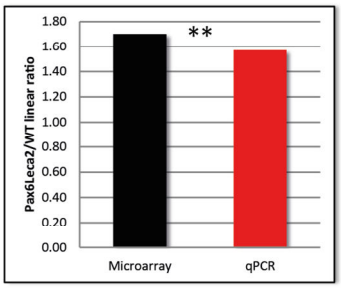


B

Probe set	Gene symbol or ID	Significant FDR<10% ration>1.4 (0.71)x	AV Pax6Leca2	AV WT
1433919_at	Asb4	3.54	255	72
1423422_at	Asb4	3.19	158	50
1440049_at	1440049_at	2.77	55	20
1438194_at	Slc1a2	2.66	827	312
1438571_at	Bub1	2.53	185	73
1454112_a_at	Haus2	2.41	291	121
1454772_at	Snrnp200	2.25	533	237
1434278_at	Mtm1	2.24	1804	806
1428077_at	LOC100047091	1.95	490	251
1419271_at	Pax6	1.90	4536	2386
1438737_at	Zic3	1.90	414	218
1439627_at	Zic1	1.87	1758	941
1452526_a_at	Pax6	1.81	506	279
1439854_at	Hrk	1.80	382	212
1428990_at	2310047K21Rik	1.74	266	130
1433707_at	Gabra4	1.74	239	137
1444139_at	Ddit4l	1.73	210	122
1433685_a_at	6430706D22Rik (HJURP)	1.71	1914	1118
1449571_at	Trhr	1.68	162	96
1456005_a_at	Bcl2l11	1.67	912	546
1437086_at	Ascl1	1.63	680	417
1419719_at	Gabrb1	1.61	195	121
1456006_at	Bcl2l11	1.61	121	75
1439332_at	Ddit4l	1.61	189	117
1416232_at	Olig2	1.59	156	98
1458076_at	1458076_at	1.57	106	68
1435449_at	Bcl2l11	1.56	135	87
1432509_at	5033430I15Rik	1.54	210	136
1457260_at	5730409E04Rik	1.54	54	35
1419123_a_at	Pdgfc	1.52	637	419
1450857_a_at	Col1a2	1.51	112	74
1447628_x_at	Mrps5	1.50	143	95
1448194_a_at	H19	1.48	1115	754
1430798_x_at	Mrpl15	1.46	377	258
1434327_at	2610020H08Rik	1.40	62	44

C

HJURP Cortex



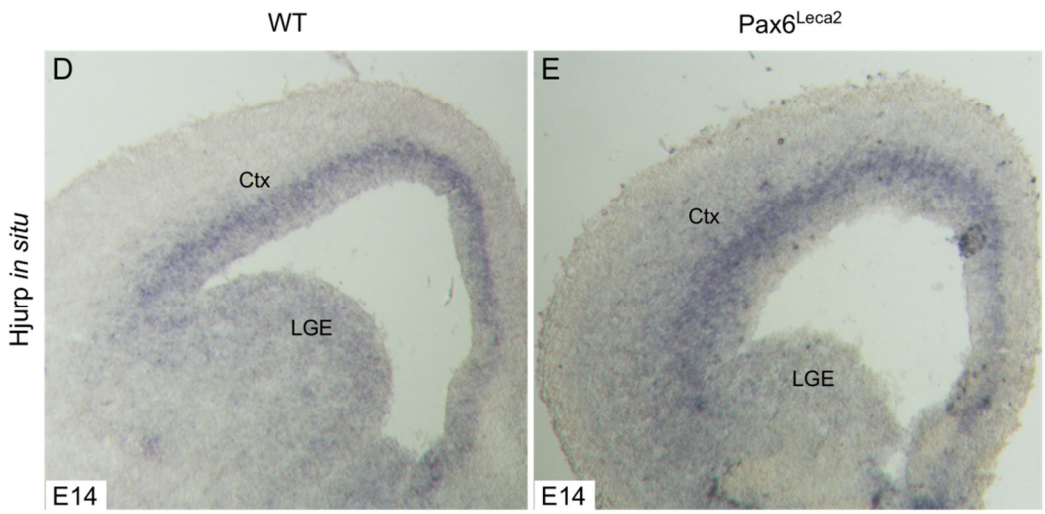


Figure 22: HJURP candidate gene

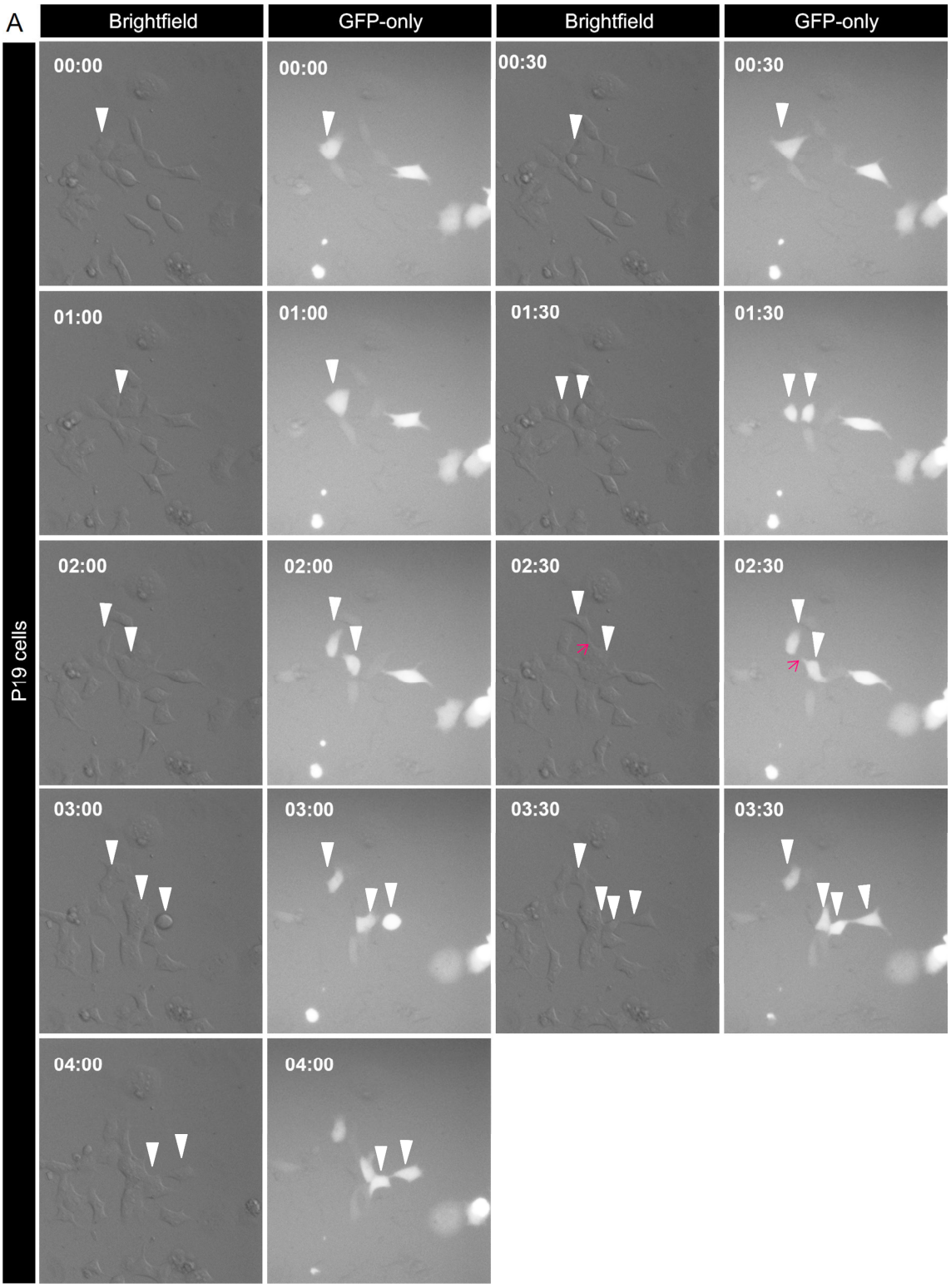
(A-E) Parts of this figure are adapted from the publication (Walcher et al., 2013). (A) Heat map illustrating the expression of the differentially regulated genes in the E14 Pax6^{Leca2} dorsal cortices in comparison with their WT littermates. Red/blue indicates higher/lower expression values (Walcher et al., 2013). (B) List of up regulated genes in the Pax6^{Leca2}, obtained with the genome-wide expression analysis performed by Affymetrix MOE430 2.0 array. Differences are determined by a filter consisting of statistical significance (FDR<10%), an average expression level exceeding 50 and at least 1.4-fold difference expression (Walcher et al., 2013). Two possible candidate genes to explain the phenotype in vivo, 6430706D22Rik also called HJURP and Bub1, are highlighted in red. (C) Histogram depicting the linear fold changes of the gene HJURP between the Pax6^{Leca2} and the WT littermates as measured by Affimetrix array analysis (black bar) and qRT-PCR (red bar). Student TTEST **P<0.01. (D-E) Micrographs depicting *in situ* hybridization in E14 sections of WT and mutants, showing the mRNA expression for the HJURP gene (Walcher et al., 2013). Abbreviations: Ctx: cortex; LGE: lateral ganglionic eminence.

4.1.5.1 Expression of Hjurp

The implication of the HJURP protein in the brain was never explored before. To further characterize the expression of HJURP in this system, *in situ* hybridization was performed. In the developing cortex (Figure 22 D-E), the mRNA is particularly localized in the VZ/SVZ. In the $Pax6^{Leca2}$ mutant (Figure 22 E) the expression of the mRNA for HJURP is increased in this cortical area. The localization of HJURP mRNA in the VZ/SVZ is in accordance with the function of HJURP that helps the incorporation of newly synthesized CENPA at the replicated centromeres during S phase (Stellfox et al., 2013; Muller and Almouzni, 2014). In this region in fact, together with the proliferating basal progenitors, there are also the radial glia cells (expressing Pax6) in the S phase of their cell cycle. Radial glia cells in fact undergo intekinetic nuclear migration which comprise that the cell nucleus migrates radially to the basal side of the cells during G1 phase of the cell cycle, undergoes to S-phase in basal position and migrates back to the apical side for G2-M phase of the cell cycle.
In the LGE of the $Pax6^{Leca2}$ mutants the increase in the HJURP mRNA appears very modest and difficult to confirm. The reason of a less clear result might reside in the characteristic heterogeneity of the progenitors present in the LGE.

4.1.5.2 HJURP in vitro

In order to recapitulate the phenotype observed *in vivo* in the mutant mice and to delineate the contribution of HJURP on it, the protein was overexpressed in vitro on P19 cells.
P19 cells are able to fast proliferate and eventually to differentiate to neurons when exposed to specific compounds like retinoic acid (Staines et al., 1994). Therefore, this cell line was used to approximate neuronal progenitors in vivo. In this system the expression of HJURP was forced via lipofection of plasmid encoding for the protein and the GFP (green fluorescent protein) that allows the visualization of the cells expressing the protein of interest. 20h after the lipofection, the time necessary to visualize the signal from the GFP, the cells were imaged for about 36h until reaching confluency.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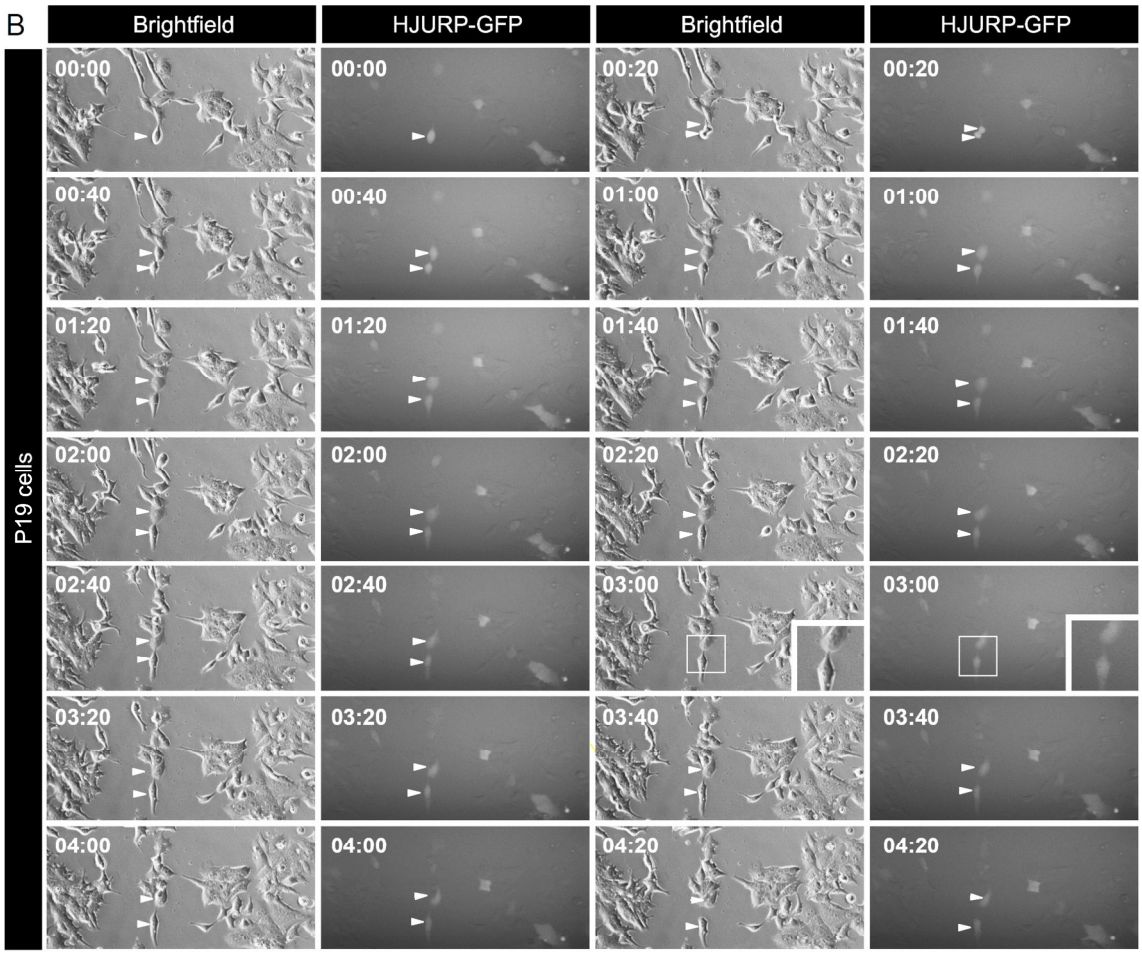
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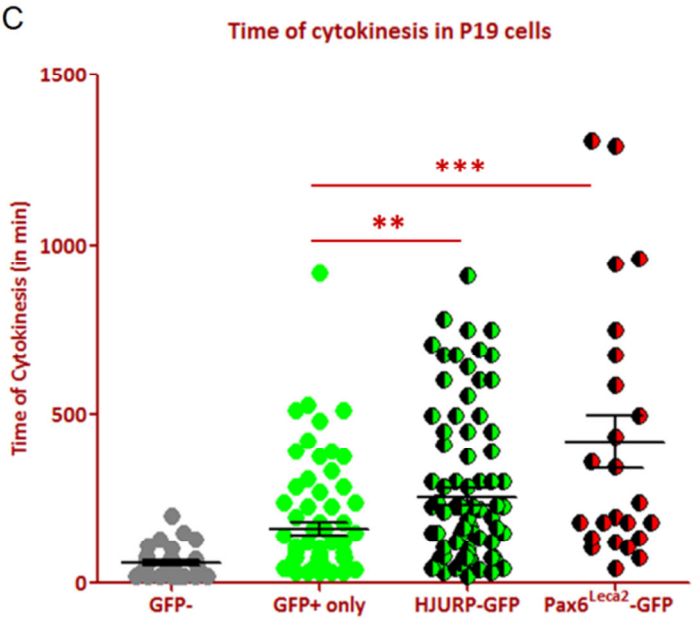

C
Time of cytokinesis in P19 cells
Time of Cytokinesis (in min)
1500
1000
500
0
***
**
GFP-
GFP+ only
HJURP-GFP
Pax6Leca2-GFP

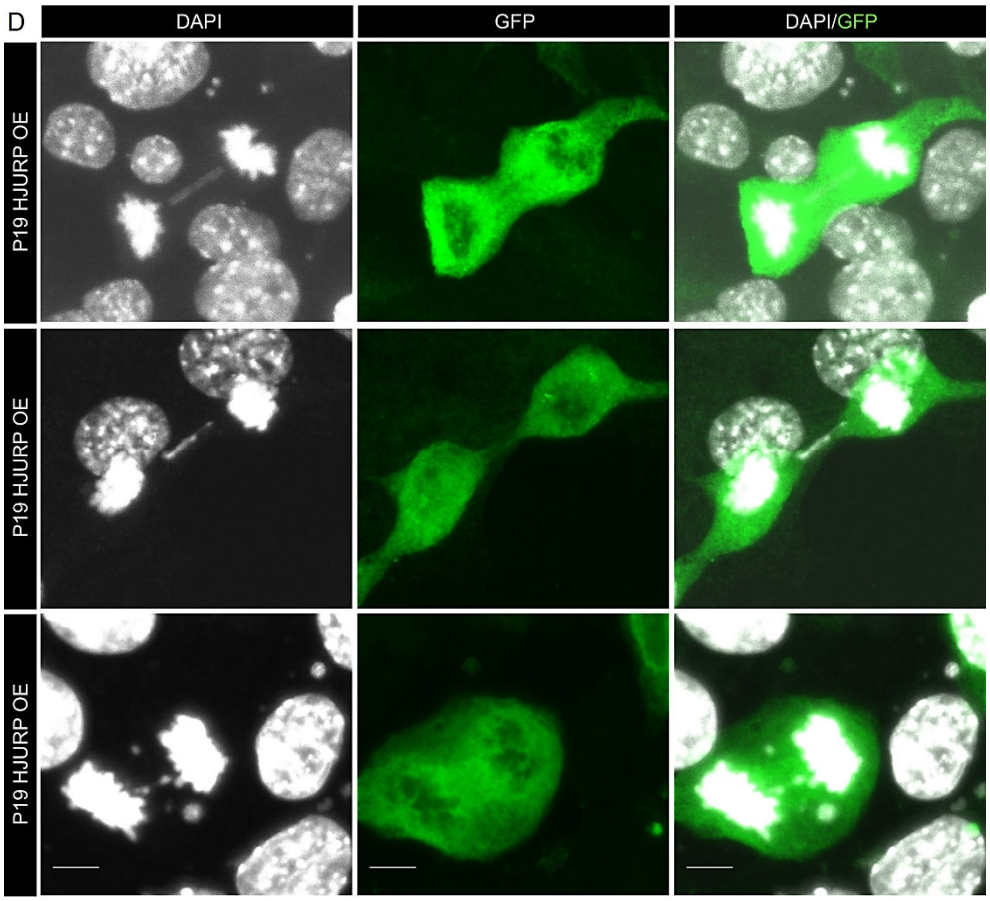


Figure 23: In vitro live imaging of P19 cells.upon HJURP overexpression
(A-B) Time series depicting the behavior of cells upon lipofection of the control plasmid (A) and the plasmid expressing HJURP (B). Pictures are acquired with an inverted Zeiss Cell-Observer Z1 video microscope. 20 x magnification. In A and B the arrow heads indicate examples of cells analyzed. (C) Dot blot depicting the time of cytokinesis as previously described for different conditions. Data are represented as mean ± SEM. ***P<0.001, Mann-Whitney test. The cells analyzed are coming from n≤3 experiment except the case of the OE of $Pax6^{Leca2}$ where n=1. (D) Micrographs depicting P19 cells upon HJURP OE immunostained 36h after lipofection. Scale bar 5µm.

In the cells expressing HJURP was measured the time of cytokinesis. This was determined has the time intercurring between the nuclear division and the complete scission of the cytoplasm of the two daughter cells; the cells in which the cytokinesis was not completed by the end of the movie where excluded from the analysis.

The overexpression of HJURP (Figure 23 C) leads to a lengthening of the time needed to complete the cytoplasmic division of the cells. The cell cytokinesis, upon HJURP overexpression, appears impaired and longer than in the wild type condition. The cells remain in contact possibly because the DNA in the two nuclei is not completely segregate in the daughter cells so that they are blocked from proceeding with the last step of cytokinesis (Figure 23 D).

P19 cells were imaged also after overexpression of the RED domain mutant form of Pax6. In this case a similar effect on cell cycle progression was detected (Figure 23 C). This supports the idea of the existence of a tight connection between the RED domain of Pax6 and the HJURP protein, in fact following the cells expressing HJURP during their division, unravels very similar behaviors than the mutation of the Pax6 RED domain per se.

The current model (Figure 24) starts hypothesizing an inhibitory capacity of the RED domain in normal conditions. In the Pax6^{Leca2} the mutation of the RED domain impairs this inhibitory function, contributing to the increase of the HJURP mRNA. As a consequence, the increment in HJURP leads to a prolongation of the cytokinesis in the apical progenitors cells of the developing telencephalon. What remains to be elucidated in this regard is the direct implication of Pax6 RED domain on the increase of HJURP expression.

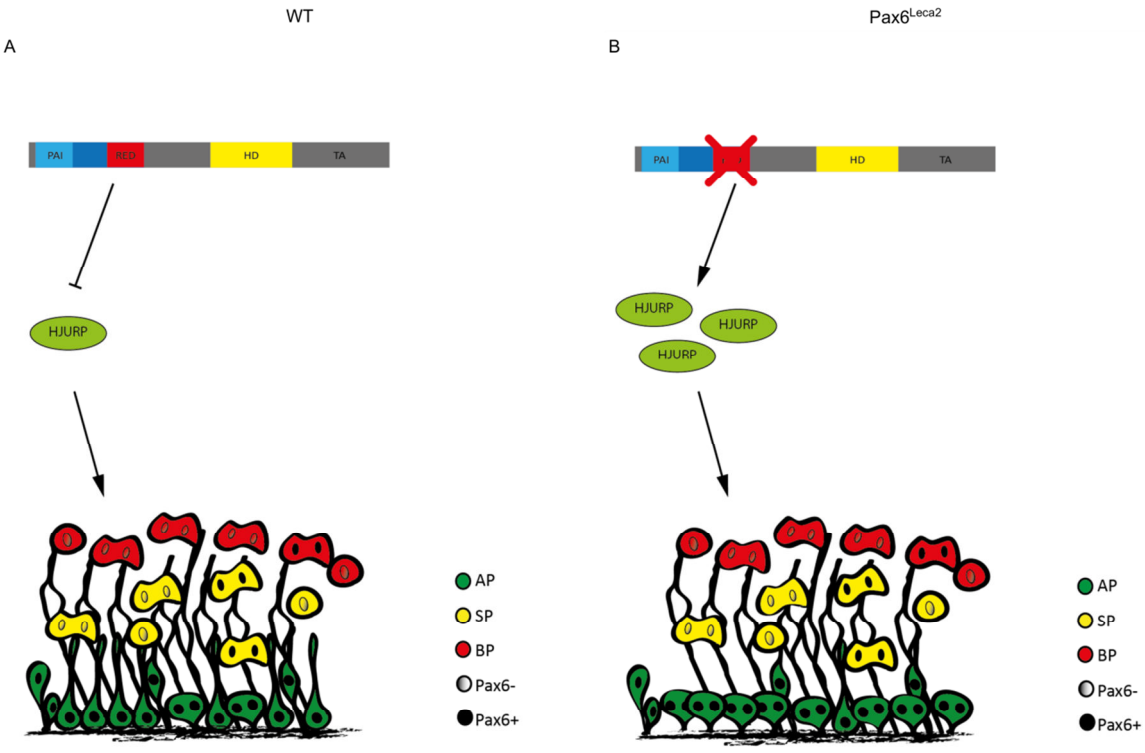


Figure 24: Working model
(A-B) Scheme depicting the model suggested by the results obtained from the analysis of the RED domain mutation in the developing brain (A) WT, (B) Pax6^{Leca2}.

4.2 Adult brain

The LGE region of the ventral telencephalon gives rise, at postnatal stages, to the cells populating the subependymal zone (SEZ), one of the regions where neurogenesis persists during adult life (Merkle and Alvarez-Buylla, 2006).

The progenitors cells present in the LGE of the Pax6^{Leca2} mice are facing defects in cell cycle progression, this motivated the assumption that the impairment encountered by the progenitors of the LGE might have repercussion on the cells originating from there. In addition, giving the fact that the cells populating the SEZ niche express the Pax6 protein in normal condition (Hsieh, 2012), we used the Pax6^{Leca2} animals as a tool to investigate the role of the RED domain of Pax6 in the SEZ region of the adult brain.

4.2.1 Pax6^{Leca2} in adult brain

To investigate the repercussion of the RED domain mutation on the adult neurogenesis, eight to ten weeks old Pax6^{Leca2} mice were used. Differently from many Pax6 mutants which die at birth due to the loss of a protein that has pivotal roles in various systems, Pax6^{Leca2} mutants are able to survive after birth and reach adulthood.

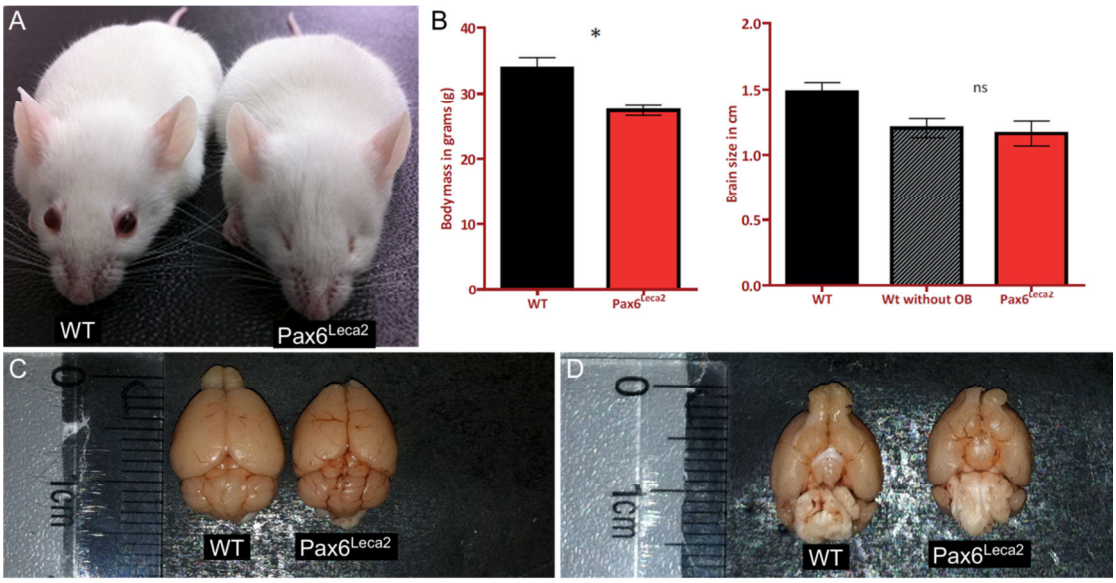


Figure 25: Gross morphology of Pax6^{Leca2}
(A) Examples of animals of the two genotyping (Walcher et al., 2013). (B) Histogram depicting the difference in body weight and brain size between WT (black bar) and Pax6^{Leca2} (red bar). Data are reported as mean ± SEM *P≤0.05; ns: non-significant. Unpaired T Test and Upaired TTest with Welch's correction. (C-D) Gross morphology of the brain in the mutants and their WT littermates seen from the top (C) and from the bottom (D) (Walcher et al., 2013).

These animals are blind, evident sign of distinction between the homozygotes for the missense mutation and their WT littermates (Figure 25A) and they have a smaller body mass (Figure 25 B). Looking at the gross morphology of the brain, Pax6^{Leca2} mice have a smaller olfactory bulb (OB) even if the brain size is unchanged (Figure 25 B-D).

At the cellular level, in the adult SEZ-RMS system is evident an accumulation of cells in the RMS which starts to be visible as early as E18 in the developing LGE (Figure 26).

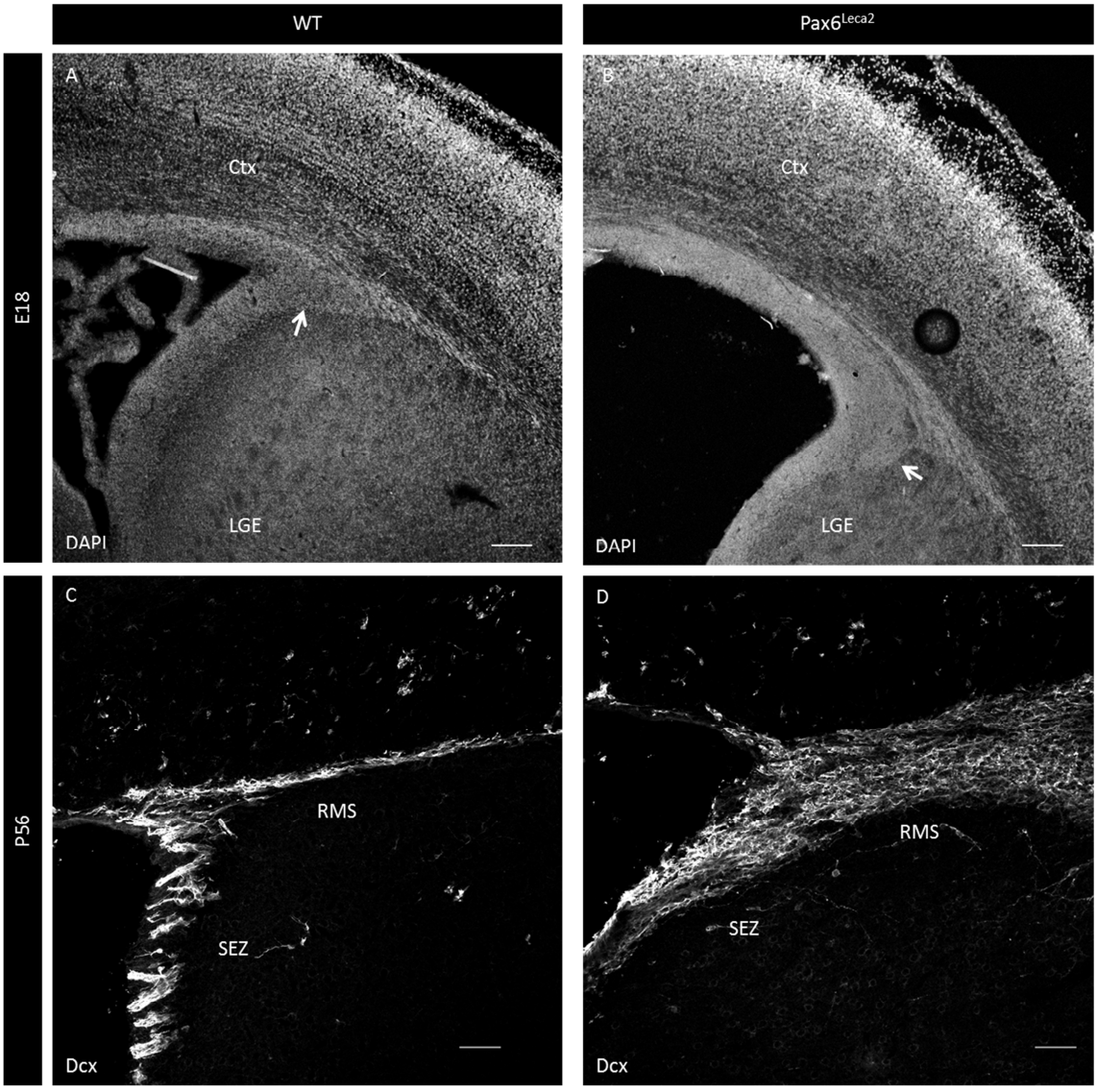


Figure 26: Cells accumulation in the Pax6^{Leca2} background

(A-B) Micrographs depicting the ventricular area of E18 WT animals (A) and their mutant littermates (B).
(C-D) Micrographs depicting the expression of the doublecortin (Dcx) in the SEZ and RMS of 8 weeks old Pax6^{Leca2} mice (D) and their wild type littermate (C). To notice the increase of Dcx+ cells in the SEZ and RMS of the Pax6^{Leca2} animals. Scale bar 100µm.

Two possible hypotheses arouse in order to elucidate the cause of this cell accumulation:

1. The RED domain of Pax6 is relevant for the proper lineage progression in the SEZ neurogenic region. Pax6 protein is expressed in the progeny of the stem cells lying in the SEZ lateral wall, namely the transit amplifying progenitors (TAPs) and the neuroblasts (Hack et al., 2005; Kohwi et al., 2005; Brill et al., 2008); impairments in their proliferation or differentiation could be the cause of the accumulation of cells.
2. The RED domain of Pax6 has roles in conferring the migratory ability to the cells in the SEZ-RMS system. The cells in the RMS, in fact, are characterized by their capability to tangentially migrate (Ghashghaei et al., 2007) in order to reach the olfactory bulbs. Losing the ability to migrate could cause the aggregation of cells in this particular region.

The attempts directed to the understanding of these possibilities will be discussed in the following paragraphs.

4.2.1.1 Neurogenic progression in the Pax6^{Leca2} mutant

To investigate the involvement of the Pax6 RED domain in the regulation of the cell proliferation in the adult SEZ we evaluated the proliferative capacity of TAPs and neuroblasts in adult (8 weeks old) Pax6^{Leca2} mutants and their age-matching littermates.

To elucidate if any of the two populations was affected, I analysed the number Dcx-positive neuroblasts in S-phase of cell cycle based on incorporation of the DNA-base analogous BrdU (one hour before sacrificing the animals, 0.5 ml of a 10 mg/ml BrdU solution are injected intraperitoneally). This approach followed by the colocalization with Dcx allows to discriminate the proliferating neuroblasts (BrdU+ and Dcx+) from the TAPs themselves (BrdU only) and locate the origin of the impairment causing accumulation of cells in the SEZ-RMS area.

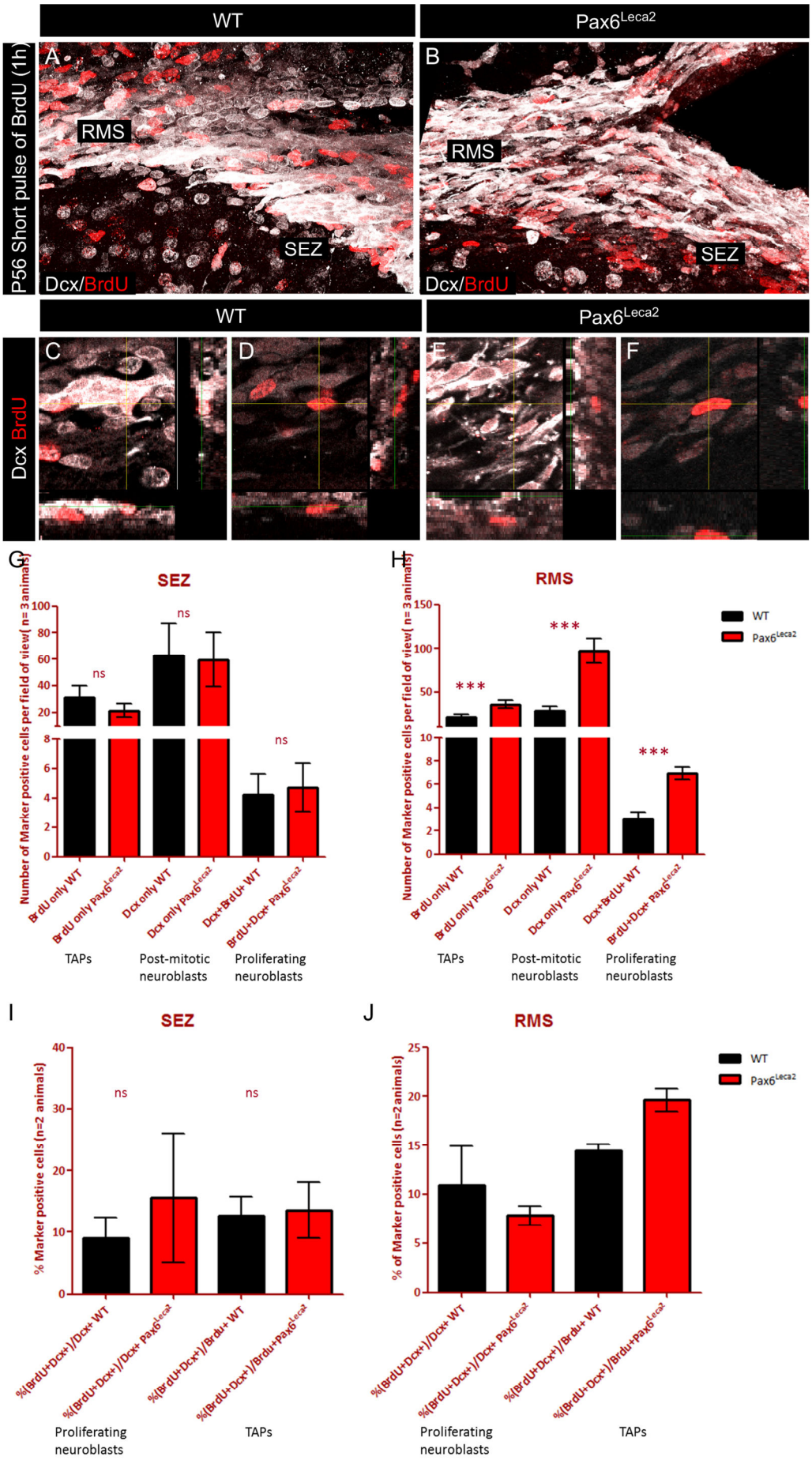

WT
Pax6Leca2
P56 Short pulse of BrdU (1h)
A
B
RMS
SEZ
Dcx/BrdU
C
D
E
F
Dcx BrdU
G
SEZ
Number of Marker positive cells per field of view(n= 3 animals)
ns
BrdU only WT
BrdU only Pax6Leca2
Dcx only WT
Dcx only Pax6Leca2
Dcx+BrdU+ WT
BrdU+Dcx+ Pax6Leca2
TAPs
Post-mitotic neuroblasts
Proliferating neuroblasts
H
RMS
***
WT
Pax6Leca2
I
SEZ
% Marker positive cells (n=2 animals)
%(BrdU+Dcx+)/Dcx+ WT
%(BrdU+Dcx+)/Dcx+ Pax6Leca2
%(BrdU+Dcx+)/Brdu+ WT
%(BrdU+Dcx+)/Brdu+Pax6Leca2
Proliferating neuroblasts
TAPs
J
RMS
% of Marker positive cells (n=2 animals)

Figure 27: Neuroblasts and TAPs proliferation analysis
(A-B) micrographs representing high magnification pictures of the SEZ-RMS area of the Pax6^{Leca2} and WT animals at postnatal stage P56. (C-F) orthogonal picture magnification of cells expressing BrdU and Dcx and BrdU only in WT (C-D) and Pax6^{Leca2} (E-F). (G-H) histograms depicting the amount of BrdU+ cells, Dcx+ and of double positive cells for Dcx and BrdU in the SEZ and RMS. Fields of view are pictures taken with a 40x magnification 800*800 pixels (2-3 sections per animals n (number of animal)=3). (I-J) histogram depicting the proportion of double positive cells among all the dividing cells and among all the postmitotic neuroblasts in SEZ and RMS. Data are represented as mean ±SEM. Mann-Whiney test for significance ns: non-significant; **P<0.01; ***P<0.001.

In the SEZ the TAPs population (BrdU only cells), similarly to the neuroblasts, is not affected by the RED domain mutation. No difference between the mutant animals and the WT was detectable in this population in the SEZ area.

In the RMS the condition is different. Looking at the absolute number of the cells positive for the markers is evident that the BrdU only cells are increased (from an average of 21.06 ± 3.223 cells in the WT to 35.88 ± 4.219 in the Pax6^{Leca2}).

The hypothesis was that the increase in number in the TAPs population would account for the increase of the population of the neuroblasts responsible for the enlargement of the RMS in the Pax6^{Leca2}.

To investigate this idea we followed the previous approach (short BrdU pulses) and focus the attention on the proliferating neuroblasts which directly generate from the TAPs (Doetsch et al., 1999).

In the SEZ the absolute number of proliferating neuroblasts (Dcx+BrdU+) and of postmitotic neuroblasts (Dcx+ only) is unchanged when the two genotypes are compared. The difference in neuroblasts number between Pax6^{Leca2} and the WT is evident only at the RMS level.

In the RMS, the number of post mitotic neuroblasts (28.65 ± 4.336 cells in the WT, 97.19 ± 14.42 the Pax6^{Leca2}) and proliferating ones is increased upon RED domain mutation (3.000 ± 0.6301 cells in the WT, 6.938 ± 0.5513 in the Pax6^{Leca2}). This difference nevertheless, disappears when the double positive population (the proliferative neuroblasts) is put in relation with total population of the post mitotic neuroblasts (Figure 27) and in relation to all the proliferating cells.

Since no relevant difference could be detected with the short pulse of BrdU paradigm explaining the enlargement of the RMS, the hypothesis is that the bulge of cells residing in the RMS is originated earlier in development and for poorly understood reasons remains in the area being composed by early generated cells and newly

supplied cells by the SEZ. In the SEZ of Pax6^{Leca2} animals, in fact, neurogenesis is proceeding in a similar manner than in the WT.

The possibility that in the SEZ of the Pax6^{Leca2} mutants the progression in the cell cycle might be delayed and other phases, rather than the S phase, might be affected remains still open.

In the SEZ and RMS system the gene HJURP is in fact expressed in both TAPs and neuroblasts. HJURP is the gene that, as shown in the previous chapter, is a possible candidate explaining at the molecular level the phenotype seen in the RED domain mutant animals having impairments in the cell cycle progression.

Investigating the data obtained from the transcriptome analysis performed on cells isolated from the SEZ niche (Beckervordersandforth et al., 2010), we could reveal the presence of HJURP also in the SEZ niche. This gene is primarily present in the stem cells (hGFAP/GFP and Prominin+) and then maintained in neuronal lineage having very high levels of expression in the progeny of the stem cells (HGFAP/GFP+ only) which, in this analysis, is composed by the TAPs and the neuroblasts.

With the *in situ* hybridization the HJURP expression was highlighted in SEZ and in the RMS (Figure 28). The HJURP expression is high in the RMS of the Pax6^{Leca2} animals where the cells show a blockage during the path through the OB.

The direct connection between HJURP and the enlargement of the RMS is still not understood and matter of speculation. The HJURP expression is nevertheless representing a bridging point between the adult and the embryonic phenotype.

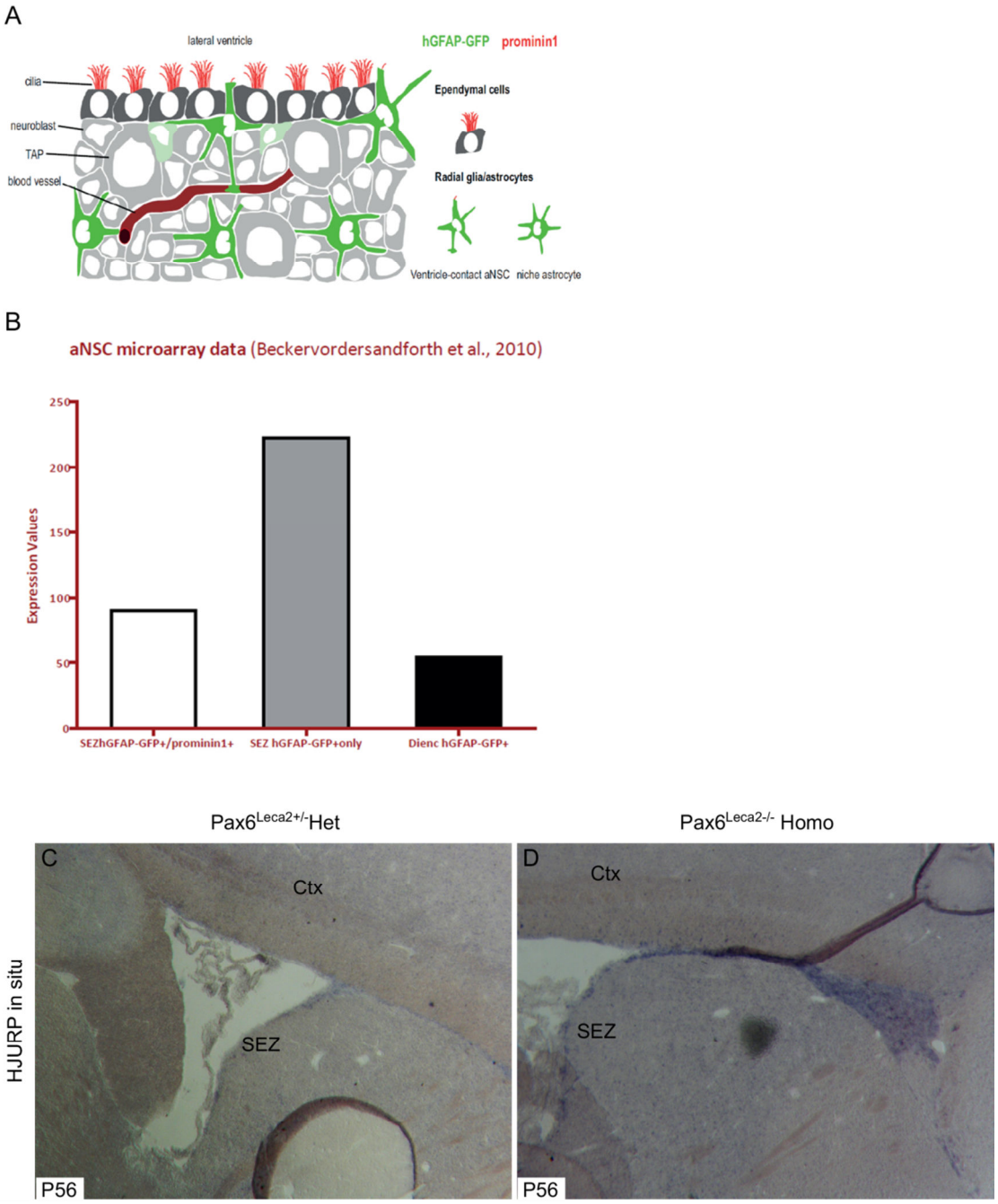


Figure 28: HJURP expression in the adult brain

(A) Scheme adapted from the original publication (Beckervordersandforth et al., 2010), representing the cell type examined by FACS sorting isolation present in adult SEZ niche of the lateral ventricle. (B) Histogram depicting the expression levels of HJURP in adult SEZ cells FACS sorted and analyzed via microarray analysis performed with Affymetrix MOE430 2.0 arrays (as described in the publication (Beckervordersandforth et al., 2010). hGFAP/GFP+ Prominin+: putative stem cells; hGFAP/GFP+only: stem cell progeny comprising of TAPs, neuroblasts and niche astrocyte; Diencephalic hGFAP/GFP+: astrocytes outside the niche used as comparison. (C-D) Micrographs depicting *in situ* hybridization in adult brains of $Pax6^{Leca2}$ homozygotes and heterozygotes, which highlight the expression of the HJURP mRNA in the SEZ and in particular in the RMS, where the neuroblasts reside. Abbreviations: Ctx: cortex; SEZ: subependymal zone.

4.2.1.2 Migration capacity of Pax6^{Leca2} neuroblasts

Neural precursors originating from the SEZ niche are characterized by their ability to migrate tangentially to the OB. Once in the OB they switch to a radial movement in order to reach their final location and get integrated into the OB system.

One further possibility arising to explain the accumulation of cells in the RMS is that the neuroblasts upon mutation of the RED domain are incapable to move tangentially along the stream.

To investigate the migratory properties of the neuronal progenitors, the SEZ area was isolated and small explants cultivated. In these conditions cells migrate from the explant allowing the analysis of the total number of migrating cells, the distance they travel and their behavior while migrating. After the migration experiment, explants can be fixed and subjected to immunohistochemical analyses to further elucidate the effect of the mutation.

Explants were isolated from the SEZ of P15 animals and placed on the matrigel for 3 days at 37°C. The distance that the Dcx-positive cells traveled from the explants was measured (Figure 29).

Dcx-positive neuroblasts in the Pax6^{Leca2} mutant migrated the same distance from the tissue explants as the Dcx-positive neuroblasts from their age-matched controls (WT: mean of the migrating distance 69.90 ± 7.926 µm; Pax6^{Leca2}: mean of the migrating distance 60.49 ± 4.321µm). From the analysis of the distances of migration of all the neuroblasts analyzed was evident that the migratory ability of the neuroblasts in the mutant explants was not impaired. In addition, Pax6^{Leca2} mutant neuroblasts formed chain structures indistinguishable for the chain structures in their age-matching siblings (Figure 29).

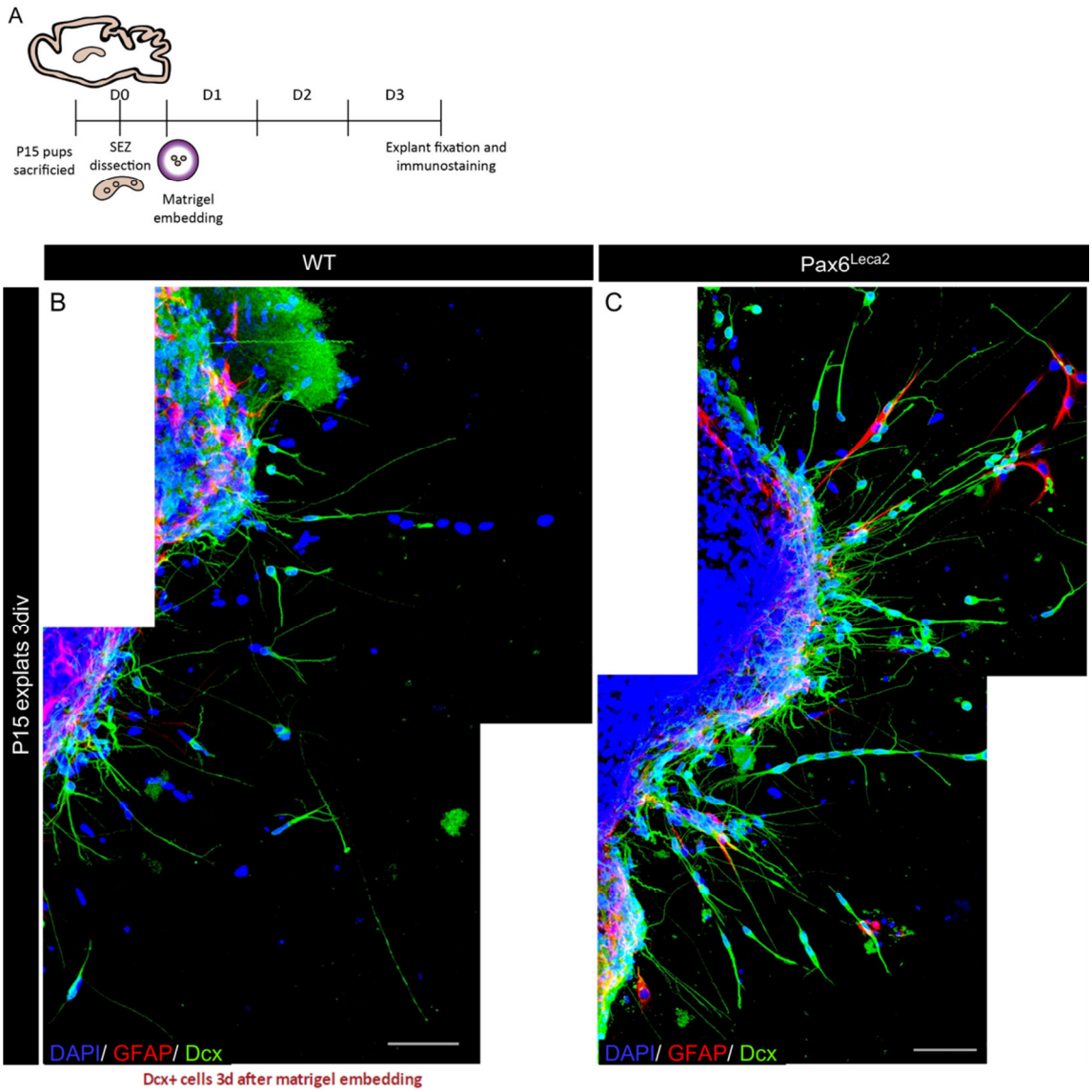

A
D0
D1
D2
D3
P15 pups sacrificied
SEZ dissection
Matrigel embedding
Explant fixation and immunostaining
WT
Pax6Leca2
B
C
P15 explats 3div
DAPI/ GFAP/ Dcx
DAPI/ GFAP/ Dcx
Dcx+ cells 3d after matrigel embedding
D
Distance from the explant (µm)
300
200
100
0
ns
WT
Pax6Leca2

Figure 29: Neuroblasts migration ability in Pax6^{Leca2} adult brains
(A) Scheme depicting the paradigm pursued as described in the method part 2.2.1.8. The experiment was repeated two times independently with a total of 73 cells Dcx+ in WT animals and 144 cells in Pax6^{Leca2}analysed. (B-C) Micrographs depicting SEZ explants from P15 mice embedded in matrigel and immunostained after 3days in vitro for neuronal markers Dcx, astrocytic marker GFAP and DAPI. Scale bar 50µm.
(D) Dot blot representing the distance in µm of every cell from the original explant 3days after matrigel embedding. Data are showed as mean ± SEM of the distance travelled by the cells in the two genotypes. No-significant difference was detectable between the two groups (unpaired TTEST p=0.2568 and Mann-Whitney test p=0.6220).

It is known in fact that neuroblasts migrating through the RMS do so aggregating themselves in so called neuronal chains. This phenomenon of cells aggregation is spontaneous and autonomous and probably initiated with the contribution of the cerebrospinal fluid (CSF) (Ghashghaei et al., 2007).

Furthermore, the neuroblasts migration via the RMS is a highly directed movement and there is no dispersion of any cell into the surrounding tissue because the cells are ensheath into an astroglial tube (Jankovski and Sotelo, 1996; Lois et al., 1996) and their route is constrained by blood vessel along the RMS (Snapyan et al., 2009).

To have insight on the presence of these physical substrates that topographically define the RMS in the Pax6^{Leca2} mutants, we performed immunohistochemistry.

In both genotypes the glial tube and the blood vessels delimiting the path of migration are present along the RMS (Figure 30) and the Pax6^{Leca2} do not present major differences compared to their WT littermates. Therefore, we conclude that the accessory structures (glial tubes and blood vessels) necessary to allow the proper migration of the cells are all in place.

These essays suggest that the accumulation of these cells cannot be explained by the migratory inability of the neuroblast or absence of any structure that allows the migration per se. This supports the idea that the RED domain mutation of Pax6 is not impairing their migratory capacity. One possibility still unexploited, which is only suggested in this work, is that a further analysis could be conducted *in vivo* in order to prove that, even in the physiological environment, the migratory performances of the neuroblasts are not defective.

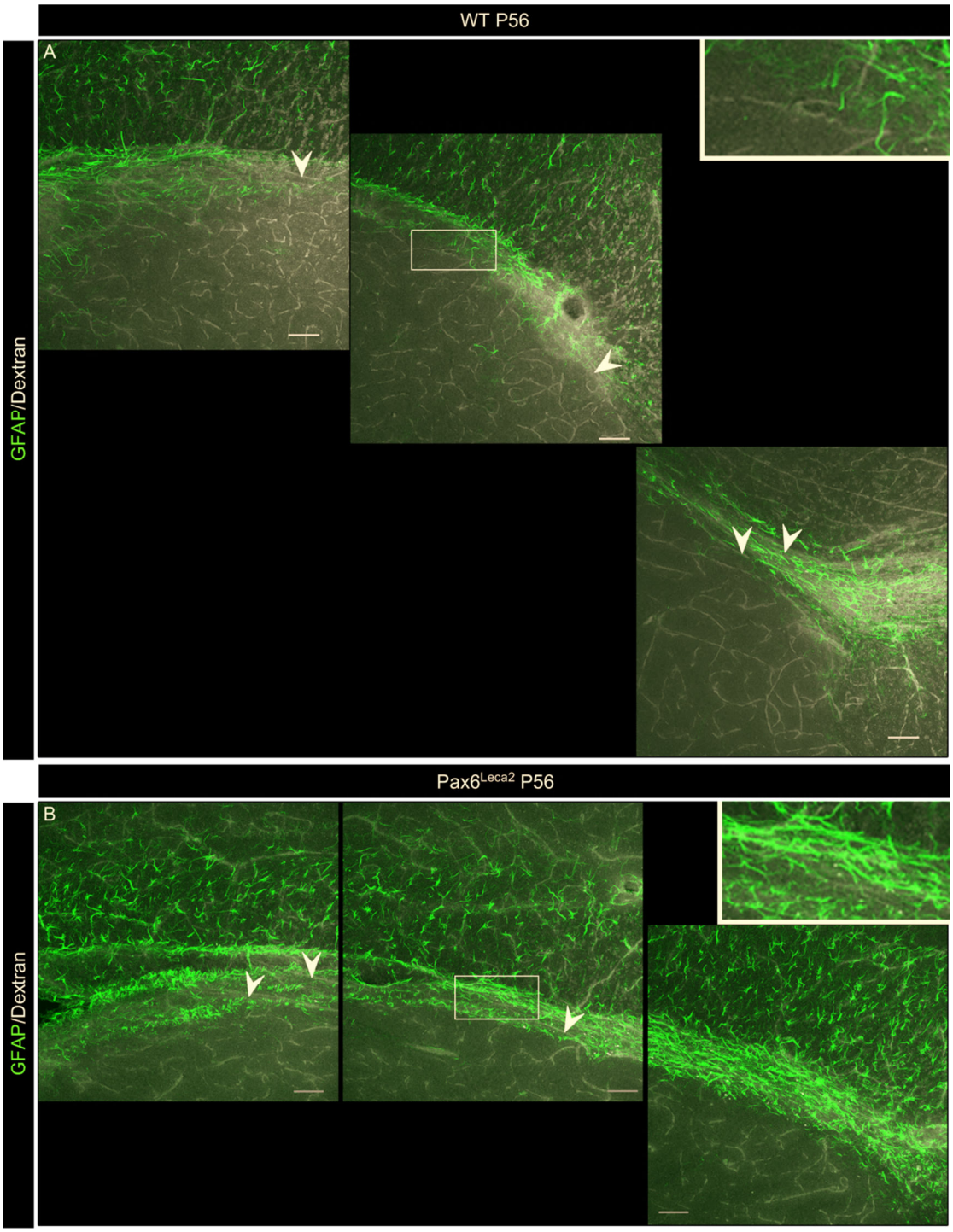


Figure 30: Migrating neuroblasts accessory structures

(A-B) Micrographs depicting the rostral migratory stream in WT (A) and mutant sections (B). In green is represented the glial tube (GFAP positive), in grey the blood vessels highlighted via fluorescent Dextran administered via tail injection. The white arrowheads highlight the blood vessels. In the insert zoom of the RMS showing that none of the two structures are impaired. Scale bar 50 µm.

4.3 Domain specific functions of Pax6

Pax6 is defined as master regulator gene. This definition underlines its capability to govern the lineage specification process regulating multiple downstream genes either directly or via a cascade of gene expression changes The Pax6 function as fate determinant has been previously elucidated in the adult SEZ system as well as in the developing cortex, underlying the crucial role of the N-terminal PD domain (Heins et al., 2002; Hack et al., 2004; Haubst et al., 2004; Hack et al., 2005; Ninkovic et al., 2013).

4.3.1 Embryonic and Adult neurospheres

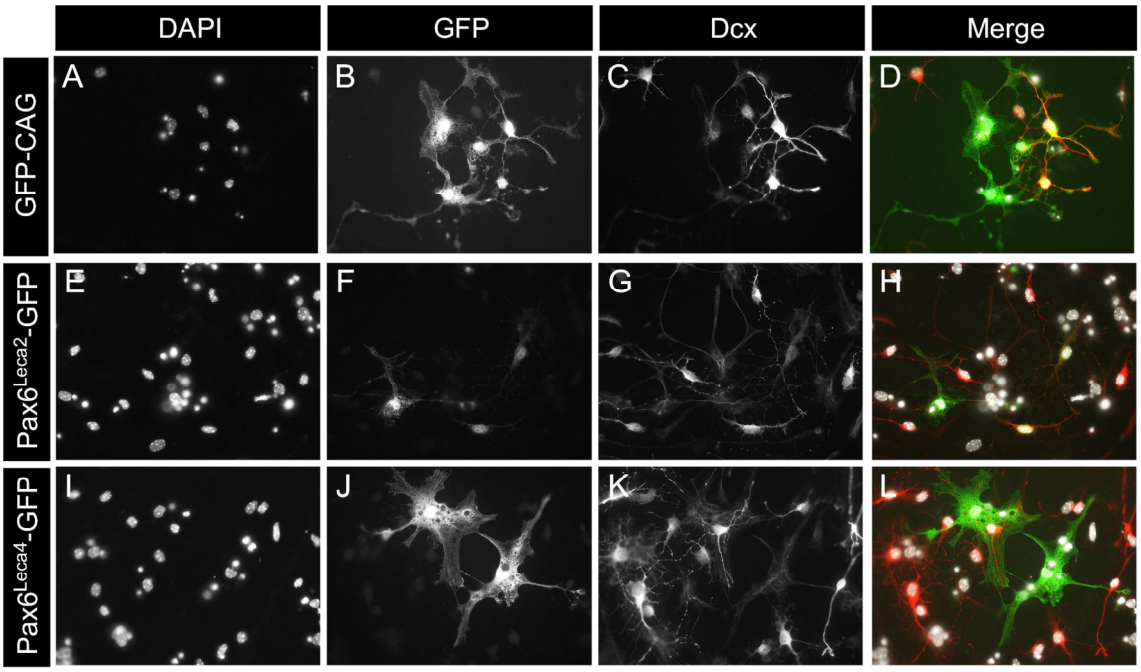
In order to understand whether the disruption of the binding capacity of the PD domain to the DNA is impairing the function of Pax6 in directing neuronal fate determination, gain-of-function experiments were performed in neurosphere derived culture.

This culture type allows quantifying the neurogenic capacity of a defined factor. Neurosphere derived cultures are, in fact, gliogenic cultures which give rise, independently from the region of origin of the cells cultured, to about 80% of glia cells (GFAP+ cells) and to 20% of neurons (Dcx+ cells) (Hack et al., 2004).

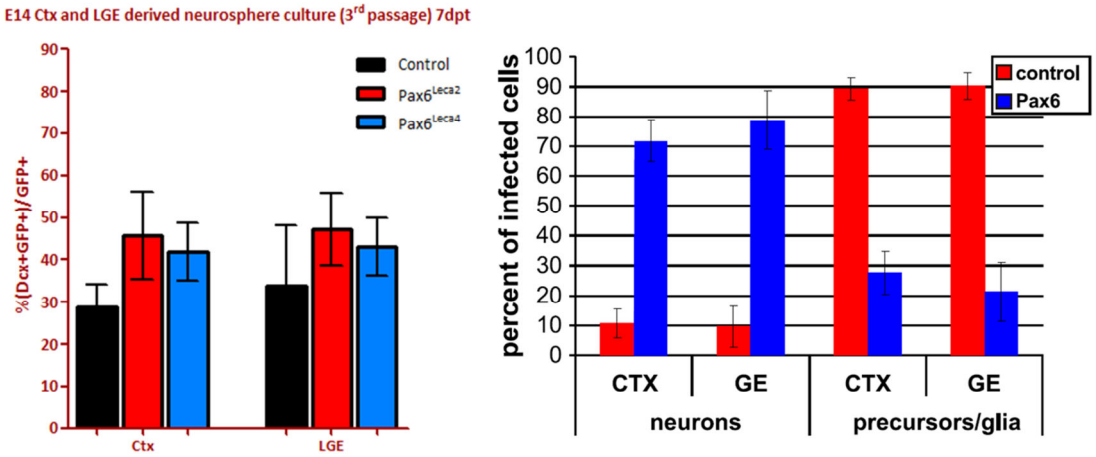
When these cells are challenged under certain conditions, e.g. the overexpression of neurogenic factors like Pax6, the outcome is reverted with the majority of cells being part of the neuronal type (Hack et al., 2004).

In this analysis neurospheres derived from E14 Cortex and LGE, as well as from adult SEZ, were exposed to Pax6 and its two mutant forms ($Pax6^{Leca2}$ and $Pax6^{Leca4}$), via viral vector transduction. The different regions and developmental stages were chosen in order to validate whether the response of the cells upon exposure to Pax6 depends on the context of origin of the cells themselves.

After the third propagation passage, where the cells were growing in suspension in medium supplied with growth factors (EGF and FGF2), the cells were plated and transduces with viral vectors expressing Pax6, $Pax6^{Leca2}$ and $Pax6^{Leca4}$.


DAPI
GFP
Dcx
Merge
GFP-CAG
Pax6Leca2-GFP
Pax6Leca4-GFP
A
B
C
D
E
F
G
H
I
J
K
L

M


E14 Ctx and LGE derived neurosphere culture (3rd passage) 7dpt
%(Dcx+GFP+)/GFP+
Control
Pax6Leca2
Pax6Leca4
Ctx
LGE
percent of infected cells
control
Pax6
CTX
GE
CTX
GE
neurons
precursors/glia

N

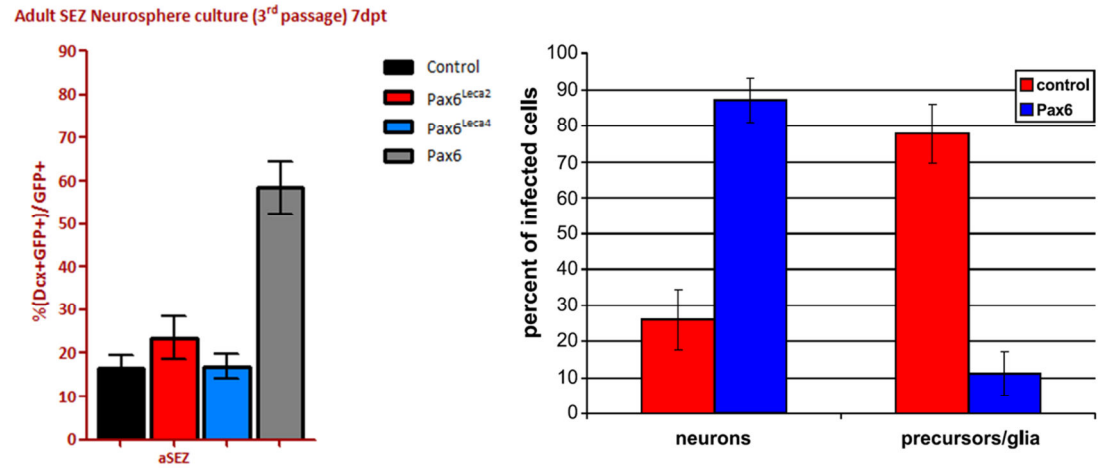

Adult SEZ Neurosphere culture (3rd passage) 7dpt
%(Dcx+GFP+)/GFP+
Control
Pax6Leca2
Pax6Leca4
Pax6
aSEZ
percent of infected cells
control
Pax6
neurons
precursors/glia

Figure 31: Embryonic ad adult neurosphere
(A-L) Example of cells derived from neurospheres culture after transfection of control (A-D), $Pax6^{Leca2}$ (E-H) and $Pax6^{Leca4}$ viruses (I-L). (M-N) Histogram depicting the number of neurons (Dcx+cells GFP+cells) among the viral transduced cells (GFP+cells) in the neurosphere cultures derived from the E14 Cortex and the E14 LGE (M) and the adult SEZ (N). (M-N) Histograms are compared with data derived from (Hack et al., 2004), figure 6 of the original publication panel E (M), panel D (N). Data are reported as mean± SEM. Unpaired T Test for significance. Non-significant difference is detected between the cells transfected with a control virus and the Pax6 mutant forms for the PAI and the RED domain in E14 derived cells as well as in the adult derived cells.

To measure the neurogenic capacity of the Pax6 PD domain mutants, the number of neurons generated from the glial cells was assessed. When the glia cells are exposed to the canonical form of Pax6, regardless if they are originating from the cortex, the ganglionic eminence (Hack et al., 2004) or the SEZ, they give rise to 60-80% of neurons. Differently, exposing the glia cells to the RED domain or PAI domain mutant forms did not change the fate of the cells maintaining the number of neurons generated similar to the control condition (Figure31).

These results suggested that, differently from the overexpression of the full Pax6 protein, the mutated form for the RED domain and the mutated form for the PAI are not able to direct the glia cell to the neuronal fate. This is valid for cell originating from the developing and the adult brain.

The activity of only one subdomain of the PD DNA binding domain is not sufficient to switch the fate of the glial cells to the neuronal one.

However, the lack of capability of RED domain mutation to direct glia cells to neurogenesis is in contrast with the previous results obtained from primary culture, composed by cells already primed to the neurogenic lineage, where the Pax6 RED domain mutation was still capable to induce neurogenesis and failed uniquely when the PAI domain was impaired (Walcher et al., 2013).

These results suggest that Pax6 neurogenic capacity is cells specific. The transcription factor Pax6 needs the full activity of its PD domain to induce neurogenesis from glia cells. In this system both the subdomain PAI and RED are necessary to conduct neurogenesis.

5 Discussion

During brain development the control of the proliferation of cells and their fate specification is crucial to preserve the equilibrium in every region of the brain, despite the high dynamicity of the developing process per se.

In the telencephalon, the transcription factor Pax6 plays a pivotal role coordinating the proliferation of progenitors and their fate specification (Osumi et al., 2008; Georgala et al., 2011a).

This work was aiming to the understanding of the role of the RED domain of Pax6 in controlling the cell cycle progression of the progenitors located in the ventral telencephalon (LGE) and in the understanding of the relevance of the functionality of this domain for the proper progression of the neurogenic process in the postnatal subependymal zone that is originating from the LGE. Towards these ends the proliferative capacity of the different progenitors in the LGE and the neurogenic progression in the adult SEZ were analysed in domain specific mutant mice $Pax6^{Leca2}$.

The data collected with this work suggest that in the ventral telencephalon the RED domain mutation of Pax6 brings to a prolongation of the cytokinesis in the apical progenitors cells. The impairments in the cytokinesis prompt to a reduction of cell survival. In addition, the RED domain mutation induces the upregulation of the chaperone protein HJURP (Holliday junction recognition protein). The upregulation of HJURP in vitro recapitulates the phenotype observed in the progenitor cells of the $Pax6^{Leca2}$ animals. Differently, in the postnatal neurogenic niche, the impairments in binding of the RED domain of Pax6 do not induce failure in the progression through the neurogenic lineage. This suggests that the accumulation of neuroblasts, evident in the postnatal brain of $Pax6^{Leca2}$ animals, has developmental origin rather than emerging at late stages.

5.1 The Pax6 RED subdomain is responsible for the cell cycle progression of apical progenitors

The RED DNA binding domain of the transcription factor Pax6 modulates the progression of the cell cytokinesis in the embryonic apical progenitors of the LGE. In

the LGE, the exclusive affection of the APs offered the opportunity to resolve the importance of the RED domain for the regulation of cell cycle progression without additional influence of the cells in the subapical region. In the APs of the LGE, the impairment in the RED domain capacity to bind to the DNA leads to a prolongation of the cell cytokinesis, unraveling a novel role for this subdomain.

Pax6 controls the proliferation of the apical progenitor cells of the cerebral cortex. The non-functional Pax6 protein leads to alterations in the spindle orientation of the cells and consequently of their cleavage plane (Asami et al., 2011). The latter generates daughter cells delaminating from the ventricular side which contribute to the increase of cells in active mitosis located in subapical positions (Asami et al., 2011). The increase in this population of cells is in fact one of the most prominent phenotypes upon disruption of Pax6 protein (Warren et al., 1999).

The PD domain of Pax6 is involved in controlling the proliferative capacity of the RG. The exclusive impairment of this domain, in fact, resembles the phenotype of the $Pax6^{Sey}$ mutant (Haubst et al., 2004). In particular in the cortical progenitors, the two subdomains of the PD domain of Pax6 antagonize in the regulation of proliferative genes. Impairing the PAI domain leads to the downregulation of pro-proliferative factors (Walcher et al., 2013) suggesting that it might promote proliferation in physiological condition. Conversely when the RED subdomain is defective, the numbers of PH3 positive progenitors is increased (Walcher et al., 2013), suggesting that it might inhibit proliferation in normal condition.

The increment of PH3 positive cells upon RED domain mutation is a general phenotype for the apical progenitor cells expressing Pax6, independently from their location in the dorsal or ventral side of the telencephalon. In the LGE, the AP cells population encounters an increment of about 30% if compared with the WT situation (Figure 14), in the cortex the AP cells almost doubled in comparison to the WT (Walcher et al., 2013).

The behavior of the non-apical progenitor cells is different. Upon RED domain mutation, in fact, in the cerebral cortex the basal progenitors increase (Walcher et al., 2013) whereas in the LGE this increment is not occurring either in the subapical progenitors or in the basal progenitors. The different behavior of the non-apically

dividing cells, in the dorsal and ventral telencephalon, could reside in the different response of the progenitors generating them to the RED domain mutation. In the LGE SP and BP are originating from the AP that are composed by RG always dividing asymmetrically having often, as first progenitor generated, a short neural precursor (SNP) also dividing apically (Pilz et al., 2013). In the LGE, SNPs are able to further proliferate within the ventricular region before undergoing differentiation (Gal et al., 2006; Stancik et al., 2010; Tyler and Haydar, 2013) and probably compensate for the impairments caused by the mutation. The SNPs in the cortex are rather different. The same population directly generates post mitotic neurons (Pilz et al., 2013) leaving to the RG task of generating non-apically diving progenitors. The non-apically dividing progenitors of dorsal and ventral region might respond differently to the RED domain mutation because different is the population that is originating them.

The increment of PH3 positive mitotic figures is accompanied by a reduction in the cell survival (Figure 16), suggesting that the cell cycle of the progenitors was affected. This is a common feature between dorsal (Walcher et al., 2013) and ventral regions pointing to the necessity of a functional RED domain to properly control the cell cycle progression of the progenitors.

Ex vivo live imaging revealed the indisputable evidence that the last phase of the cell division is prolonged upon RED domain mutation and the progenitor cells encounter difficulties in concluding their cytokinesis (Figure 19). All the cells dividing during the time lapse are affected, but it is difficult to predict which are the identity of the cells and their stage of differentiation. The fate of the cells after scission of the two daughter cells is still a speculation. In fact, some of them might die as a consequence of not proper DNA segregation but most of them might still be able to conclude successfully the scission into two daughter's cells considering that no major defects are detected in the region at the moment of the analysis and at later stages.

This is the first report suggesting the direct implication of Pax6 in the regulation of cell cytokinesis. Other transcription factors like the zinc finger transcription factor Sp2 (specificity protein 2) are reported as regulators of cell cycle progression, in particular Sp2 is suggested to be able to regulate centrosomal proteins in the M-phase progression (Liang et al., 2013). Knowing the relevance in this context of genes

encoding for centrosomal associated proteins and knowing that Pax6 is also relevant in the regulation of such genes (Holm et al., 2007) with for example its capability to directly target the Spag5 (Asami et al., 2011), I searched for possible targets in the microarray of RED domain mutants. Nonetheless, since centrosomal proteins are not significantly differentially regulated in the RED domain context, I moved searching for targets likable to the found phenotype.

The evidence that the point mutation in the RED domain (R128C) is causing upregulation of the Pax6 mRNA itself (Figure 12), suggests that this is acting as a dominant negative mutation This is also further corroborated by two different reporter assays in vitro (Walcher et al., 2013; Xie et al., 2013), highlighting that the $Pax6^{Leca2}$ mutation enhances transcriptional activation and that in physiological condition the RED domain has inhibitory functions.

From these assumptions, the set of upregulated genes in the RED domain mutants in comparison with the WT was analyzed. In this group the Holliday junction recognition protein (HJURP) was detected. HJURP gene changes directly with the mutation of the RED domain. The PAI domain mutation and the non-functional Pax6 do not interfere with the expression of the protein HJURP (Walcher et al., 2013).

HJURP is a CENP-A specific chromatin assembly factor, important for the deposition of CENP-A at the centromere (Stellfox et al., 2013). According to the working model, the $Pax6^{Leca2}$ mutation leads to the disruption of the inhibitory function of the RED domain which leads to the upregulation of this chaperone protein.

The in vitro upregulation of this protein recapitulates the impairments in cytokinesis evident in the RED domain mutant cells. In this work it is suggested that cells with mutant Pax6 having HJURP upregulated cannot conclude their cytoplasm scission due to the presence of DNA lagging in intercellular bridge. HJURP dysregulation has been in fact described as the cause of malfunctioning of the kinetochore which results in chromosome instability (Kato et al., 2007; Mishra et al., 2011).

The direct transcriptional control of this chaperon protein exerted by the RED domain of Pax6 is still speculative but nevertheless opens the possibility of a new level of regulation of the Pax6 protein acting at the centromere level.

5.2 PAI and RED domains are necessary for Pax6 neurogenic function

Neurogenesis is linked to proliferation and in the brain, at different developmental stages, depends on Pax6. In the developing cortex, losing this protein causes the decrease of cortical neurons (Schmahl et al., 1993; Haubst et al., 2004; Tuoc et al., 2009); in the adult brain, deletion of Pax6 in the neurogenic zone (SEZ) leads to fate conversion of cell towards a glia fate (Ninkovic et al., 2013). In order to accomplish to this function at the molecular level Pax6 utilizes the PD domain (Haubst et al., 2004). In more details, the improper DNA binding capacity of the PAI subdomain leads to the reduction of the neuronal output and concomitantly to the reduction of the proliferation capacity of the progenitors. The RED domain mutants, on the contrary, do not have defects in neurogenesis (Walcher et al., 2013), indicating that the neurogenic fate specification of the progenitors in vivo is achieved prominently by the activity of the PAI DNA binding domain.

The analysis conducted in this work highlighted the importance of both the subdomains of the PD to fate convert already committed cells to the neuronal fate. Previously was shown that in primary culture derived cells (cells fully committed to the neuronal fate from developing cortex) the neurogenic potential of the PD domain of Pax6 is abolished only when the PAI subdomain is impaired (Walcher et al., 2013). In this condition the activity of the RED subdomain is sufficient to regulate neurogenesis. When the cells are in a different context and committed to glia fate, the PAI and RED domain simultaneously need to be available to convert the cells to the neuronal fate.

In embryonic derived neurospheres from dorsal and ventral telencephalon when the RED domain of Pax6, or the PAI domain, is impaired the neurogenic capacity of Pax6 is lost. When the neurosphere derived cells are transfected with the viral vectors expressing both the two mutant forms of Pax6, in the culture are generated a similar amount of neurons than in the control conditions (Figure 31). In contrast to the full length of Pax6 which is giving rise to about 80% of neurons among the transfect cells in these cultures (Hack et al., 2004), in the PAI and RED domain mutant the number of neurons is settled around 40% in the embryonic derived cells, and 20% in the adult derived cells. The incapability of both the subdomain mutations to fate convert the glia

cells is valid for the embryonic derived neurospheres as well for the adult derived ones. The comparable reaction of the cells when exposed to neurogenic factors like Pax6, independently from the region of origin or the developmental stage of the cells cultivated, it is supported by previous results (Hack et al., 2004). What is known is in fact that when cells are treated with growth factors (like EGF and FGF), as in the case of neurospheres, they are induced to lose their dorsal and ventral identity as well as the age difference. Notably, the region specific differences in gene expression among cells from cortex or GE are reduced and even neurospheres from adult region contain the same expression levels of the same transcription factors with only few exceptions (Hack et al., 2004).

Thus these results suggest that Pax6 neurogenic capacity is cells context specific and the transcription factor Pax6 needs the full activity of its PD domain to induce neurogenesis and convert fate specification. The lack of capability of RED and PAI domains to direct glia cells to neurogenesis, complements the previous results obtained from primary culture (Walcher et al., 2013), giving new insides on domain-specific functions.

The context specific response of the RED domain in regulating the neurogenic function of Pax6 suggests an interesting hypothesis that this mutation impairs the interaction with chromatin remodelling factors. Pax6, in fact, has been proposed as a recruiter of chromatin remodelling factors to gene loci and Brg1, a chromatin remodelling factor, has been shown to directly interact with Pax6 (He et al., 2010; Ninkovic et al., 2013).

Supposedly, in already committed cells the chromatin is already accessible to be bound whereas when the reprogramming machinery has to be ignite by Pax6 is necessary that the RED domain is fully active to remove the suppression of neuronal genes and be able to convert the fate of the cells.

The mutation $Pax6^{Leca2}$ is located in the third α-helix of the RED subdomain (Xu et al., 1999). This causes a change from a positively charged arginine to a neutral cysteine which is known to be able to disrupt the helix conformation in different proteins (Lund et al., 2008). The conformational changes in the secondary structure of the Pax6 are predicted not only to disrupt the interaction with DNA but also might impair protein-protein interaction.

The fact that Pax6 cannot create an interaction with remodelling factors is an intriguing hypothesis that opens further fields of investigations.

5.3 Pax6 RED domain affects the development of the postnatal neurogenic niche

In the adult brain, at physiological conditions, the stem cells residing in the subependymal zone (SEZ), via multiple step of fate restriction, progress through the neurogenic process. Neuroblasts, young neurons generated in this area, move along the rostral migratory stream to reach the olfactory bulbs.

In the $Pax6^{Leca2}$ background, in the area where the neuroblasts enter in the rostral migratory stream, there is an accumulation of cells. These cells express the neurogenic marker doublecortine (Dcx), suggesting that they derive from neurogenic process that might be impaired at different levels. Nevertheless, from the analysis conducted in this work, there is no evidence supporting that the cells accumulate at postnatal stages. In the $Pax6^{Leca2}$ in fact, neither the neuroblast production nor their intrinsic capability to migrate are impaired.

These suggestions all point to the fact that the RED domain functionality is not determinant for the progression of the neurogenic process in the adult SEZ, or could be replaced by the PAI subdomain.

The hypothesis that is supported is that the accumulation of cells located in the RMS has a different developmental origin. The SEZ-OB system is in fact originating from the LGE (Merkle and Alvarez-Buylla, 2006).

In the LGE region of the mutant animals, has described above, the apical progenitors are facing a prolonged cytokinesis and the protein HJURP is taking part on this process. In the cells present in the RMS of $Pax6^{Leca2}$ mice HJURP mRNA is present and I cannot exclude a role of this protein in the regulation of the cell cycle process, at least in a subpopulation of cells residing in the SEZ-RMS region.

In addition, an evident phenotype of the RED domain mutant animals is the presence of a reduced OB and this can also be due to improper cell cycle progression in the LGE.

The OB develops very early during embryogenesis from a predetermined region of the rostral telencephalon (Lopez-Mascaraque et al., 1996). The OB system is composed by

projection neurons and interneurons. The second group is composed by granule cells and periglomerular cells. These cells are largely generated during early postnatal life and their production is maintained lifelong (Rosselli-Austin and Altman, 1979; Ming and Song, 2005).

However, was found that the LGE is the primary source of early generated OB interneurons (Tucker et al., 2006). These cells, originating from the LGE, migrate via a primordium of RMS into the prospective OB between E12.5 and E14 (Tucker et al., 2006). Their capability to migrate is acquired in the LGE environment between E9.5 and E11 (Tucker et al., 2006). Supposedly in the LGE of the $Pax6^{Leca2}$ animals, the impairment in cell cycle progression might lead not only to depauperate the numbers of cells reaching the OB but also to decrease their migratory fidelity.

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Affidavit

Eidesstattliche Versicherung/Affidavit

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation "Transcription factor Pax6 regulates cell cycle progression and cell fate determination: the modular logic of complex transcriptional control„ selbstständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe

I hereby confirm that the dissertation "Transcription factor Pax6 regulates cell cycle progression and cell fate determination: the modular logic of complex transcriptional control" is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

München, 15.06.2015
Munich, 15.06.2015

Stefania Petricca

List of Contributions

The following people contributed material or raw data to this work.
Dr Sven Falk provided movies of wild type embryonic ventral telencephalon brains (E14). Example pictures derived from these movies are used in the Figure 18 panels A and B. The analysis of these movies are summarised in Figure 19.
Andrea Steiner Mezzadri provided the cells used for the analysis in Figure 31.
Dr Tessa Stahl (born Walcher) provided cDNA for the analysis in Figure 12 panel E and E14 brain sections for the analysis in Figure 16.

Munich, 15.06.2015

Stefania Petricca Prof Dr Magdalena Götz

Acknowledgments

"Every step I have taken in my life has led me here, now" Alberto Garutti

Reaching this page just one thought echoes in my mind: "It is over!"

This though is accompanied by thousands feelings: excitement, fear of the future but mostly gratitude for the wonderful time I had in the last years.

First and foremost I owe my deepest gratitude to Jovica Ninkovic and Magdalena Götz for their continuous support, patience, motivation and enthusiasm. This thesis would have never been possible without your guidance.

I am very grateful to my Thesis Advisory Committee, for important comment and suggestions in the past years.

I would like to show my gratitude to all the people in the Graduate School of Systemic Neurosciences: was wonderful being one of you.

A very special thanks goes to all the members of Götz group: technicians, scientists, students, but mostly friends. Your presence made this journey special and I will take my next steps jealously guarding a piece of all of you in my heart.

Lastly my greatest thanks goes to my family. My parents, my sisters, my "brothers", Tommaso, Alberto e Marta, you are all the pillars of my life…and especially you, Matteo. This is all for you. Your strength and constant believe in me gave me the determination to continue without giving up. Since this journey brought me also to you then it was definitely worth doing it.

Grazie a tutti.

Curriculum Vitae

Name: Stefania Petricca

Place and date of birth: Sora, 17/07/1984

Nationality: Italian

E-mail: petriccastefania@gmail.com

EDUCATIONAL BACKGROUND

March 2015	**Lüdwig Maximilian University - München** PhD in Biology (Helmholtz Zentrum München, Institute of Stem Cell Research)
September 2010	**Lüdwig Maximilian University - München** Fast-track student, Graduate School of Systemic Neurosciences
December 2008	**"La Sapienza" University of Rome - Rome** Bachelor of Sciences in Biology
2003	**High school "Liceo Socio-Psico Pedagogico V. Gioberti"** Italy Diploma Mark100/100

PROFESSIONAL BACKGROUND

October 2010	**Helmholtz Zentrum München, Institute of Stem Cell Research**
March 2015	PhD student
January 2009	**Helmholtz Zentrum München, Institute of Stem Cell Research**
September 2009	Internship project

LIST OF PUBBLICATIONS

Ninkovic J, Pinto L, **Petricca S**, Lepier A, Sun J, Rieger MA, Cvekl A, Favor J and Götz M.	*The transcription factor Pax6 regulates survival of dopaminergic olfactory bulb neurons via Crystallin αA.* **Neuron 2010***.*
Ninkovic J, Steiner-Mezzadri A, Jawerka M, Akinci U, Masserdotti G, **Petricca S**, Fischer J, von Holst A, Beckers J, Lie CD, Petrik D, Miller E, Tang J, Wu J, Lefebvre V, Demmers J, Eisch A, Metzger D, Crabtree G, Irmler M, Poot R, Götz M.	*The BAF Complex Interacts with Pax6 in Adult Neural Progenitors to Establish a Neurogenic Cross-Regulatory Transcriptional Network.* **Cell Stem Cell. 2013**
Rosa-Eva Hüttl, Simone Eckstein, Tessa Stahl, **Stefania Petricca**, Jovica Ninkovic, Magdalena Götz, Andrea B Huber	*Functional dissection of the Pax6 Paired Domain: Roles in neural tube patterning and peripheral nervous system development.* **Developmental Biology (in revision)**