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THE APICAL POLARITY COMPLEX PROTEIN, PALS1, REGULATES CELL FATE IN THE DEVELOPMENT OF CEREBELLUM AND NEOCORTEX

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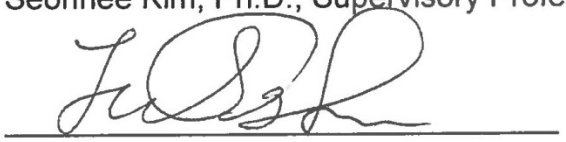

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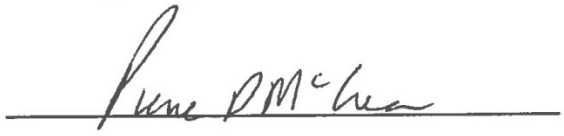

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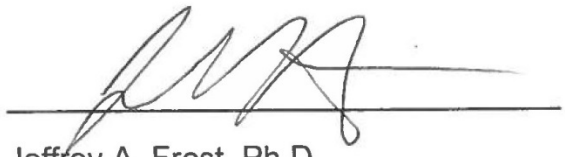

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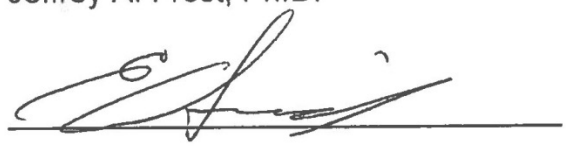

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THE APICAL POLARITY COMPLEX PROTEIN, PALS1, REGULATES CELL FATE IN THE DEVELOPMENT OF CEREBELLUM AND NEOCORTEX

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DEDICATION

I dedicate this work to my family, Minkyeong Jo and Joshua S. Park, whose love supported me throughout this entire period. I also dedicate this work to my parents who have supported my all of my endeavors. There is the only word to describe their concern and care for me: love. I love all of them forever.

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It would not be possible to complete my thesis without sincere support and advice from my mentor, colleagues and family members. I sincerely appreciate each of them. I owe the deepest gratitude to my mentor Dr. Seonhee Kim, who accepted me as a graduate student without lab experience. I have learned a lot of biological techniques and deep thinking that helped me with the scientific questions that I have confronted for 5 years.

I also would like to show my appreciation to our committee members in the past and present. A special thanks to my supervisory committee members, Dr. Pierre McCrea, Dr. Jeffrey A. Frost and Dr. Eric Swindle for all their help with scientific advice and suggestions.

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Finally, I appreciate my family: Minkyeong Jo, my lovely wife, and Joshua S. Park, my precious son. They have been my support to live, and they are the strongest motivation that I have to continue science. I sincerely love my family.

THE APICAL POLARITY COMPLEX PROTEIN, PALS1, REGULATES CELL FATE IN THE DEVELOPMENT OF CEREBELLUM AND NEOCORTEX

Jun Young Park

Supervisory Professor: Seonhee Kim, Ph.D.

Through their biased localization and function within the cell, polarity complex proteins are necessary to establish the cellular asymmetry required for tissue organization. Well-characterized germinal zones, mitogenic signals, and cell types make the cerebellum and neocortex excellent models to address the critical function of polarity complex proteins in the generation and organization of neural tissues. Here we report a focal distribution of Pals1, a central component of the apical complex, in progenitors. Our genetic analyses revealed that Pals1 deletion in the brain developed a remarkably undersized and disrupted layer structure of cerebral cortex and cerebellum. Furthermore we demonstrated that Pals1 is not only essential for brain organogenesis, but is also required for maintaining a cycling pool of progenitors in germinal zones and preventing premature differentiation. Interestingly, we did not detect profound changes in the downstream effects of well-established mitogenic/morphogenetic signaling through Shh and Notch in the Pals1 mutant. However, the localization of other apical complex proteins and tight junction proteins was severely affected by the absence of Pals1, which likely resulted in impaired cell adhesion and compromised tissue integrity. Importantly, we have found a critical function of Pals1 in regulating mitosis as Pals1 deletion causes the delay of mitotic progression and incomplete chromosome segregation. Additionally, we uncovered a crucial downstream factor mediating Pals1

function, Pttg1, which is known as an essential protein for sister chromatid segregation during mitosis. Thus, our study identifies Pals1 as a new intrinsic factor required for the proliferation and differentiation of neural progenitor cells by ensuring normal progression of mitosis.

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ABBREVIATIONS

VZ	ventricular zone
RGP	radial glial progenitor
NEC	neuroepithelial cell
H&E	hematoxylin and eosin
PP	preplate
MZ	marginal zone
IPC	intermediate progenitor (=basal progenitor)
BP	basal progenitor
SVZ	subventricular zone
FL	fibrous layer
CP	cortical plate
SP	subplate
NES	nuclear export signal
NLS	nuclear localization signal
URL	upper rhombic lip,
PCL	Purkinje cell layer
EGL	Extracellular granular layer
BG	Bergmann glia
CP	choroid plexus
HS	hemisphere
VS	vermis

Chapter One:

Background and Introduction

Introduction

Cell polarity is fundamental to many aspects of cell and developmental biology, and is involved in developmentally integral events, such as the process of proliferation, differentiation and morphogenesis in both unicellular and multicellular organisms. The epithelial cell layer is an intensively polarized layer of cells that contacts with the outside environment. The loss of adhesion and cell polarity results in tumors, apoptotic cell death and abnormal development of organs. Polarity complex proteins are known to be an intrinsic mechanism responsible for the generation of the apico-basal axis in the cell and for the maintenance of apical junctions (Assemat et al., 2008). The role of these complex proteins in setting up cell and tissue polarity which is required for various cellular process including asymmetric cell division, directed migration and growth during development is well established from *Caenorhabditis elegans* to mammals. Furthermore, fundamental roles of polarity complex proteins are identified in brain development.

The mammalian cerebellum and cortex have a very specialized germinal zones which share specific epithelial characteristics. An increase in neuronal number during brain development is a highly regulated process defined by the number and cell division mode of progenitors in germinal zones (Gotz & Huttner, 2005). Several neuronal and glial progenitors are generated from these germinal zones during the brain development, depending on different stages or expression of different fate determinants. Although many intrinsic and extrinsic factors have been identified to influence cerebellum and cortical development, we address Pals1 as an intrinsic molecule essential for mitotic progression and the maintenance of the progenitor pool. With such evidence, we

support Pals1's importance in the regulation of cell fate decisions during brain development.

1. Polarity Complex Proteins and their interactions

The formation of epithelial structure in tissue refers to the asymmetric distribution of several protein complexes, leading to cell polarity and cell-cell junctions. Polarized epithelial cells can result in differentiating two sides of membrane domains by sequestering different biomolecules: the apical domain facing the external environment and basal domain interfacing with adjacent cells and connective tissue (Figure 1). Through genetic studies, a set of genes were discovered in the *C. elegans* and *Drosophila melanogaster* model organisms, which were termed polarity complexes. These polarity complex proteins include the following three: Par3/Par6/aPKC (Par complex), Crumbs/Pals1/Patj (Crbs complex), and Scribble/Lgl/Dlg (Scrib complex). All of the following complexes have been confirmed to be responsible for determining epithelial polarity, and they often interact each other to regulate polarity via specific interacting domains (Figure 2). Such responsibilities include the development of the apico-basal polarity and the maintenance of apical junctions, such as tight junctions and adherens junctions, in mammalian epithelial cells. Each member of the core polarity complex proteins will be fully explored in extensive detail regarding their essential interacting partners and their individual roles in the maintenance of cell polarity. In this thesis, through referring back to known aspects of polarity complex proteins and their developmental implications, a conclusion will be made regarding the identified functions of the studied Pals 1 protein in various systems, including the developing cerebellum

and the neocortex, focusing on different types of progenitors and their regulation of proliferation and differentiation.

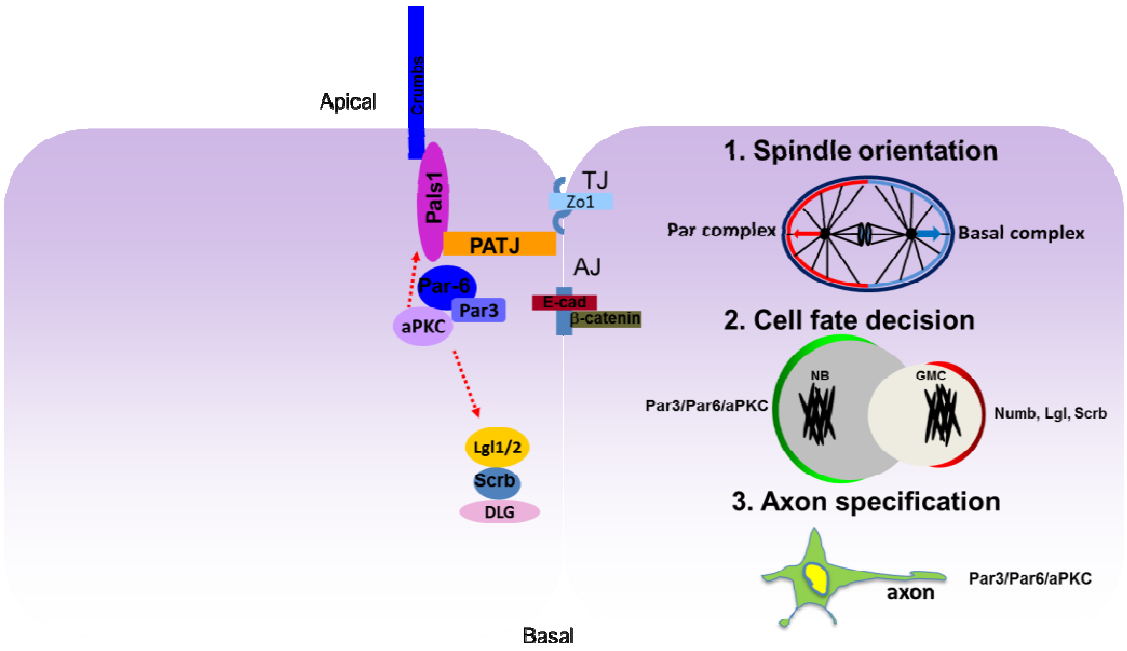


Figure 1. The localization of apico-basal complex proteins and their function in polarized cells. Apico-basal complex proteins are localized in a distinct location, where they can not only interact with each other but also interact with junctional molecules, such as Zo1 at tight junctions and E-cadherin and β-catenin at adherens junctions, to regulate cell polarity in the cell. The localization of polarity complex proteins is also involved in spindle orientation, cell fate decision, and axon specification.

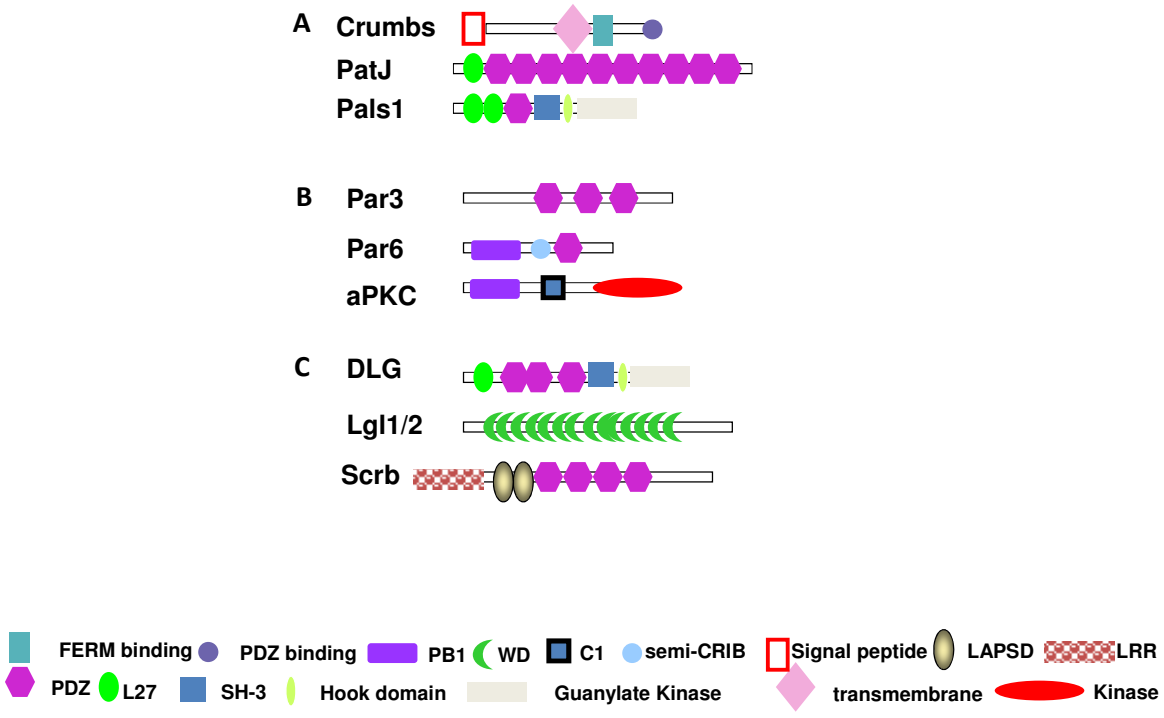


Figure 2. The structure of polarity complex proteins. A. Crb complex, B. Par complex, and C. Scrb complex. The various domains are shown as different symbols.

1.1 Par complex proteins overview

The *Par* complex proteins are the first identified genes that are involved in cell polarization during asymmetric cell division in the zygote of *C. elegans*. As its name implies, the Par proteins are integral factors for the distribution of determinants and cell polarity. For example, removal of Par3 protein provides insight on the protein's function resulting in asymmetrically distributed proteins in the partitioning of early fate determinants (Moghadam B et al., 1995). Kemphues et al. identified six *par* genes (Par1-6) through genetic screens. Mutation of these genes results in abnormal cleavage in dividing cells and also abnormal distribution of P granules (Kemphues et al., 1988). In addition, the seventh *par* gene named aPKC3 was discovered by Tabuse et al., and its knockdown resulted in a similar phenotype as seen in *par3* knockdown (Tabuse et al., 1998). With this positive link, Par complex protein, Par6/Par3/ aPKC3, was born.

1.1.1 Mammalian Par6

There are three Par6 proteins identified in mammals encoded by separate genes: Par6A/C, Par6B and Par6D/G. All of them have three conserved domains which mediate their interactions with other members of the complex (Assemat et al., 2008). All Par6 proteins bind to other polarity complex proteins, such as Par3, Pals1 and Crb3 through the PDZ domain (Hurd et al., 2004, Joberty et al., 2000; Lemmers et al., 2004; Lin et al., 2000). Par6 has a Phox/Bem 1(PB1) domain, which is able to bind with aPKC, followed by Cdc42/Rac interaction binding motif, which can bind to the Cdc42 or Rac GTPases (Bose et al. 2006).

Although three Par6 proteins are ubiquitously expressed, they show slightly different expression in the tissue. Par6A expression is enriched in the brain, and both Par6B and Par6G are highly expressed in the kidney (Gao & Macara, 2004). Activated Par6-aPKC localized at the leading edge in migrating astrocytes and keratinocytes directs microtubules required for polarized migration (Cline and Nelson, 2007).

Par6 proteins are able to bring polarity protein to specific locations, such as tight junctions, and out compete interactions with the other polarity complex protein (Suzuki & Ohno, 2006). For example, interaction between Par6-Pals1 interrupts Pals1-Patj binding and interferes the formation of the tight junction (Wang et al., 2004), suggesting that Par6 is essential for tight junction formation. In contrast with this idea, Par6B overexpression delays tight junction formation, although there was no effect on adherens junctions formation (Gao et al., 2002). A mutation of the PDZ domain in Par6B is satisfactory to disrupt tight junction, suggesting the importance of its interaction with other polarity complex proteins in tight junction. Interestingly, despite the fact that Par6G is also involved in the formation of tight junctions, unlike Par6B, it does not interrupt tight junction formation when overexpressed (Gao & Macara, 2004).

Although the exact molecular mechanism underlying the functions of Par6 has not yet been elucidated, the current known major role of Par6 is transient interaction with aPKC to maintain tight junctions, and physical interaction with Scribble polarity complex, Lgl (Yamannaka et al., 2003). Phosphorylated Lgl detaches from the aPKC-Par6 complex due to competition of Par3, leading to the aPKC-Par6-Par3 complex (Yamanaka et al., 2003).

1.1.2 Mammalian Par3

Two *Par3* genes, *Par3A* and *Par3B*, have been identified in mammals. The Par3A protein has three different isoforms each having three PDZ domains. However, each of them have different molecular weights: 180kDa, 150 kDa, and 100 kDa. Even more, amongst the three Par3A isoforms, all have aPKC binding domains except the 100kDa isoform protein. Par3A binds with Par6 via PDZ domain to interact with aPKC (Lin et al., 2000), but Par3B is not able to interact with aPKC (Kohjima et al., 2002; Gao et al., 2002). Although Par3A has been known as a scaffolding protein that interacts with Par6 and aPKC, Par3A can form a homodimer via its N-terminus which leads to the apical localization of Par3A (Mizuno et al., 2003).

Par3A expression is high in the heart, kidney, and brain (Lin et al., 2000). Par3B expression is high in the kidney, lung and skeletal muscle. Par3A and Par3B can be colocalized with Zo1 at tight junctions (Assemat et al., 2008).

The proper localization and function of Par3 is regulated by the presence of aPKC, which has a capability to interact with and phosphorylate Par3A (Pieczynski and Margolis, 2011). The knockdown of Par3A delays apical protein localization to the membrane, leading to mislocalization of apical domain and formation of multiple lumens in MDCK cells when there is a point mutation in S827/829A which lacks the ability interacting with aPKC (Horikoshi et al., 2009). In epithelial cells, Par3A may be stabilized by binding with the junctional adhesion molecules (JAM) via its first PDZ domain, and they are colocalized to the area of cell-cell contact (Ebnet et al., 2001). The functional study addresses the role of Par3 essential for the growth and elongation of

the primary cilium through the interaction with Kinesin-2 in MDCK cells (Sfakianos et al., 2007). In addition, Par3 can induce neuronal polarization via direct regulation of microtubules in mouse hippocampal neurons (Chen et al., 2013).

1.1.3 Mammalian aPKC

Two aPKC genes, *aPKCλ/ι* and *aPKCζ*, have been identified in mammals (Selbie et al., 1993; Ono et al., 1989). Both of these proteins have similar molecular weights (75 kDa) (Bose & Wrana, 2006). *aPKCλ/ι and aPKCζ* can be separated from conventional PKCs since they have a unique PB1 domain interacting with Par6. Unlike classical PKCs, they do not have a C2 domain, and they only have a partial C1 domain. In turn, this leads to the inactivation of aPKC*λ/ι and* aPKCζ by Ca2+, diacyl-glycerol and phorbol esters (Hirano et al., 2004). The only conserved domain from the PKC proteins is the catalytic domain located in the C-terminus (Akimoto et al., 1994; Moscat & Diaz-Meco, 2000). This domain is known to phosphorylate Par3 (Nagai-Tamai et al., 2002) and Lgl (Plant et al., 2003).

Both aPKC*λ/ι* and aPKCζ are highly expressed in the brain and lung. In addition, aPKCζ is observed in the testis and kidney. The aPKC proteins localize at tight junctions with other Par complex proteins in MDCK cells.

Unlike other polarity complex proteins that do not have kinase activity, aPKC proteins have serine threonine kinase catalytic activity. The overexpression of kinase deficient aPKC mutant results in the disruption of tight junction formation in MDCK cells, followed by mislocalization of Par6 and Par3 (Suzuki et al., 2001). Noda et al. have identified that Par6 proteins not only bind with aPKC*λ/ι and* aPKCζ, but also directly

interact with GTP-bound Rac through CRIB-like motif and Cdc42. When the constitutively active form of Rac is expressed with aPKCs and Par6 in HeLa or COS-7 cells, these proteins co-localized to membrane ruffles, where the leading edge of polarized cells occur. In turn, this suggests that Par6 functions as an adaptor protein that links activated Rac and Cdc42, as well as aPKCs (Noda et al., 2001). When Cdc42 is activated, phosphorylation of aPKC occurs, and as a result, activation of aPKC also occurs. This series of events leads to tight junction formation (Standaert et al., 1999; Gopalakrishnan et al., 2007).

1.2 Crbs complex proteins overview

The first discovery of *Crbs* gene is derived from *D. melanogaster* (Jurgens et al., 1984). Mutations in the *Crbs* gene severely disrupts the embryonic cuticle and epithelia derived from the ectoderm, as well as leading to apoptosis during embryogenesis (Tepass et al., 1990). The *Drosophila* embryo forms a monolayer structure sharing many characteristics of epithelial structure, such as high polarization. At later stages of the *Drosophila* embryo development, the lateral plasma membrane is divided by three distinct domains: Sub-apical complex, Adherens junction and Septate junction. The mutations of *Crbs* in *Drosophila* embryos result in the failure of formation of adherens junctions, severely disrupted epithelial structure, and in some cases induced cell death (Tepass et al., 1990).

The Crb protein is mainly localized in the apical domain of epithelial cells. The molecular weight of *Drosophila* Crb is 200 kDa. The small cytoplasmic domain has an integral function in Crb, and mutation of this domain shows complete loss of function

(Wodarz et al., 1993). Conversely, overexpression of Crb leads to the expansion of the apical domain, while the basolateral domain is decreased (Wodarz et al., 1995). Furthermore, single layered epithelium transforms to mutilayered tissue when Crb is overexpressed (Klebes & Knust, 2000).

Stardust (Sdt), Drosophila homologue of Pals1, is expressed in embryonic epithelia from the onset of gastrulation. The *Sdt* mutant shows the loss of epithelial integrity and cell shape, causing failure to concentrate the scattered spot adherens junctions into a continuous zonula adherens (ZA). As a result, cells lose adhesion, and the epithelium becomes a mutilayered structure. The developmental defects induced by *sdt* mutations are homologous to those associated with *crb* mutations, suggesting the idea that these genes are linked in a common pathway. Studies on double mutant by Knust et al. show that *sdt* acts downstream of *Crb* (Knust et al., 1993) and binds to the four C-terminal amino acids of Crb through its PDZ domain (Bachmann et al., 2001).

Dpatj has been known as a component of Crb complex with Crb and Sdt in *Drosophila* (Roh et al., 2002; Pellikka et al., 2002). The discovery of Dpatj was from the study of *dlt* (Disk Lost) locus. Bhat et al. addressed a newly identified protein that interacts with Crb through four PDZ domains. They showed that this protein is essential for epithelial cell polarity (Bhat et al., 1999). Subsequent studies have identified that the *dlt* locus actually encodes several genes, and mutations on *dlt* would disrupt the *Drosophila* Codanin-1 homolog, which is a cytoplasmic proteins. Finally, this newly identified protein is Dpatj (Pielage et al., 2003). The depletion of Dpatj induces loss of

polarity (Pielage et al., 2003). Dpatj is known to bind at the N-terminus of L27 domain of Sdt, therefore, a second polarity complex has emerged.

1.2.1 Mammalian Crb

There exists only one *Crb* gene in *D. melanogaster*. However, three *Crb* genes, have been identified in mammals: Crb1, 2, 3 with sizes of 154, 134, and 13 kDa, respectively (Medina et al., 2002). These three Crb proteins have highly conserved transmembrane and cytoplasmic domain (Assemat et al., 2008). Crb1 contains 3 Laminin A/G domains, and Crb2 has 19 and 14 EGF-like domains, respectively in its extracellular domain (Hollander et al., 1999; Katoh, 2004). In contrast, Crb3 has only a short extracellular domain, which does not have any other domains that interact with other proteins (Medina et al., 2002). The cytoplasmic tail of Crbs has two motifs: a FERM binding domain and a PDZ binding domain (Makarova et al., 2003).

Crb1 is mainly localized in the retina, and Crb2 is highly expressed in the retina and brain (Hollander et al., 2001), but *Crb3* is expressed in skeletal muscles and in epithelial tissues (Fogg et al., 2005, Makarova et al., 2003).

CRB1 mutations are involved in Leber congenital amaurosis (LCA) and retinitis pigmentosa (RP) which are retinal diseases. This suggests that Crb1 may impair cell adhesion and the formation of the retina layer structure to contribute to these disorders (Hollander et al., 1999 & 2001). Interestingly, Crb 1 and Crb2 show significantly similar homology in their molecular structure but Crb2 mutations have not been identified in patients with these pathological conditions (Hurk et al., 2005). As Crbs has also been known to stabilize apical junctions in *Drosophila*, mutations of the FERM binding motif

and loss of the ERL1 severely disrupts the formation of tight junctions in MCF10 cells (Fogg et al., 2005). Besides the common role of Crb in the formation of tight junctions, Crb3 has unique function that is essential for the formation of cilia via the kinesin motor protein. When Crb3 was knocked down in MDCK cells, ciliogenesis is profoundly impaired (Fan eta l., 2002).

1.2.2 Mammalian Pals1

PALS1 is a scaffolding protein that has multiple protien interaction domains. The strong mRNA expression of Pals1 has been identified in the placenta, kidney, apical side of neocortex, and its protein localization is observed in the tight junction (Kamberov et al., 2000).

We will discuss more detail in chapter 1.4 regarding the role of Pals1 with the significance of Pals1 in the thesis.

1.2.3 Mammalian Patj/Mupp1

Two homologues of the *Drosophila* Dpatj are identified in mammals: Patj (Pals1 associated tight junction protein) and Mupp1 (multi PDZ domain protein) (Roh et al., 2002).

Patj protein is 196 kDa and is an interacting protein with Pals1 and localizes at tight junctions together with Pals1. Patj is characterized by having one L27 domain and 10 PDZ domains that interact directly with ZO3 and Claudin1 via its 6 and 8 PDZ domains (Roh et al., 2002). In addition, Pals1 and Patj interact with each other via the L27 domain (Roh et al., 2002). On the other hand, the size of Mupp1 is 219 kDa and

interacts with the serotonin 5-hydroxytryptamine type 2 receptor (Ullmer et al., 1998). Mupp1 contains one L27 domain and characterized with 13 PDZ domains. Mupp1 binds with Pals1 via L27 domain as Patj does. Mupp1 and Patj are structurally similar having multiple PDZ domains and the ability to bind Claudin1, a tight junction protein (Assemat et al., 2008).

Patj is mainly expressed in epithelial tissues such as bladder, testis, colon, heart, kidney, brain, and skeletal muscle (Lemmers et al., 2002; Philipp & Flockerzi, 1997). Similarly, the expression of Mupp1 is observed at tight junctions (Hamazaki et al, 2002) and also identified in the brain, heart, placenta, liver kidney and skeletal muscle (Ullmer et al, 1998).

The disruption of Patj by overexpression or downregulation with RNAi induces destructive localization of ZO family proteins and Occludin, leading to the idea that Patj is essential for the stabilization of tight junctions (Lemmers et al, 2002; Michel et al, 2005; Shin et al, 2005). Specifically, the results from a Patj knockdown support that Crb3 and Pals1 are not localized at the tight junction, suggesting that Patj stabilizes the Crb3 complex (Michel et al,2005). Although its functional role has not been elucidated, it has been reported that Mupp1 binds with several tight junction molecules such as JAM (junctional adhesion molecules), CAR (Coxsackievirus-adenovirus receptor) and Claudins in addition to Pasl1 and Crb. This leads to the idea that Mupp1 may serve as a scaffolding protein (Hamazaki et al, 2002; Coyne et al, 2004; Jeansonne et al, 2003; Poliak et al 2002).

1.3 Scrib complex proteins overview

In *Drosophila*, the defect of *Scrib* was characterized by the disruption of cell adhesion and polarity. The *Scrib* mutant shows a corrugated cuticular surface, whereas wild type cuticle shows a smooth and continuous shape (Bilder & Perrimon, 2000). Scrib protein has 195 kDa moclular weight and as a member of the LAP (LRR and PDZ) protein family, contains 16 leucine rich repeats (LRR) and four PDZ domains (Bilder et al., 2000).

The expression pattern of Scrib at early embryonic stage is at zonula adherens, whereas in mature epithelial cells it expresses at the septate junction. The mutants of *Scrib* do not induce the loss of entire cell polarity, instead, apical proteins are mislocalized (Bilder & Perrimon, 2000).

There are two other Scrib complex components: Lgl (Lethal giant larvae) and Dlg (Discs large). *Lgl* and *Dlg* were originally identified as tumor suppressor genes in *Drosophila* during larvae formation. The mutations of *Lgl* and *Dlg* led to tissue-specific tumors (Stark & Bridges, 1926).

The molecular weight of Lgl is a 130 kDa that has short domains consisting of WD repeats (Lutzelschwab et al., 1987). *Lgl* mutations induces defective embryonic and larval development (Mechler et al., 1985), such as neoplastic overgrowth of larval brains and imaginal discs (Woodhouse et al., 1998).

The molecular weight of Dlg is a 102 kDa and as a member of MACUK proteins consists of one L27 domain, three PDZ domains, one SH3 domain, and one GUK domain. The mutations of *dlg* induce overgrowth of the imaginal discs (Woods & Bryant, 1991).

Although there is no physical interaction among Scrib complex proteins, relative localization and their similar loss of function suggest that Scrib, Lgl, and Dlg may form a biochemical complex to control apical and basolateral membrane domains (Bilder et al., 2000).

1.3.1 Mammalian Scrib

Mammalian Scrib is a 175 kDa protein and contains a large cytoplasmic multi-domain (Bilder & Perrimon, 2000), and is a member of LRR and LAP family of proteins due to the 16 LRRs and 4 PDZ domains (Bilder et al., 2000). It has been known that the LRR repeats direct Scrib protein's target to the basolateral membranes (Navarro et al., 2005).

The expression of Scrib is highly expressed in the skin, placenta, breast and intestine, but low in the liver, kidney and skeletal muscles (Navarro et al., 2005).

Scrib directly interacts with ZO2 through its PDZ domains (Metais et al., 2005), but Scrib localization does not overlap with ZO2 molecules at tight junction. Instead, Scrib colocalizes with adherens junction molecule such as *β*-catenin and N-cadherin (Navarro et al., 2005). Although Scrib does not colocalize with tight junction molecules, the downregulation of Scrib induces delayed assembly of tight junctions and defective morphology in MDCK cells (Qin et al., 2005).

1.3.2 Mammalian Dlg

Dlg is a member of MAGUK and have 5 family members (Dlg1-5). However, in this chapter, we will focus on Dlg1 due to its close relation to *Drosophila* Dlg and that it is a well-studied molecule in epithelial cells (McLaughlin et al., 2002). Besides being a

part of the MAGUK family, having three PDZ domains, one SH3 domain, one hook domain and a GUK domain, an L27 domain is located at N-terminus of Dlg1. Dlg1 binds with multiple proteins including membrane palmitolyated protein (MPP) family members such as MPP2, MPP3, and MPP7 (Karnak et al., 2002), suggesting that Dlg1 functions as a scaffolding protein.

Dlg1 mRNA expression was detected in the lung, kidney, liver, brain and skeletal muscle (Laprise et al., 2004).

It has been shown that Dlg1 deficient mice demonstrate delayed embryonic growth and die after birth (Caruana & Bernstein, 2001; Iizuka-Kogo et al., 2007). In addition, the loss of Dlg1 demonstrated that Dlg1 is only expressed in adherens junction, and this induced the disruption of polarity and nephrogenesis, led by delayed mesenchyme to epithelial transition (Iizuka-Kogo et al., 2007). The SH3, Hook, and GUK domains that are localized in C-terminus are known to be essential for development. The truncation of the C-terminus induces defective cleft palate (Caruana and Bernstein, 2001).

1.3.3 Mammalian Lgl

Multiple Lgl proteins are identified in mammals: Lgl1, Lgl2, Lgl3 (Syntaxin-binding protein 5) and Lgl4 (Syntaxin-binding protein 5-like). The molecular weight of each protein is 115 kDa, 113 kDa, 120-130 kDa, and 130 kDa, respectively. They are characterized with repeated WD40 domains that may be interacting with Scrib via these domains (Kallay et al., 2006).

The localization of Lgl1 and Lgl2 is observed in the basolateral membrane, which is below the adherens juctions, and both proteins are expressed in the stomach and brain (Assemat et al., 2008).

The overexpression of Lgl1 and Lgl2 shows disruption of the formation of junctional complexes during the polarity establishment phase. Despite this disruption, there is no effect in confluent MDCK cells. In order to be localized in basal membrane, Lgl1 and Lgl2 need to be phosphorylated by aPKC to release the interaction between Par6 and aPKC, as non-phosphorylated Lgl1 is localized in the apical domain (Yamanaka et al., 2003). Although the overexpression of aPKC at the apical side does not change cell proliferation, it triggers cytoplasmic accumulation of Lgl in Drosophila epithelia, suggesting that Lgl may be involved in tissue homeostatsis (Grifoni et al., 2007)

Collectively, polarity complex proteins are inter-connected and share their functions to maintain cell polarity and tissue integrity. The primodia of many organs shares the characteristics of epithelium, and this suggests that cell polarity is an essential factor for the proper organogenesis. This leads to the idea that cell polarity is integral to the maintenance of epithelia in the primodia, and the failure to establish cell polarity during development may induce defective cell-cell adhesion, eventually malformation of organs.

1.4 Functional studies of Pals1

Pals1 is a 77 kDa protein working as a scaffolding protein, mediating other polarity complex proteins such as Par6, Crumbs, and Patj via direct or indirect

interaction . The yeast two-hybrid screen using the intracellular domain of CRB1 on a retinal cDNA library as bait showed that Pals1 was the only interacting protein identified (Roh et al., 2002). Pals1 downregulation induces defects in tight junction and cell polarity (Straight et al., 2004), followed by mislocalization of E-cadherin in MDCK cells (Wang et al., 2007). In addition, Pals1 depletion causes loss of Patj, but Crb expression is not significantly changed by the removal of Pals1 (Straight et al., 2004; Wang et al., 2007).

1.4.1 Gene structure of Pals1, a member of p55-like MAGUK

Membrane-associated guanylate kinase (MAGUK) proteins are scaffolding proteins having Src homology 3 (SH3), PSD-95 Discs Large-zona occludens-1 (PDZ), and guanylate kinase (GUK) domains (Roh et al., 2002). A subfamily of MAGUK proteins are divided by DLG-like, ZO1-like, p55-like, and LIN2-like, depending on the presence of additional domains (Tseng et al., 2001). Pals1, known as membrane-associated palmitoylated protein 5 (MPP5), is a subset of p55-like MAGUK in the human gene. It contains highly conserved domains from flies to mammals: evolutionarily conserved region 1(ECR1), a bipartite L27 (L27N & L27C), PDZ, SH3, and GUK domains (Figure 3). Pals1 is an adaptor protein that contains several domains to interact with other proteins. Pals1 can bind directly to the ERL1 motif of Crb1 via PDZ domain and can link with CRB3 as well through its PDZ domain (Roh et al., 2002, Makarova et al., 2003). In addition, L27N can interact with Patj and L27C with Lin7. Direct protein-protein interaction between Pals1 and Par6 has been known to require the ECR1 of Pals and PDZ of Par6, respectively (Wang et al., 2004). Sequence analysis

of Pals1 addressed two prospective NLS motifs located between the ECR1 and L27 domains and between the SH3 and GUK domains, respectively. This suggests that Pals1 may be located and play a significant role in the nucleus, despite Pals1 localization and function at the apical junction is mainly considered in the most of previous studies. Together, domain analysis of Pals1 demonstrated that Pals1 can bring Crb and patj together to form the Crb tripatate complex and mediate physical interaction with Par complex.

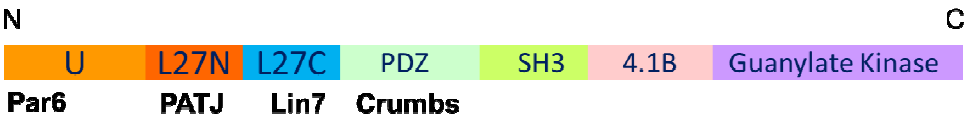
1.4.2 The significance of Pals1 during development

Although previous studies revealed the role of Pals1 as a scaffolding protein in Crb complex proteins for the maintenance of cell polarity and adhesion in epithelia, only limited number of studies has been done for the role of Pals1 during development. As the mutations of *Crbs* and *Dpatj* impair morphogenesis of photoreceptor cells and induce light-dependent retinal degeneration in *Drosophila*, mutations in *Sdt* also lead to the light-dependent retinal degeneration depending on its isoforms (Berger et al.,2007). Rohr et al. reported that heart and soul (Has)/protein kinase C iota (PRKCi) and Pals1 ortholog, nagie oko (Nok)/MPP5 are necessary for the polarized epithelial organization and coherence of myocardial cells during heart cone formation in zebrafish. The zygotic mutations of Nok induces myocardial defects at later stages such as incomplete heart tube elongation and failure of myocardial cell expansion. Thus, they identified that Nok is essential for cardiac morphogenesis by regulating the polarized epithelial organization, coherence of the myocardium and expansion of myocardial cell size to form an elongated tube from the heart cone (Rohr et al., 2005). Using deletion constructs of

Nok to define essential domains for cell polarity and cardiac morphogenesis, the deletion of the PDZ domain completely recapitulates all Nok mutant phenotype (Bit-Avragim et al., 2008), suggesting the critical importance of interaction with Crb proteins.

Unlike the *Crb* family which is comprised of three different genes, Pals1 is a single gene in mammals. Pals1 mRNA expression is high in the placenta and kidney, and moderate in the brain, heart and skeletal muscle (Kamberov et al., 2000). During eye development, Pals1 is known to be required for Crb1 localization in the subapical region of the Muller glia (Rossum et al., 2006). Conditional deletion of Pals1 in the developing retinal progenitor cells inhibits the assembly of Crb complex proteins and leads to the neural cell death, adhesion defects, tissue polarity disruption and photoreceptor degeneration, followed by visual impairment and mimicking the human LCA phenotypes (Cho et al., 2011).

A



B	NLS1	NLS2
Drosophila Std	RRRREEE	
Zebrafish nagie oko	RRRREEE	KKTKKKRKK
Mouse Pals1	RRRREEE	KKNKKKRKK
Human MPP5	RRRREEE	KKNKKKRKK

C	NES
Zebrafish nagie oko	LEDLLMSL
Mouse Pals1	VEDLFSSL
Human MPP5	IEDLFSSL

Figure 3. The schematic diagram of Pals1 and its conserved domain with putative NLS and NES sequences within the Sdt/nok/Pals1/MPP5 protein family. Pals1 is a member of MAGUK protein family and contains evolutionarily conserved domains: ECR1, a bipartite L27, PDZ, SH3, and GUK. Protein-protein interactions of Pals1 with other polarity complex proteins take place through the major domains: ECR1. (B,C) NLS and NES sequences, which are highly conserved in different species. Two prospective NLS motifs are located between the ECR1 and L27 domains and between SH3 and GUK domains in mouse. NES sequences are located within the N-terminal of L27N domain.

In the developing cortex, Pals1 deletion leads to the precocious progenitor cells withdrawal from the cell cycle, inducing premature differentiation to neurons during early neurogenesis followed by massive apoptotic cell death, resulting in significantly reduced size of the brain (Kim et al., 2010). Although previous studies have elucidated the role of Pals1 in different species and organs, it still remains to be elucidated how and why the depletion of Pals1 induces cell death and progenitor depletion and to identify the underlying mechanisms that cause these phenotypes.

1.5 Cerebellum development

The cerebellum is the primary center for motor coordination and essential for brain organization in cognitive processing and sensory discrimination (Schmahmann 2004). Impaired neuronal defects in the cerebellum usually results in motor dysfunction and ataxia such as Zelweger syndrome, paroxysmal ataxia, and Fryns syndrome in humans (Volpe & Adams, 1972). The volume of the cerebellum occupies only 10% of the total brain, but contains 80 to 85% of human neurons (Herrup & Kuemerle 1997). The cerebellum comprises an outer cortical structure, white matter, and a set of cerebellar nuclei (CN) beneath the white matter. The CN can project efferent fibers to other organs such as the thalamus, brainstem, and spinal cord (Paxinos 1995) to mediate motor movements and balance.

Although the cerebellum is a morphologically complex organ, the cerebellar cortex is histologically homogeneous and divided into three distinct cellular layers. First, the molecular layer (ML) contains two major interneurons, the basket and stellate cells.

Second, the Purkinje cell layer (PCL) is occupied by the cell bodies of Purkinje cells (PC) whose dendrites arborize in the ML and Bergmann glia (BG). Third, the granule cell layer (GCL) is located in the deepest area from the cerebellar cortex, and contains mainly GCs and other interneurons such as Golgi cells, Lugaro cells and unipolar brush cells.

The neuroepithelium that gives rise to the cerebellum undergoes sequential stages of structural and genetic transformations to generate a complex foliated structure with different molecular coding (Sillitoe & Joyner, 2007). In mice at E9, the cerebellum arises from dorsal rhombomere 1(r1) of the developing hindbrain (Millet et al. 1996, Wingate & Hatten 1999, Zervas et al. 2004). From E9 to E12, the wing-like morphology is generated by forming medial-lateral axis of the cerebellar primordium (Sgaier et al. 2005), and this medial-lateral axis is maintained until adulthood. At E17, four fissures separate cerebellum into five folds along with the anterior to posterior axis. The cerebellum acquires a three-dimensional structure AP folds intersected by ML molecular. Rudimentary synaptic connections and the circuitry of the cerebellum has been established by the animal's birth. By postnatal day 16, the cerebellum completes the organization of all cell types in the layers (Sillitoe & Joyner, 2007).

1.5.1 Progenitors in cerebellum development

The cerebellum has two spatially distinct germinal zones: the ventricular zone (VZ) and the upper rhombic lip (URL). A genetic fate-mapping study in mice has addressed that GC precursors originate from the dorsal portion of rhombomere 1 (Machold & Fishell 2005, Sgaier et al 2005, Wang et al 2005). However, GC precursors

are only generated from upper rhombic lip (URL) which is located adjacent to the fourth ventricle (Wingate & Hatten 1999). In addition, other cerebellar neurons (unipolar brush cells, projection neurons of the deep nuclei) and noncerebellar glutamatergic neurons also arise from the URL (Carletti & Rossi 2008). The specification of GC precursors in the URL is regulated by several transcription factors such as Atoh1 (Ben-Arie et al 1997), and secreted factors, FGFs and BMP, diffusing from the boundary of mesencephalon/rhombencephaon and the dorsal midline, respectively (Chizhikov et al 2006, Machold et al 2007, Basson et al 2008). GC precursors start tangential migration over the surface of the URL at around E13.5 in mouse (Miale & Sidman 1961) to form the external granular layer (EGL). Similarly like migrating interneurons in telencephalon, migrating GC precursors have a unipolar morphology and do not follow radial glia (Metin et al 2008, Ryder & Cepko 1994, Gilthorpe et al 2002). Time-lapse experiments showed that GC precursors are saltatory during the migration, alternating phases of forward movement with pauses (Gilthorpe et al 2002, Rieger et al 2009).

The second germinal zone of the cerebellum is the ventricular zone (VZ). The genetic inducible fate mapping (GIFM) using *Ptf1a*-Cre allele showed that PCs, and three of the GABAergic interneurons (Golgi, stellate, basket) are generated from VZ, and possibly astrocytes (Hoshino et al., 2005; Pascual et al., 2007). *Ascl1CreER* allele GIFM discovered different time stages that GABAergic neurons and glial cells were generated by administration of tamoxifen. They described that PCs and GABAergic deep cerebellar nueclei are the first born cells from VZ from E10 to 11.5 and E10.5 to 13.5, respectively. A subset of Bergmann glia are produced from E13.5 to 14.5, and

other majority of GABAergic interneurons are generated from E13.5 to early postnatal stages (Sudarov et al., 2011).

1.5.2 Neural progenitors & major signaling pathway in cerebellum development

The generation, proliferation and differentiation of cerebellar progenitors is a tightly regulated process. Several signaling pathways are essential for the proper regulation to maintain the progenitor pool. I address two signaling pathways, Shh and Notch, that have been known to be critical for the maintenance of neurogenesis and gliogenesis during cerebellar development.

1.5.2.1 Shh signaling pathway in cerebellum

The regulation of GC precursor (CGNP) proliferation and cerebellar foliation is controlled by sonic hedgehog (Shh) signaling pathway. Hedgehog was first identified in *Drosophila* embryo, and mammalian hedgehog comprises three proteins: Sonic hedgehog, Indian hedgehog, and Desert hedgehog (Vaillant & Monard, 2009). The Shh signaling pathway activates its target genes through the activity of the Gli transcription family such as Gli1, Gli2, and Gli3. When Shh is absent, Patched 1 (Ptc) transmembrane protein inhibits Smoothened (Smo) transmembrane protein G coupled receptor. This induces Gli3 cleavage and transformation to a transcriptional repressor form to repress their target genes (Figure 4). Upon binding of Shh to PTC, this inhibition between Ptc and Smo is released, and Gli3 is not cleaved, leading to the initiation of transcriptionally active Gli1 and Gli2 transcription factors (Fuccillo et al., 2006).

PCs express Shh mitogen from around E17 onward, as a result, massive proliferation of GCs are induced by Shh expressed from PCs (Sidman et al., 1962;

Caddy & Biscoe, 1979; Wetts & Herrup, 1982; Herrup, 1983). The first study shown on the role of Shh in GC proliferation was in culture experiments. It was shown that the treatment with Shh antibodies into the cerebellum reduces GC proliferation (Dahmane & Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya & Scott, 1999). Conditionally inactivated Shh in the mouse using loxP-Cre system showed that Shh is necessary for GCP proliferation and cerebellar foliation (Lewis et al., 2004). The Gli2 downstream factor of Shh signaling play a role as a main activator for Shh induced proliferation of GCPs (Corrales et al., 1999), and SMO transmembrane protein functions as a critical component of the receptor complex in Shh signaling (Murone et al., 1999).

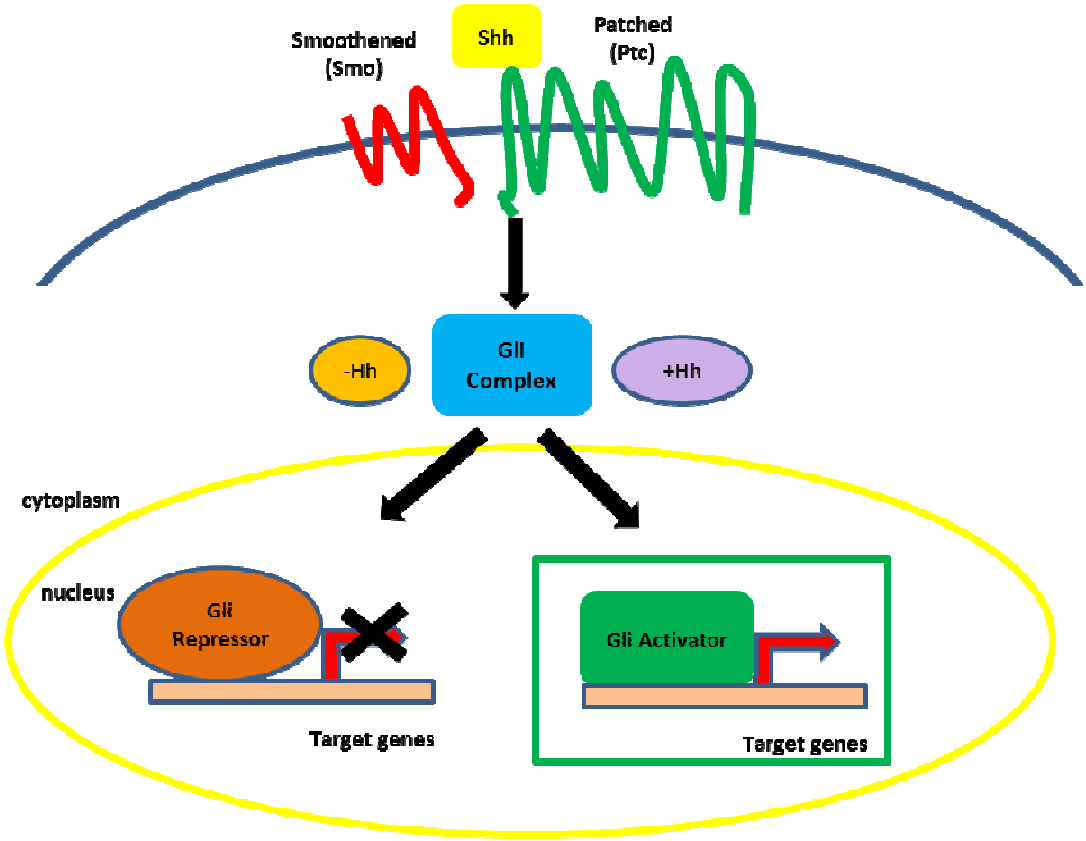


Figure 4. Schematic diagram for Shh signaling. In the absence of ligand, Ptc inhibits Smo to repress target genes. In the presence of ligand, this inhibition is released, and Gli activator induces target gene expression.

1.5.2.2 Notch signaling pathway in cerebellum

Notch is an evolutionarily conserved signaling pathway throughout the animal kingdom. Mammalian Notch receptors are composed of four proteins from Notch1 to Notch4 (Bolos et al., 2007), and they contain a large extracellular domain that is involved in ligand binding and a cytoplasmic domain that is important for signal transduction. The extracellular domain has epidermal growth factor (EGF)-like repeats that are essential for binding with Notch ligands (Fehon et al.,1990; Rebay et al., 1991), followed by three cysteine-rich LIN12/Notch repeats (LNR) to inhibit signaling in the absence of the ligands. Upon ligand binding to Notch extracellular domain, this induces proteolytic cleavage of extracellular domain. This generates the translocalization of Notch intracellular domain (NICD) to the nucleus to activate target gene expression shown in Figure 5 (Oswald et al., 2001).

Glutamatergic interneurons and astrocytes, including Bergmann glia, stem from the same germinal neuroepithelia (Carletti & Rossi, 2008; Hoshino, 2012), leading to the idea that the two lineages might be related. This idea is supported by studies of Notch signaling in the cerebellar primordium, indicating that both cerebellar neurons and glia may be generated by common ancestors. Notch is expressed in the VZ, starting from around E10 in the mouse (Machold et al., 2007). The ablation of Notch in the cerebellum results in premature neuronal differentiation and undergoes apoptotic cell death arising from the VZ (Lütolf et al., 2002; Machold et al., 2007), leading to the reduced size of cerebellum (Lütolf et al., 2002). In contrast, the Notch intracellular

domain is constitutively activated by retroviral expression and promotes the genesis of astrocytes at the expense of neurons (Machold et al., 2007).

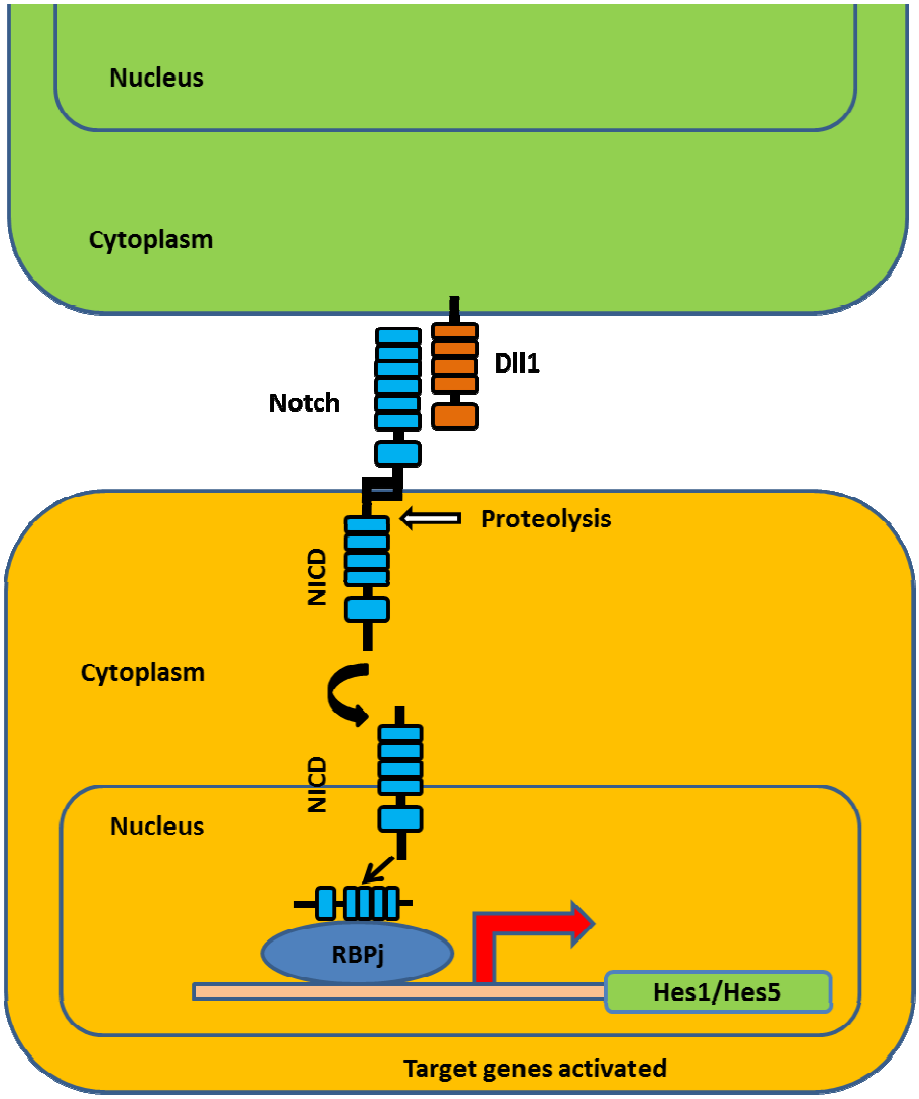


Figure 5. Canonical Notch signaling pathway. Upon binding with ligand, the Notch receptor undergoes proteolysis. This leads to the translocalization of Notch intracellular domain to the nucleus to activate target genes.

1.5.3 Cell polarity and cerebellum development

Although the role of polarity complex proteins in dendrite development, axon guidance and neuronal migration are well known (Solecki et al., 2006), the intrinsic mechanism for the study of polarity complex proteins during cerebellum development remains unclear. Famulski et al. have reported the role of one of polarity complex proteins, Pard3A, during granule cell migration from its germinal zone. Cerebellar granule neurons are regulated by proteasomal degradation of Pard3A when they exit the germinal zone, mediated by the Seven in Absentia homolog (Siah) E3 ubiquitin ligase. The overexpression of Pard3A and Siah knockdown induces precocious radial migration of granule cells from EGL pool. Pard3A recruits junctional adhesion molecule C (JAM-C), known as a tight junction component that interacts with Pard3A directly to the neuronal cell surface, leading to the acceleration of adhesive interactions to exit the germinal zone (Famulski et al., 2010).

Although the function of polarity proteins in EGL migration has been explored, there have been no studies performed to determine their function in cerebellar progenitor proliferation and differentiation. The deletion of Pals1 in cerebellum may reveal the significance of polarity proteins in cerebellar progenitors to answer this question.

1.6 Cortical development and developmental disorder

The cerebral cortex is the largest structure of the brain and plays a key role in memory, attention, language, and consciousness. The coordinated process of

progenitor self-renewal, differentiation, and cell death during the cortical development is essential to generate the ultimate size of the cerebral cortex.

The identification of the cortical VZ and SVZ as the germinal zone for the genesis of projection neurons and astrocytes has addressed several important questions regarding the composition of the progenitor pool. One crucial piece of information came from the studies on birth dating using tracers, which defined that cortical projection neurons and astrocytes originate in a temporal order. During early stages of neocortical development, a preplate that comprises of the earliest born neurons forms between the VZ and the meninges at the pial surface. Subsequent splitting of the preplate generates two other areas called marginal zone and subplate by migrating neurons that are born and layer themselves in an inside out fashion. Early generated neurons form layer VI and V first, followed by layer IV, III, and II by temporally sequential orders (Figure 6). At the end of neurogenesis, progenitors generate astrocytes (Qian et al., 2000; Pinto and Gotz, 2007). Two different models could explain how this temporal order is established. One model is that a common progenitor might generate the different types of neurons and astrocytes in a temporal order. The other is that multiple types of progenitors may coexist to generate a specific neuronal types and astrocytes on a specific time line (Franco & Muller, 2013).

Abnormalities in the development of the cerebral cortex is associated with severe mental and physical disabilities (Walsh, 1999; Francis, 2006). Microcephaly is a neurodevelopmental disorder characterized by a small cerebral cortex at birth, and the consequences of microcephaly is strongly associated with neurological defects such as

mental retardation and seizure (Mochida, 2001; Wood et al., 2005; Cox et al., 2006). The key players that have been identified in this phenotype are Abnormal Spindle-like microcephaly-associated protein (ASPM), Cyclin-dependent kinase 5 regulatory subunit associated protein 2 (CDKRAP2), Centromere protein J (CenPJ), and Microcephalin/MCPH1 (autosomal recessive primary microcephaly 1) (Cox et al., 2006; Fish et al., 2006; Paramasivam et al., 2007). These genes are highly expressed in neural progenitors during cortical development. All of the proteins encoded by these genes are localized to the mitotic apparatus, and some of them are known to be essential for the proper cell division of neural progenitors (Fish et al, 2006). This suggests the significance of cell division during cortical development, and the malfunction of proper cell division may be the presumed causation of microcephaly.

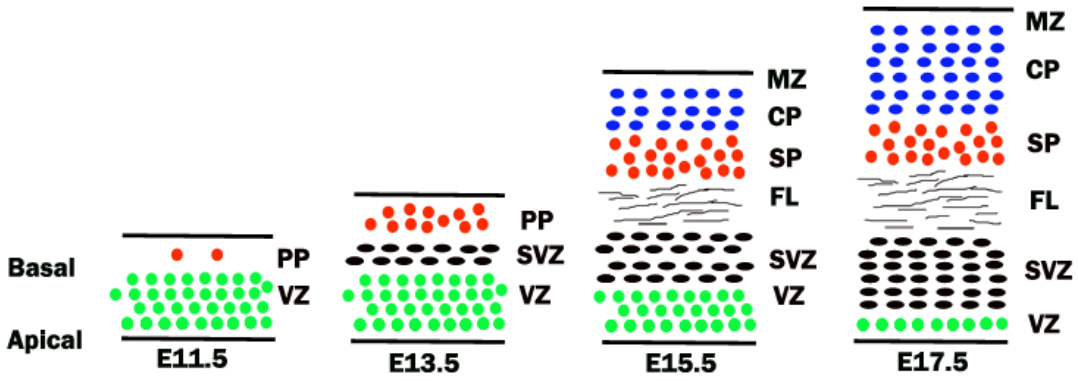


Figure 6. Schematic diagram for neocortical development. Until E11.5, the majority of NECs undergo symmetric cell division in the apical region (shown as green). Around E13.5, RGPs (shown as green) start asymmetric cell division and generate one progenitor by self-renewal and one IPC. Generated IPCs move upward from the VZ and form the SVZ for symmetric terminal division (shown as black). At E15.5, progenitor pool in VZ decreases, whereas more neurons (shown as red and blue) generated, and early cortical layers are formed. At E17.5, extensive neurogenesis occurs, but RGP pool is significantly reduced. Furthermore, glial cells start to be generated.

1.6.1 Progenitors in cortical development

During corticogenesis, neural progenitors undergo symmetric and asymmetric cell division (Caviness et al, 1995). Symmetric cell division generates two progenitor cells, while asymmetric cell division produces one progenitor and a neuron, or another type of neural progenitor (Cotz & Huttner, 2005; Huttner & Kosodo, 2005). The proliferation of neural progenitors tightly regulated by cellular and molecular events, and these different cellular and molecular events lead to the balance of two neuronal types, early born and late born neurons, to maintain progenitor pools in the neocortex.

All neocortical neurons and glial cells originate from neural stem cells derived from anterior neuroectoderm. Neural stem cells contain several epithelial characteristics such as polarity and adhesion, and these cells are known as neuroepithelial cells (NECs). At E8, during neurulation in the mouse, anterior NECs undergo symmetric cell division to expand the neural stem cell niche by rapid proliferation, leading to the formation of forebrain (Smart, 1973). By E9, the anterior neural tube closes to form the lateral ventricles, and the NECs form a pseudostratified neuroepithelial structure lining with lateral ventricles. NECs have apico-basal polarity and are tightly anchored at the ventricular surface by tight junctions and adherens junctions (Aaku-Saraste et al., 1996; Zhadanov et al., 1999; Manabe et al., 2002). They reach to the basal lamina at the pial surface via integrins (Graus-Porta et al., 2001; Radakovits et al., 2009). The onset of neurogenesis starts at E9 to E10. During neurogenesis, NECs transform their characteristics to another distinct progenitor type: radial glial cells (RGCs). During this transition, NECs lose epithelial characteristics, instead, they acquire glial characteristics.

However, their processes still maintain contacts with VZ and pial surfaces. The prominent changes at this transition is that they lose tight junctions (Aaku-Saraste et al., 1996) and the expression of astroglial genes, such as astrocyte-specific glutamate transporter (GLAST), brain lipid-binding protein (BLBP) and tenascin-C (Hartfuss et al., 2001; Heins et al., 2002; Noctor et al., 2002). In addition, RGCs are the progenitors for most neurons and glial cells generated in the neocortex (Miyata et al., 2001; Noctor et al., 2001; Noctor et al., 2002). While NECs typically undergo symmetric cell division to expand the progenitor pool, RGCs divide asymmetrically to generate one daughter cell and a neuron or another type of progenitor (Noctor et al., 2004). Time-lapse images have shown that only 10-20% of dividing RGCs generate neurons directly (Attardo et al., 2008; Kowalczyk et al., 2009), but most RGCs expand their niche by producing RGCs or intermediate progenitor cell (IPC) (Noctor et al., 2004) or by dividing symmetrically to expand progenitor pools. IPCs have distinct characteristics from NECs and RGCs. The most significant difference is that IPCs undergo symmetric terminal division to generate two neurons (Miyata et al., 2004; Noctor et al., 2004). IPCs express Tbr2 transcription factor, while Pax6, the RGC-specific transcription factor, is downregulated (Englund et al., 2005). Furthermore, IPCs migrate to a more basal area to form an anatomically distinct proliferative region, subventricular zone (SVZ). IPCs lose their contact with the ventricular zone and switch their shape from the radial morphology of RGCs to a multipolar shape (Miyata et al., 2004; Noctor et al., 2004).

1.6.2 Molecular signaling regulating cortical progenitor cell division

Several signaling pathways are involved in RGP proliferation. The major signaling pathways that are involved in corticogenesis are Notch, Wnt, and Shh signaling pathways.

1.6.2.1 Notch signaling pathway

Notch signaling has been known to suppress proneural genes, whereas Numb/Nmb-like, Notch antagonists, drives neuronal differentiation. During asymmetric RGPs division, higher Notch expression retains RGPs as progenitors, whereas low Notch expressing RGPs show higher expression of proneural genes and initiates differentiation (Dong et al., 2012; Shimojo et al., 2008). Therefore, the interaction between Notch and Numb/Numb-like is essential for the maintenance of RGPs during neocortical development (Franco & Muller, 2012). In the developing mouse cortex, it has been known that oscillating Hes1 expression in RGPs also induces oscillation of *Delta* and *Ngn2* expression, suggesting differential Hes1expression levels may determine RGCs proliferation and differentiation (Paridaen & Huttner, 2014). However, one important point that has not been elucidated yet is how cells change their response to Notch signaling during neurogenesis as Notch signaling is still active in differentiated neurons. Although several notch repressors have been identified, there iss no evidence how these repressors are specifically upregulated when cells undergo neurogenesis. A recent study addressed increased Bcl6 expression during neurogenesis. Bcl6 changes Hes5 promoter activity by the modification of Notch dependent composition of the complex, and this recruits deacetylase Sirt1 and silences Hes5 permanently (Tiberi et

al., 2012). This epigenetic clue can be an answer that this epigenetic switch leads to stable Hes5 inactivation to stabilize neuronal differentiation.

Polarity protein, Par3, can regulate the localization of cell fate determinants Numb/Numb-like. Asymmetric distribution of Par3 in RGPs induces unequal inheritance of Numb/Numb-like in their progeny, leading to the cell fate in which one daughter cell remains a progenitor and the other becomes IPC or neuron (Bultje et al., 2009).

1.6.2.2 Wnt signaling

In canonical Wnt signaling pathway, β-catenin is major downstream effector molecule. In the absence of Wnt, β-catenin is degraded by Ubiquitin E3 ligase following β-TrcP mediated ubiquitination and phosphorylation by casein kinase Iα (CKIα) and GSK3β (Nelson and Nusse, 2004). These proteins form a larger destruction complex with Axin and adenomatous polyposis coli (APC), and all play an essential role in ubiquitin mediated β-catenin degradation by proteasome. In the presence of Wnt signals, they bind to the receptor Frizzled and induce ternary formation with LRP5/6, ultimately leading to the dissociation of β-TrCP from the destruction complex (Li et al., 2012). This leads to the accumulation of β-catenin in the cytoplasm and translocation to the nucleus where it can bind with T cell factor/lymphoid-enhancing factor (TCF/LEF) and activate target genes.

Wnt signaling has dual roles in neurogenesis. Wnt signaling accelerates symmetric RGP division by continuous β-catenin expression, leading to the delay of BP formation (Wrobel et al., 2007). During late neurogenesis, Wnt signaling promotes BP

formation and induces neuronal differentiation through N-myc upregulation (Kuwahara et al., 2010; Munji et al., 2011; Fang et al., 2013). In the chick neural tube, N-myc was highly expressed in RGPs that undergo neurogenesis (Zinin et al., 2014).

1.6.2.3 Shh signaling

Shh signaling is integral for proper dorsoventral patterning in the central nervous system. During neurogenesis, the Gli3 repressor acts as a cell fate determinant that is necessary for BP generation and neuronal differentiation. The elevation of Gli3 repressor appears during neurogenesis for BP generation and neuronal differentiation, but active Shh signaling downregulated (Wang et al., 2011). During the development of the neocortex, the activation of Shh enhances symmetric cell division for RGPs mediated by Notch transcription factor Hes1 (Dave et al., 2011). This suggests that the crosstalk between different signaling pathways is essential for the regulation of neocortical development.

1.6.3 Polarity proteins in cortical development

The first study of polarity complex proteins demonstrating importance of brain development is the genetic study of Lgl1. The knockout of Lgl1 in mice results in rosette-like structures of neuroepithelium, similar to the rosettes derived from human primitive neuroectodermal tumors. Lgl1-/- newborns generate severe hydrocephalus and die neonatally. Neural progenitors fail to exit the cell cycle to differentiate and thus show continuous proliferation. Dividing cells, in the absence of Lgl1, fail to localize Notch inhibitor Numb asymmetrically, leading to the failure of asymmetric cell divisions, which may explain hyperproliferation without differentiation. This suggests that the

mammalian Lgl1 is essential for the regulation of proliferation and differentiation in progenitors, and tissue organization (Klezavitch et al., 2004).

The second functional study of polarity complex proteins in brain development came from the manipulation of Par3 in cortical progenitors. Although there is no genetic mutant studies for Par 3, this study addresses the importance of mammalian Par3 (mPar3) in dividing RGPs. During interphase, mPar3 is enriched at the lateral membrane domain in the endfeet of RGPs, whereas mPar3 starts to be dispersed asymmetrically as the cell cycle progresses. The loss or ectopic expression of mPar3 results in the failure of asymmetric cell division and generation of two neurons or two RGPs, respectively. In addition, differential expression levels of mPar3 regulates Notch signaling through the maintenance of Numb/Numb like; thus, notch signaling acts downstream of mPar3 function in asymmetric cell division(Bultje et al., 2009).

The third study was done with Mpp3, which is also known as Dlg3, and interacts with Pals1 (Kantardzhieva et al., 2006). *Mpp3* conditional knockout mice specifically delete Mpp3 expression in cortical progenitor cells and result in gradual deletion of the apical complex proteins, leading to the disruption of adherens junctions. Although cortical morphology and cell integrity in VZ are maintained, the loss of Mpp3 shows randomized spindle orientation and ectopic localization of mitotic cells. In addition, neuronal migration is retarded and shows ectopic cortical layers at later stages. These results suggest that Mpp3 is not only essential for the maintenance of apical complex proteins and adherens junctions but also crucial for proper neuronal migration for cortical development (Dudok et al., 2013).

Pals1 is an important scaffolding protein that functions as a bridge connecting other apical complex proteins such as Crb and Patj. Since Pals1 exists as only one gene unlike other polarity complex genes, it removes the possibility of redundancy. Also, it is possible that the deletion of Pals1 may not only disrupt the function of Crb complex proteins but also interacts with other molecules during development. The cerebellum and cortex are major organs in central nervous system (CNS) that are involved in memory, attention, motor control and other higher functions. In addition, the epithelial structure of primordium in these organs serve as an excellent model to study the role of Pals1 during the development.

To this end, Pals1 flox/flox;Emx1-Cre mice have been generated and characterized previously with respect to cortical development (Kim et al., 2010). Pals1 flox/flox;Emx1 Cre exhibit significant reduction of the cortex and near absence of neocortex through precocious differentiation of progenitors into neurons, which are removed via apoptotic cell death. Although previous work demonstrated the importance of Pals1 function in progenitor proliferation, it is unclear whether Pals1 functions in neurogenesis and its underlying molecular mechanism. To explore Pals1 function in neurogenesis and cell division modes of RGPs, asymmetric versus symmetric, we have utilized hGFAP-Cre to delete Pals1 in radial progenitors of the dorsal cortex and progenitor cells for the majority of cerebellar cells, except early born neurons, such as DCN and PCs . Through analyzing this valuable mouse model, my research is to investigate the role of Pals during cerebellar development as a cell fate determinant in the Chapter 3 and the role of Pals1 in neocotical development as a major mitosis regulator in Chapter 4. Based on the results, I will address future directions in Chapter 5.

Chapter Two:

Materials and Methods

Materials and Methods

2.1 *Mice*

Animals were handled in accordance with protocols approved by the IACUC of Temple University School of Medicine. *Pals1*$^{f/f}$ and hGFAP-Cre mice (Zhuo et al., 2001) (Jackson laboratory) were bred for generation of CKO and genotyped by PCR as previously described (Kim et al. 2010). SmoM2 mice were obtained from Jackson laboratory, which were bred with *Pals1*$^{f/f}$ and hGFAP-Cre mice and genotyped by PCR described previously (Jeong et al., 2004).

2.2 *Histology and immunohistochemistry*

Embryos at various developmental stages were decapitated and fixed in 4% paraformaldehyde (PFA) in PBS. For postnatal animals, brains were dissected out and fixed in 4% PFA in PBS. Fixed tissues were embedded in paraffin to prepare 7μm parasagittal sections. Hematoxylin and eosin (H & E) staining of tissue sections was performed as previously described. Tissue sections were deparaffinized and rehydrated through an ethanol series and distilled H_2O for immunofluorescence and immunohistochemistry. After 30 minutes of antigen retrieval with boiling citrate buffer, sections were stained with the following antibodies: mouse α-BrdU (1:50; Becton Dickinson Immunocytometry Systems), rabbit α-phospho-histone H3 (PH3) (1:250; Millipore), rabbit α-Pals1 (1:250, Upstate), mouse α-PKCλ (1:250; BD Biosciences), mouse α-Reelin (G10, 1:250; Millipore), rabbit α-BLBP (1:250; Millipore), Dab1 (gift from Dr. Howell), rabbit α-S100β (Novus Biologicals), rabbit α-Pax2 (1:250; Covance), mouse α-p27 (1:250; BD Biosciences), rabbit α-Pax6 (1:250; Covance), mouse α-Pax6 (1:250; Developmental Studies Hybridoma Bank), mouse α-Calbindin (1:250; Sigma), rabbit α-

GFAP (1:250; Thermo Scientific), rabbit α-Par3 (1:250; Millipore), mouse α-Zo1 (1:250; BD Biosciences), mouse α-Lim1+2 (1:250; Developmental Studies Hybridoma Bank,, rabbit α-Cleaved Notch1 (1:250; Cell Signaling), mouse α-N-Cadherin (1:250; BD Biosciences), rabbit α-Cleaved Caspase-3 (1:250; Cell Signaling), and rabbit α-Crumbs (1:250; gift from Dr. Malicki). rabbit α-Tuj1 (1:250; Millipore), rabbit α-pS6 (1:250; Cell signaling) mouse α-phospho-vimentin (4A4) (1:250; MBL), rabbit α-Ki67 (1:1000, Leica biosystem), rabbit α-parvalbumin (1:250; Millipore), mouse α-NeuN (1:250; Millipore), rabbit α-Cux1 (1:250; Santa Cruz), rabbit α-rabbit α-Foxp1 (1:250; Abcam), rabbit α-Ctip2 (1:250; Abcam), chicken α-GFP (1:250; Aves labs), mouse α-Yap (1:250; Abcam), rabbit α-CyclinD1 (1:250; Santa Cruz), rabbit α-Aurora B (1:250; Abcam), and rabbit α-Hes1 (1:250; from Nardin Brown).

. Species-specific Alexa Fluor 488 (1:500, Invitrogen) and Cy3 (1:500, Jackson Immunochemical) secondary antibodies were used for fluorescence detection, and nuclear DNA was counterstained with Hoechst 22358 (1:1000, Invitrogen). Alternatively, after incubation with the primary antibody, biotinylated anti-mouse or anti-rabbit secondary antibody was applied and incubated with peroxidase-conjugated avidin. Staining was visualized with a DAB (Diaminobenzidine) Substrate System (Sigma).

2.3 *Western blot*

Whole cerebellums were dissected at postnatal day 0 to prepare protein lysates, and meninges were removed from the tissue. Protein lysates were homogenized in an ice-chilled cell lysis buffer (20mM Tris-HCL (pH 7.5), 150mM NaCl, 1mM Na_2EDTA, 1mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1mM beta-glycerophosphate, 1mM Na_3VO_4, and 1□g/ml leupeptin) containing 5% Protease Inhibitor Cocktail P8340

(Sigma). Using an SDS-PAGE gel, protein lysates were separated and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat dry milk, and primary antibodies were applied on the membranes at 4℃ overnight. The primary antibodies included mouse α-Reelin (1:500; Millipore), rabbit α-Dab1 (1:500; Cell Signaling), rabbit α-phospho-Dab1 (1:500; Cell Signaling), mouse α-Yap (1:250; Abcam), mouse α-Pttg1 (1:1000; Cell Signaling), rabbit α-Tbr2 (1:1000; Millipore), mouse α-Tuj1 (1:1000; Millipore), rabbit α-Tbr1 (1:1000; Millipore) and α-GAPDH (1:500; Cell Signaling). Expression signals were detected by chemiluminescence (ECL Kit; GE Healthcare).

2.4 In situ *mRNA hybridization*

Fixed whole brains were washed with PBS and immersed in 30% sucrose for cryoprotection and embedded in an OCT compound (Tissue-Tek). Frozen samples were sectioned at 20μm thickness. Paraffin samples were deparaffinized, rehydrated and dried at room temperature for 15 to 20 minutes. *In situ* hybridization was performed using antisense digoxigenin-labeled riboprobes by the *in vitro* transcription of cDNAs: Pals1, Gli1, Gli2, N-myc, Pttg1, Notch1, Hes1, and Hes5. *In situ* hybridization was carried out as previously described (Hui and Joyner, 1993).

2.5 *Bromodeoxyuridine (BrdU) administration and cell number quantification*

BrdU (Sigma, St. Louis, MO) dissolved in PBS was administered for 30 minutes before sacrifice at 50□g/g body weight through intraperitoneal (IP) injection. For the analysis of the proliferation of EGL and VZ progenitors, the total number of BrdU+ cells in the EGL or VZ, respectively in the image was counted manually using Photoshop and ImageJ at 3 midsagital levels of each animal (n=3), after the acquisition of the images

with an Axiophot microscope (Zeiss, Germany). The same method was applied to count Pax2, Sox9 and Olig2 positive cells.

2.6 *Brain size analysis*

To measure the size of the cerebellum, we analyzed at least 3 animals per genotype. Images of fixed brains were taken at the same magnification using Axiophot (Zeiss, Germany) to measure the width and length of the cerebellum. The images were analyzed and quantified in Photoshop or ImageJ, and statistical analysis was performed. For the circumference, three histological pictures of midsagittal sections (vermis) were used for each animal to measure in the imageJ. Three to five animals for each genotype were used for measurement of the width, length and circumference.

2.7 *Real time PCR experiments*

For Real-time PCR analyses, Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR system with LuminoCt® SYBR® Green qPCR ReadyMix™ (Sigma-Aldrich) were used. Reaction mixtures included 10 □l of 2X SYBR Green qPCR Ready Mix, 300nM of each primer, and 1 □l of previously reverse-transcribed cDNA template. The cDNA template was synthesized after a reverse transcription reaction when total RNA was isolated from E17.5 cerebellum (N=3 for WT and CKO). The thermocycler parameters were 95℃ for 10 min, followed by 40 cycles of 95℃ for 15 s and 60℃ for 1 min. All reactions were performed in triplicate. We used Gli1(5'-GGAAGTCCTATTCACGCCTTGA-3', 5'-CAACCTTCTTGCTCACACATGTAAG-3') Gli2 (5'-TACCTCAACCCTGTGGATGC-3', 5'-CTACCAGCGAGTTGGGAGAG-3') N-Myc (5'-AGGAAGCACTCCCCCATATT-3', 5'-GTCATCTTCGTCCGGGTAGA-3') Cyclin D1 (5'-TTGACTGCCGAGAAGTTGTG-3', 5'-CCACTTGAGCTTGTTCACCA-3')Pals1 (5'-

CTTCGCACACAGTCCTTGAA-3', 5'- CTAACAACGCCCGAAGTCTC-3') Acin 5'-CTGAACCCTAAGGCCAACC-3', 5'-CCTGGATGGCTACGTACATG-3') primers.

2.8 *Statistical analysis*

The f-test was performed to determine if the values were of equal or unequal variance before the t-test. The statistical significance was determined by a student's *t*-test for brain size analysis, cell counting of Tbr1+, Cux1+, BrdU+, Pax6+, BrdU+/Pax6+, Ki67-BrdU+, and Pax2+ cells and proportion of p27+ cells. $p<0.05$ was determined to be statistically significant.

2.9 *Plasmids and* In utero *electroporation*

For pCAG:Pals1-GFP construct, pCAG-GFP empty vector was digested with Xba1 and XhoI. Full length Pals1 sequences were obtained from PCR using pCAG-Pals1 as a backbone DNA and ligated into digested pCAG-GFP. pCAG:H2B-GFP, pCAG:Cre-GFP, pCAG-GFP constructs were obtained from Addgene, and pCAG-Yap construct was gifted from Raehee Park. Pttg1 gene is synthesized by Genewiz and subcloned into pCAG vector. Timed pregnant mice were anesthetized and their uterine horns were exposed. Plasmid DNA (1-2µg/µl) was injected into the lateral ventricles of embryos using pulled micropipettes. For electroporation, 5 pulses separated by 900ms were applied using a BTX ECM830 pulse generator at 45V for E13.5 embryos. After the surgery, embryos were allowed to develop in utero until the indicated time for the analysis.

2.10 Cell culture and immunohistochemistry

Before spreading cells, 12-mm circle coverslips (Carolina Biological) were prepared by washing with 70% ethanol overnight at room temperature. Next day, coverslips were coated with 200 μg/ml poly D-lysine (Sigma) and washed three time with distilled water after 5 hours incubation. Each coverslip was placed on each well in 24-well cell culture dish (Nest Biotech). All cells were grown in a humidified 37℃ incubator with 5% CO2. All cells were maintained in the conditions with 10% FBS and 1% antibiotics mixed in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (*DMEM/F-12*) media until the date for the experiment. Media has been replaced once per two days. For immunohistochemical analysis, the specimens were washed three time in 15 min intervals, followed by fixation for 15 minutes with 4% paraformaldehyde in PBS. The specimens were stained with the following antibodies: rabbit α-Pals1 (1:250, Upstate), rabbit α-CyclinD1 (1:250; Santa Cruz), and α-Aurora B (1:250; Abcam). Species-specific Alexa Fluor 488 (1:500, Invitrogen) and Cy3 (1:500, Jackson Immunochemical) secondary antibodies were used for fluorescence detection, and nuclear DNA was counterstained with Hoechst 22358 (1:1000, Invitrogen).

2.11 *Organotypic cortical slice culture and Time-lapse confocal imaging*

Timed pregnant mice were electroporated with pCAG:H2B-GFP construct and harvest after 24 hours. After screening under a fluorescent dissecting stereo microscope (Leica M205C), only GFP expressing embryos were selected for the dissection. The cortex of embryos was dissected and flipped over appearing that apical size of VZ is upside. Dissected tissues were mounted on a 35 mm petri dish (Fisher Scientific) and attached with gelatin for 30 minutes at 37 ºC. Cultures were maintained in a humidified incubator with distilled water at 37 ºC for the scanning, and consistent CO_2 was supplied. Time-

lapse images of GFP-expressing cells were acquired every 30 minutes for 14 hours. GFP fluorescence in the specimens was imaged using a Leica SP5 confocal laser scanning microscope.

2.12 Electron microscope scanning

The E13.5 brains were fixed in 3% glutaraldehyde and stored with Millonig's buffer for 5 minutes. The buffer was replaced with 2% % OsO_4 and stored for 1 h at 4℃. Tissues were washed with distilled H_2O for 5 minutes and dehydrated by a serial standard ethanol wash, followed by the incubation with 50% LX-112, propylene oxide, and 100% LX-112. For polymerization, tissues were embedded in a flat mold at 70℃ overnight. Using a Leica Ultracut-R microtome and a glass knife, tissues were cut at 500 nm and heat-fixed to glass slides and stained with Toluidine Blue. Thin sections (120 nm) were cut with diamond knife (Daitome, Hatfield, PA, USA) using same microtome and placed on a 150 mesh copper grid (EMS, Hatfield, PA, USA), followed by staining with 2% uranyl acetate for 15 minutes and rinsed with distilled H_2O. Tissues were stained with Reynold's lead citrate for 5 minutes, rinsed and dried at 70℃. Images were taken from the JOEL 1200 Transmission Electron Microscope at 60 kV and captured with the 1 k × 1 k Gatan Digital Imaging System (Electron Microscopy Laboratory, Department of Pathology, UTHSC, Houston, TX, USA).

Chapter Three:

The roles of Pals1 in cerebellar development

3.1 INTRODUCTION

A mature cerebellum comprises four major cell types: Purkinje cells (PC), cerebellar granule neurons (CGNs), interneurons and glial cells. The majority of cerebellar cells originate in two germinal zones: the ventricular zone (VZ), the lining of the apical cerebellar neuroepithelium; and the upper rhombic lip (URL), the neuroepithelium located between the cerebellar plates and the choroid plexus of the fourth ventricle (Millen and Gleeson, 2008; Zervas et al., 2005). GABAergic cerebellar deep nuclei (cn) neurons, PCs, interneurons and most of the glia cells develop from the VZ, either directly or via intermediate progenitors in the white matter. URL and its descendants generate glutamatergic cn neurons, unipolar brush border cells and granule neuron precursors (CGNPs) (Leto and Rossi, 2012). Cerebellar cells are organized into distinct layers including the molecular layer, PC layer and granule cell layer through intricate directional movements from their germinal zones (Roussel and Hatten, 2011).

Unlike other neurogenic areas in the developing brain, CGNPs in the external granule cell layer (EGL) remains proliferative throughout early postnatal ages, producing CGNs that migrate inwardly to form the internal granule cell layer (IGL). It has been shown that CGNPs expand progenitor pools through mainly symmetric cell division, and clonally related progenitors exit the cell cycle during a relatively short period of time (Espinosa & Luo; 2008). The PC plate beneath the EGL layer serves as a major source of the mitogenic signal Sonic Hedgehog (Shh), which drives CGNP proliferation during late embryogenesis and early postnatal stages (Sillitoe & Joyner; 2007). While migrating through the Shh-rich EGL toward PCs, CGNPs exit the cell cycle

and differentiate, demonstrating the exquisite balance between proliferation and differentiation that is required to coordinate granule neuron generation and clonal expansion of CGNP during development. Too much proliferation causes hyperplasia or cancer, while too little results in an underdeveloped and poorly functioning organ. If Shh signaling is deregulated in CGNP, the devastating result is medulloblastoma, which is the most common malignant brain tumor in children. Recent transcriptional profiling that identified Shh as one of the four medulloblastoma molecular subgroups (Taylor et al., 2011) has spurred research to develop novel therapeutics targeting this signaling pathway, as approximately 25-30% of medulloblastomas arise due to mutations that activate the Shh signaling pathway (Roussel and Robinson, 2013). Therefore, it is critical to investigate the cellular/molecular mechanisms involved in the decision to initiate a differentiation program rather than proliferation in the presence or activation of mitotic/morphogenetic signals such as Shh during granule cell development to further understand pathologic conditions.

Cell polarity, which includes both molecular and cytoarchitectural asymmetry within the cell, is crucial for various cellular processes, including proliferation, differentiation and directed growth and migration. Polarity complex proteins include evolutionarily conserved apical complex proteins, such as the Crb complex (Crbs/Pals1/Patj) and the Par complex (Par3/Par6/aPKC), and basal complex proteins, such as the Scribbles complex (Scribbles/Lgl/Dlg) (Assemat et al., 2008; Pieczynski and Margolis, 2011; Tepass, 2012). These proteins play an indispensable role in the establishment and maintenance of cell polarity required for proper development of many tissues. For example, recent studies have demonstrated that apical complex proteins

are necessary for the self-renewal of neural progenitor cells (Costa 2008; Kim et al., 2010, Budje et al., 2009), axon determination (Shi et al., 2003; Chen et al., 2013), dendrite development (Tanabe et al, 2010), tissue polarity (Cho et al., 2012) and neuron survival (Kim et al., 2010). Furthermore, regulation of ubiquitination of the apical complex protein Pard3A at the germinal zone is necessary for newly born CGN to execute a cell adhesion-dependent exit from the EGL (Famulski et al., 2010). However, there is little information about the function of the apical complex proteins in the regulation of CGNP proliferation and differentiation as well as cerebellar organogenesis.

Here, we genetically ablate the central component of the apical complex, Pals1, in progenitor cells during cerebellar development and provide evidence of the critical necessity of Pals1 function in cerebellar cell generation and organization. Furthermore, Pals1 deficiency causes profound premature differentiation of progenitors including CGNPs in the EGL and more importantly, it completely abrogates uncontrolled proliferation by activated Shh signaling. This suggests an essential Pals1 function in cellular fitness for proliferation in both health and disease conditions. Together, these newly described functions identify Pals1 as a critical intrinsic factor for regulating CGNP proliferation in addition to confirming its known role in the proper generation of neurons from the ventricular zone.

3.2 RESULTS

3.2.1 Pals1 is expressed in progenitors during cerebellar development

To study the function of Pals1 in cerebellum development, its expression pattern and subcellular localization were examined during embryogenesis and postnatal stages. Previous studies have demonstrated that apical complex proteins are highly concentrated at the apical junction and are essential for self-renewal of cerebral cortex progenitors (Bultje et al., 2009; Costa et al., 2008) (Kim et al., 2010). We examined Pals1 expression at embryonic day 15.5 (E15.5) in germinal zones of the developing cerebellum, depicted in Fig. 7A. Pals1 is evidently expressed in these proliferating zones (Fig. 7B), but Pals1 transcripts are substantially diminished at E15.5 in Pals1 CKO mice (Fig.7C). Prominent expression remained in the choroid plexus (CP) where Cre expression was absent (Fig. 7C', red arrow), confirming the cerebellum-specific ablation of and the specificity of the *Pals1* probe we used for *in situ* hybridization. In accordance with known patterns in other types of neuroepithelium, Pals1 proteins localize to the apical surface in the VZ and URL of wild type mice at E15.5, but are markedly reduced in the Pals1 mutant (Fig. 7D, E, F, G). The External Granular Layer (EGL) also expresses Pals1 broadly and weakly in wildtype (WT) cell bodies at E18.5, but is largely absent in the CKO.

Pals1 expression in the ventricular apical lining cells continues during early postnatal stages, such as postnatal day (P) 0 (Fig. 7J'), although proliferating cells are nearly absent from the VZ at this stage. At P0, Pals1 expression was consistently observed in the proliferating EGL and expression in the PC layer began (Fig. 7J''). At P6, Pals1 expression in the PC layer and EGL cell layer were detected at both the transcript

and protein level (Fig.7K-M'). However, Pals1 protein is only detected when the signal is amplified with DAB immunohistochemistry, resulting in concentrated spots of Pals1 staining (Fig. 7M', arrow).

To provide evidence that this immunostaining indeed detects Pals1, we compared WT and Pals1 CKO animals. In the Pals1 CKO, immunopositive signals are substantially reduced in general but remained in PCs (Fig. 7N', arrow). Pals1 expression in PCs of the CKO would be unaffected because Cre is not expressed in the early progenitors which generate them. Importantly, focally dense Pals1 staining in the CGNP is almost completely diminished in the Pals1 CKO, supporting true Pals1 expression in CGNPs. Together, these observations of Pals1 expression suggest that Pals1 may serve a critical function in the proliferation of CGNPs in the EGL in addition to progenitors in germinal zones with epithelial characteristics such as the URL and VZ.

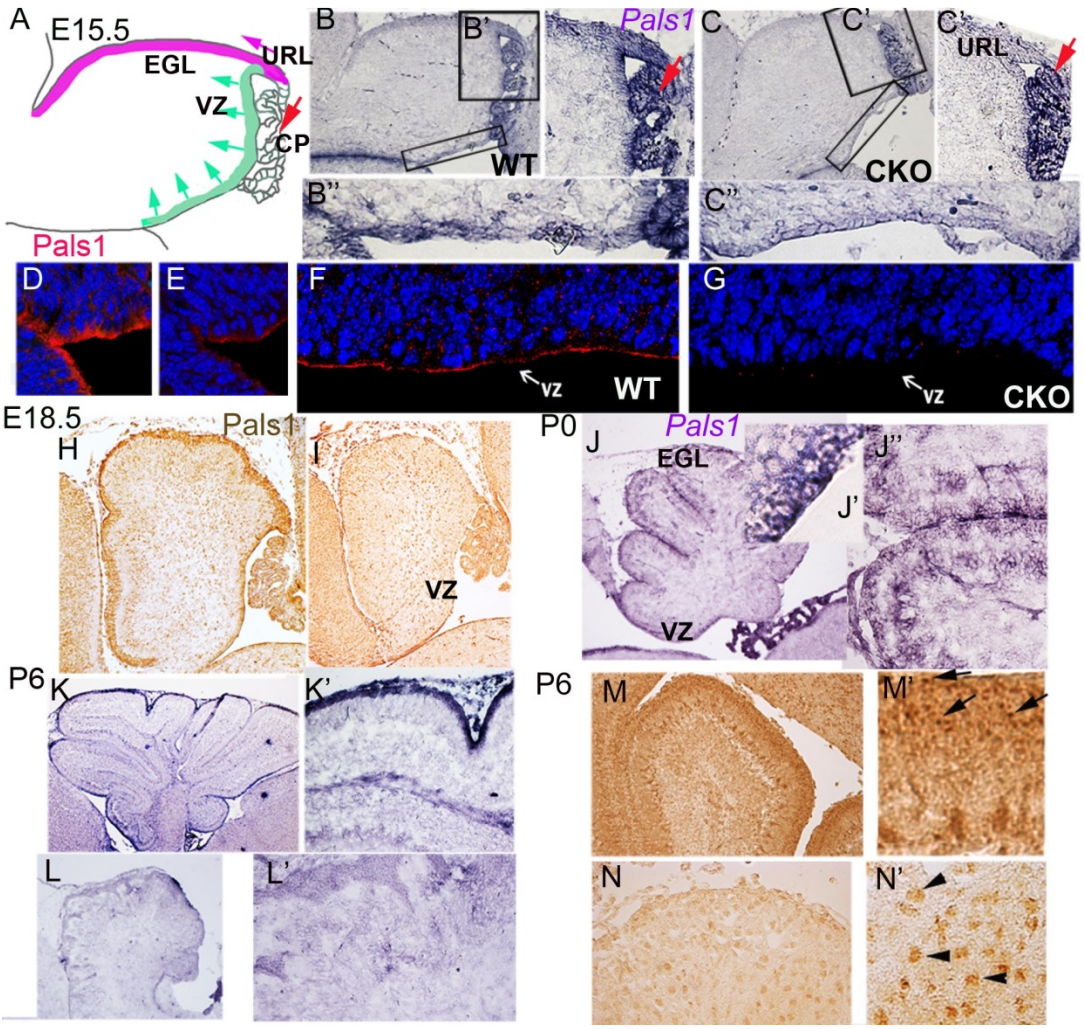


Figure 7. Pals1 is expressed dynamically during development of the cerebellum. (**A)** Schematic drawing of VZ, URL, and EGL in the developing cerebellum. (**B, C)** Pals1 mRNA expression in URL, EGL, VZ and CP in WT **(B, B', B',)** and Pals1 CKO **(C, C', C'')** mice at E15.5. Red arrow indicates Pals1 expression in CP in both WT and CKO. (**D, E)** Pals1 protein is highly expressed at the apical ventricular surface of URL, VZ of WT animals **(D, F)**, but diminished in Pals1 CKO at E15.5 **(E, G)**. **(H, I)** Continued expression of Pals1 protein in URL, EGL, VZ at E17.5 in the WT **(H)** but it is markedly reduced in Pals1 CKO **(I)**. **(J, J', J'')** At P0, Pals1 transcripts are found in VZ (**J'**) EGL and PCL (**J''**). **(K-L')** Prominent concentrated Pals1 transcripts are found in the EGL and weak expression seen in white matter and PCL at P6 **(M, N')**; Pals1 proteins are detected in the EGL and PCL layer including PC cells in the WT whereas concentrated punctate staining of Pals1 seen in the WT (arrow, **M'**) is no longer remained in EGL of CKO but staining in the cell body were maintained (arrow head, **N'**).

3.2.2 Pals1 is required for cerebellar histogenesis and foliation

Orderly generation of neurons and glia is essential in establishing the cytoarchitecture and functional circuitry necessary for cerebellar functions (Sudarov et al., 2011). Since Pals1 is expressed in the progenitor population and possibly other cerebellar cells and because it is known to play a critical role in cerebral cortex development, we studied the function of Pals1 in cerebellum development through genetic ablation. We used *hGFAP Cre* mice to delete *Pals1* from most cerebellar neurons and glia. However, progenitors for early born neurons such as PCs, DCN (Deep cerebellar nuclei) do not express Cre since *hGFAP Cre* is expressed in proliferating progenitors in the EGL, URL and VZ, starting at E13.5 (Zhuo et al., 2001).

Pals1 deletion causes the cerebellum to be dramatically undersized at P0 and onward (Fig. 8A-F). To characterize the cerebellar phenotype, size differences between wild type (WT) and CKO littermates were compared by measuring the length, width and circumference of the cerebellum at P0, P5 and P21 (Fig. 8A-J). Except for cerebellar width at P0, the measurements were considerably lower in the CKO mice, indicating the essential function of Pals1 in cerebellum development. To determine the defects in layer formation and foliation, we conducted serial histological analyses at embryonic and postnatal stages (Fig. 8L-S). Cerebellar cells form distinct layers, including a cell-sparse molecular layer (ML), a PC layer and IGL, which are organized into a foliated structure with ten lobes in the adult mouse. Our histological analysis first revealed a phenotypic difference in the Pals1 mutant at E17.5, despite the absence of gross anatomical changes at that stage. The length and thickness of the URL was noticeably smaller than in WT controls, and the dorsal surface of the cerebellar anlage, where CGNPs reside,

was hypocellular (Fig. 8L, L' M, and M'). In addition, cells in the apical lining of the developing cerebellum formed a less populated and thinner VZ in the Pals1 mutant than in WT littermates (Fig. 8L, L'', M and M'').

The cerebellum develops five cardinal lobes during late embryogenesis (around E18) (Sudarov and Joyner, 2007). About six to seven lobes have formed at the vermis in the WT by P0 (Fig. 8N). Folia formation was significantly delayed in the Pals1 CKO at P0 (Fig. 8O), and small indentations began to form in the cerebellum's surface a few days later, indicative of future fissures (data not shown). At P8, deformed lobes that were smaller and more disorganized than normal were detectable in most Pals1 CKO animals (Fig. 8P, Q). In adults, the Pals1 mutant cerebellum demonstrated fused lobes with severely disorganized layers characterized by a poorly defined molecular layer and dispersed CGNs (Fig. 8S). In addition to a global size difference and incomplete elaboration of the folia, there was an apparent striking reduction in granule cell number (Fig. 8R, S), the most abundant neuronal population in mammalian brain. Together, the results of our histological study with the Pals1 CKO revealed the essential function of Pals1 in cerebellar histogenesis and organization.

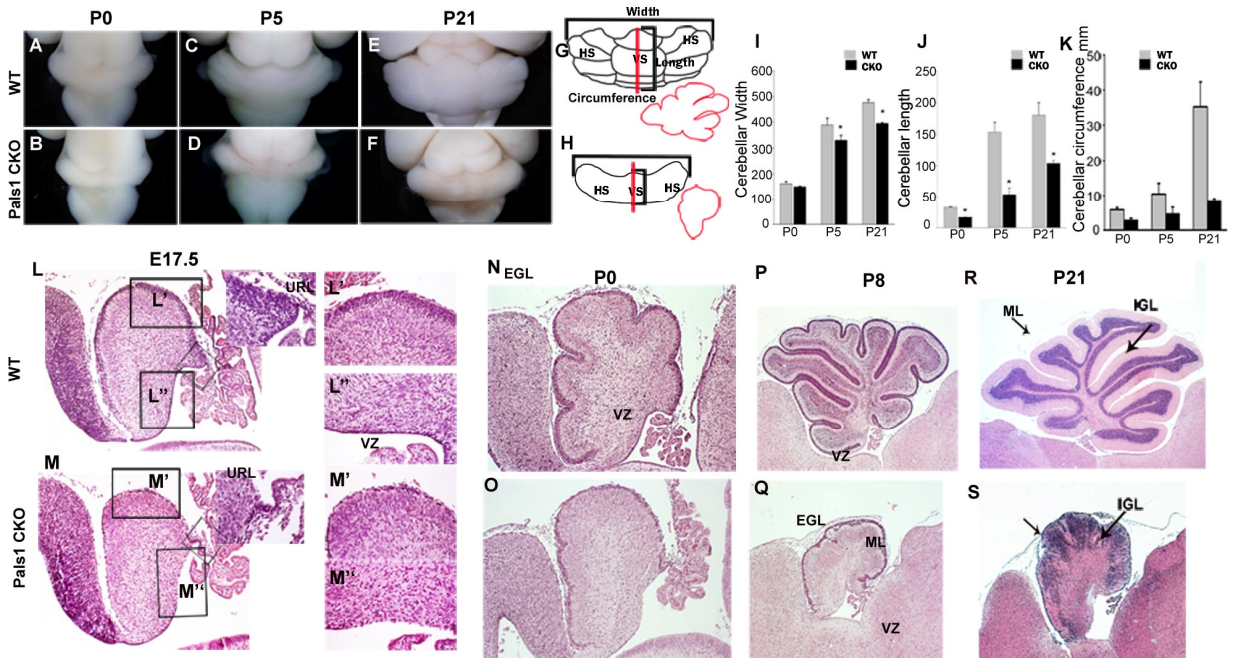


Figure 8. The function of Pals1in cerebellar histogenesis

(**A-K**), The Pals1 CKO cerebellum was smaller than that of WT littermates at P0 and later. Cerebellar width, length and circumference were measured at P0 (**A, B**), P5 (**C, D**), and P21 (**E-F**) and compared with WT (**I-K**). (**L-S**), Histological analysis using H&E staining. E17.5 Pals1 CKO mice show a diminished URL and a decreased URL-derived CGNP population in the EGL (**M'**) compared to WT (**L'**). VZ is less dense in the Pals1 CKO (**M''**) than in WT (**L''**). At P0, cerebellar lobes have not formed in the Pals1 CKO (**O**), while the WT cerebellum shows 7 distinctive lobes (**N**). Deformed foliation is detected in Pals1 CKO at P8 (**P, Q**). Undersized cerebellum with fused lobes and a disorganized layer structure is found in Pals1 CKO mice compared to WT at P21 (**R, S**).

3.2.3 Pals1 is required for cerebellar layer organization

Pals1 expression in the PC is not affected by Cre recombination since early progenitors do not express *hGFAP-Cre*. However, we sought to identify PC developmental defects, as PC migration and dendritic development rely highly on other cerebellar cells, radial glia progenitors, CGN and BG. To examine any non-cell-autonomous effect of Pals1 on PC positioning and dendrite development, we studied PC development during embryogenesis and postnatal stages. In the Pals1 mutant, a normal number of PCs appeared to be generated, but the localization was severely disrupted, shown by the broad distribution of PCs in the white matter compared to the WT at P0 (Fig. 9A and B). By P6, PCs are remained as clusters at the border of the white matter and fail to form multiple layered PC plate to single layer shown in WT (Fig. 9C, C', D and D'). Because of the striking depletion of CGNP and the subsequent reduction of CGN generation in the EGL, a prime source of Reelin, Reelin-Dab1 signaling, which is the critical signal for PC migration, have been compromised in the Pals1 mutant (data not shown). The reduced number of radial glia progenitors may also contributed to impaired migration of PCs since radial glia scaffold supports the PC migration.

Interestingly, Pals1-deficient glia cells were not only decreased in number, but were also remarkably dispersed throughout the cerebellar plate and even found on the pial surface (Fig. 9F, arrows) at P0 and thereafter. In the wild type, however, BG were mainly located underneath the EGL and formed a distinct single layer within the PC layer by P6 (Fig. 9E and I). Cerebellar BG cells are unique glial cells characterized by unipolar radial glial processes and found from late embryogenesis to adulthood. The BG cells play a pivotal role in granule cell migration, PC dendritogenesis and synaptic

regulation. Because of the severe defects in BG location and profound reduced CGN of which axon provides synaptic inputs to PC, the development of PC dendrites in the Pals1 mutant appear to be randomly directed and less elaborated (Fig. 9J, K). Thus, the failure to develop PC layer comprising compact PC and BG in the Pals1 mutant because of abnormal positioning and/or defective functioning of BGs. In the Pals1 mutant at P21, most PCs were dispersed in the cortical area and failed to form a PC layer consisting of BG and PC. Immunostaining of the Pals1 mutant with markers for both PC and BG and astrocytes (Calbindin and S100□, respectively) revealed a lack of close contact between the cell bodies (Fig. 9J and K insets).. Pals1 loss does not appear to affect the mobility of these cells, as BGs were capable of migrating out of the VZ. BGs in the Pals1 mutant also demonstrate defects in radial fiber morphology at P21, their processes were stunted or bended, and they failed to attach to the pial surface (Fig 9K-N'). These BG defects are likely contributed to the disruption of inward radial migration of CGN at later stages (Fig. 9P, R). Together, layer organization of cerebellum is severely affected by Pals1 loss with reduced number of neurons and glia as well as dispersed distribution of cerebellar cells.

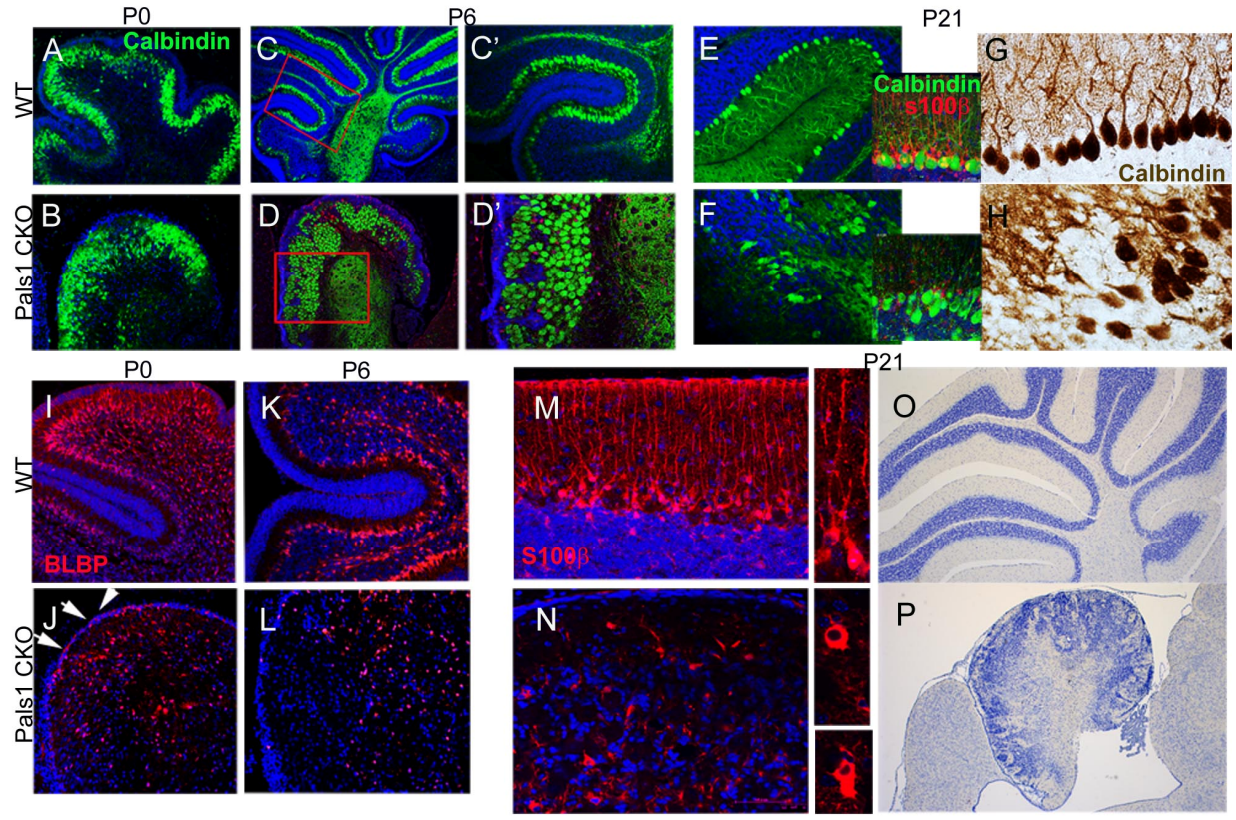


Figure 9. Pals1 deficiency causes lamination defects

(A, D'). At P0 and P6, the localization of PCs is severely disrupted in the CKO as a significant portion fail to reach the PC layer; some remain in the white matter (B, D, D'), whereas PCs form few cell layers by P6 in the WT (C, C'). **(**At P21, most PCs are dispersed in the cortical area and fail to form the PC layer. **(L, L' M, M')** mTORC1 signaling activation, measured by pS6 staining, is decreased in the Pals1 mutant at P21 (M, M'), whereas there is strong pS6 expression in the PC cell body and dendrites of the WT (L, L'). **(A-F)** BLBP positive cells **(A, A' B, B'),** almost none are detected at P0 **(C, D)**. At P0, fewer BLBP+ cells are in the cerebellum compared to WT (**E**, **F**), and some are detected even underneath the pia (**F**, arrow). **(G, H)** Comparable numbers of Olig2+ oligodendrocyte progenitors are present in the CKO and WT at P0. **(I, J)** At P6, many BG cells labeled by S100β form a layer within the PC layer in the WT, whereas BG cells in the CKO fail to form a layer. **(K-L')** At P21, smaller numbers of BG cells are present in the mutant than WT, the cells are dispersed throughout the cerebellum, and radial processes are not generated. High resolution images are shown in K' and L'. **(M, M', N, N')** GFAP immunostaining also demonstrates disorganized and randomly directed BG processes.

3.2.4 Pals1 deletion disrupts the localization of apical complex proteins in the cerebellar neuroepithelium

To understand the cellular changes leading to the severe reduction of neuronal and glial production, we examined the tissue integrity mediated by cellular junction proteins. First, we examined histological changes at E15.5, when the Pals1 protein had largely disappeared from the apical junction (Fig. 7C, G), and found tissue integrity was undisrupted in the mutant (Fig. 10A, B). Next, we examined changes in junctional components by marker analyses. The apical junction, which includes tight junction proteins such as Zo1, is located apically to the adherens junction (AJ). We found that Pals1 deletion in progenitors had some effect on the localization of the AJ components □-catenin and N-cadherin (Data not shown, Fig. 10C, D) as the reduction of their distribution at the junction is consistently observed. However, profound defects in maintaining and/or establishing the tight junction protein, Zo1, at the apical junction were detected by E15.5 (Fig. 10E-H).

Pals1 functions as a scaffold to recruit other apical polarity complex proteins via its protein–protein interaction domains such as PDZ, SH3, and L27 to assemble and maintain a macromolecular network at the apical junction. In order to analyze Pals1's scaffolding role in the VZ of the developing cerebellum, we first studied the localization of Crumbs (Crb) proteins, known to interact with Pals1, using pan-Crb antibody, which detects all three homologs of Crbs. In the absence of the Pals1 protein, Crb proteins were no longer localized at the apical junction (Fig. 10K-N). The other apical complex that interacts with Pals1 is the Par complex; this physical interaction is mediated by Pals1 and Par6 (Hurd et al., 2003) and by Crb and Par6 (Morais-de-Sa et al., 2010).

Similar to previous observations in the cortical neuroepithelium, the Par complex proteins Par3 and aPKC are not maintained at the apical surface in the Pals1-deficient cerebellum (Fig. 10I, J, M, N, O and P). Comparable defects were observed in the URL, another neuroepithelial germinal zone (Data not shown), but defects in the localization of apical polarity complex proteins in the EGL are difficult to address because, unlike in the neuroepithelium, they are not distinctly labeled. Together, these results show that, despite the absence of visible differences in histology and in the population of dividing cells, the molecular composition and distribution (or localization) at the apical junction is already considerably changed at E15.5. These defects can critically disrupt self-renewal of epithelium-derived progenitors, as seen for cerebral cortex progenitors (Kim et al., 2010).

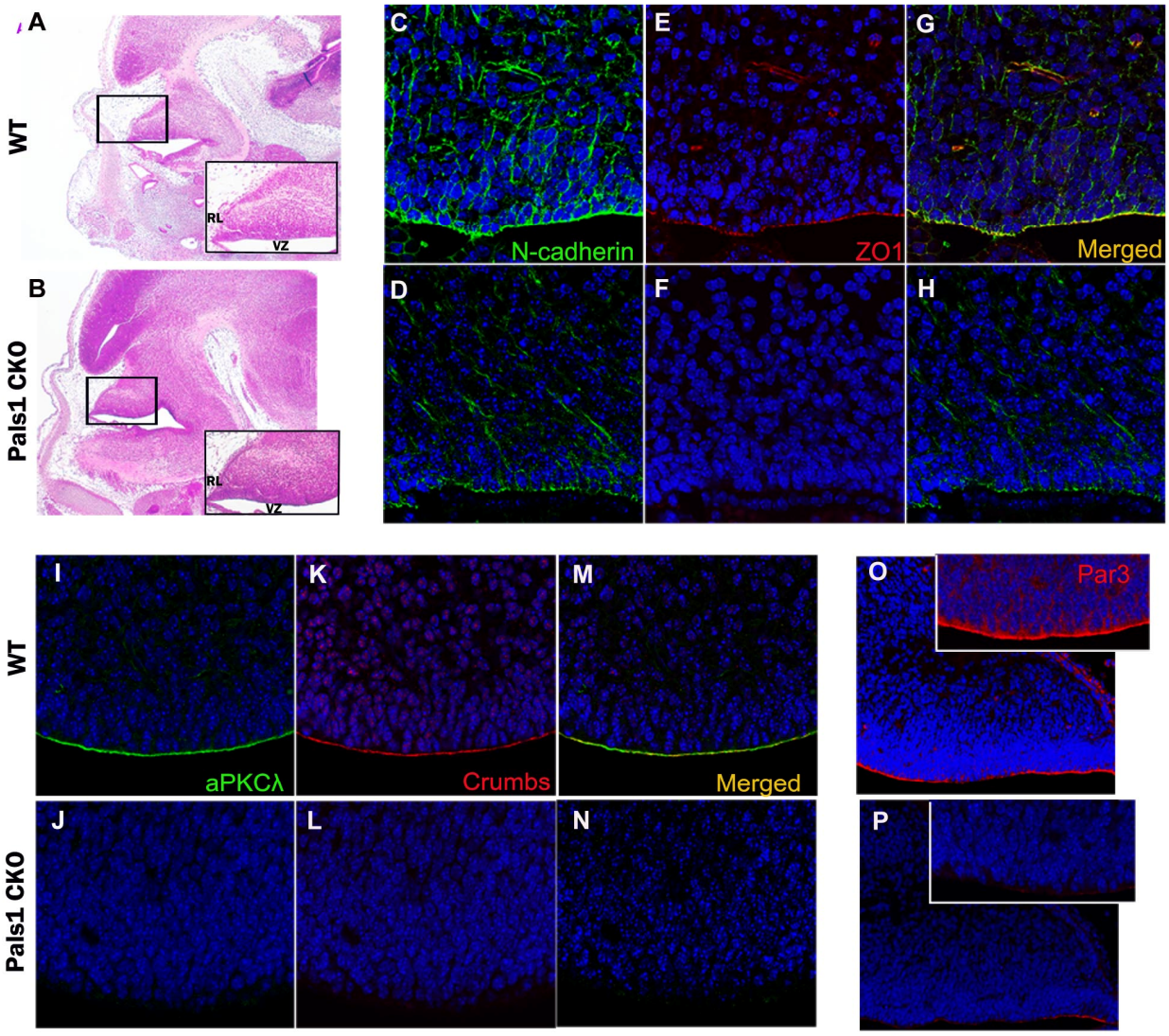


Figure 10. Pals1 is required for localization of apical junction proteins

(A, B) Histological analysis at E15.5 does not reveal any abnormalities in tissue integrity in the Pals1 mutant, which is identical to the WT control. **(C- H)** Although the AJ protein N-cadherin remains, the Zo-1 protein is strikingly reduced at the apical junction in the Pals1 mutant **(D, F, H)** compared to the WT **(C, E, G)**. **(I-N)** Apical complex proteins, such as Crb (red) and aPKC (green), are no longer detectable at the apical junction in the Pals1 mutant. **(O, P)** Par3 proteins are not well maintained at the apical junction.

3.2.5 Loss of Pals1 reduces ventricular progenitors, interneurons and glia cells

Since our histological analysis clearly demonstrated hypocellularity in the apical lining of VZ starting at E17.5, we compared numbers of dividing cells at the VZ after acute BrdU labeling at E15.5, E17.5 and P0 (Fig. 11A – F). BrdU positive proliferating cells were not significantly reduced in the Pals1 mutant at E15.5 compared to wild type, but reductions were statistically significant at E17.5 (*P=0.016).* At P0, few BrdU positive cells are found in the apical lining of both WT and CKO, as VZ is no longer mitotically active after birth (Fig. 11G). We also labeled proliferating VZ cells by *Hes1* and *5* in situ hybridization and NICD (Notch intracellular domain) immunostaining and saw a consistently reduced number of cells expressing the Notch signaling component in Pals1 mutants at E17.5 (data not shown). A previous study demonstrated that committed cerebellar interneurons can be marked by transient Pax2 expression in the white matter (Marichich and Herrup, 1999). We reasoned that reduced numbers of Pals1-deficient VZ progenitors likely leads to a decrease in Pax2 positive interneurons. As expected, we saw a striking decline of Pax2 positive cells in the white matter during the late embryonic stage and especially at P0 and P6 (Fig. 11I-P). Interestingly, at E17.5, Pax2 positive cells were shifted to the ventral side and could be found in the ventricular apical surface (Fig. 10L, arrows), indicating the possibility of premature differentiation of VZ cells to Pax2 positive interneurons or migration defects. It is noteworthy that the interneuron population at P21 was prominently decreased, as determined by a paucity of smaller Parvalbumin positive cells but a less prominent alteration in the number of larger Parvalbumin-expressing PCs (data not shown). Collectively, these results show that Pals1 is required for the proliferation of VZ

progenitor cells, analogous to its function in other neuroepithelial germinal zones. The cerebellar VZ also produces glial and their progenitor cells during mid to late embryogenesis (Sudarov et al., 2011). A recent GIFM (genetic inducible fate mapping) study with Ascl1-CreERT2 mice showed that the birth date of a subset of BG is around E14.5 (Sudarov et al., 2011). The reduction of radial glia cells in the VZ became visible at E17.5 by BLBP immunostaining but not prior to that (Fig. 11 Q, R). Furthermore, because Pals1 proteins started to disappear at the VZ surface around E15.5 in the Pals1 CKO with hGFAP Cre (Fig. 7C and E), it is plausible that the production of this early cohort of VZ-derived BGs and other glia cells was not affected in the Pals1 mutant. However, the reduction of glia cells was obvious at P0 and thereafter, indicating that the severe depletion of VZ progenitors impacted glia production. Because gliogenesis progresses in the white matter after birth, we determined whether Sox9 expressing glia cells and progenitor cells in the white matter was reduced. Sox9 positive glia progenitor and glia cells were strikingly reduced by about 70% in Pals1 CKO as compared to WT (Fig. 11S, T, W). Additionally, Olig-2 positive oligodendrocyte progenitors were also significantly reduced at P0 (Fig. 11U, V, X), although oligodendrocyte progenitors may be derived from outside the cerebellar germinal zone (Grimaldi et al., 2009). Collectively, Pals1 loss induces the reduction of proliferating cells in the ventricular zone and consequently affects the production of ventricular derived progenitors or neurons and glia cells.

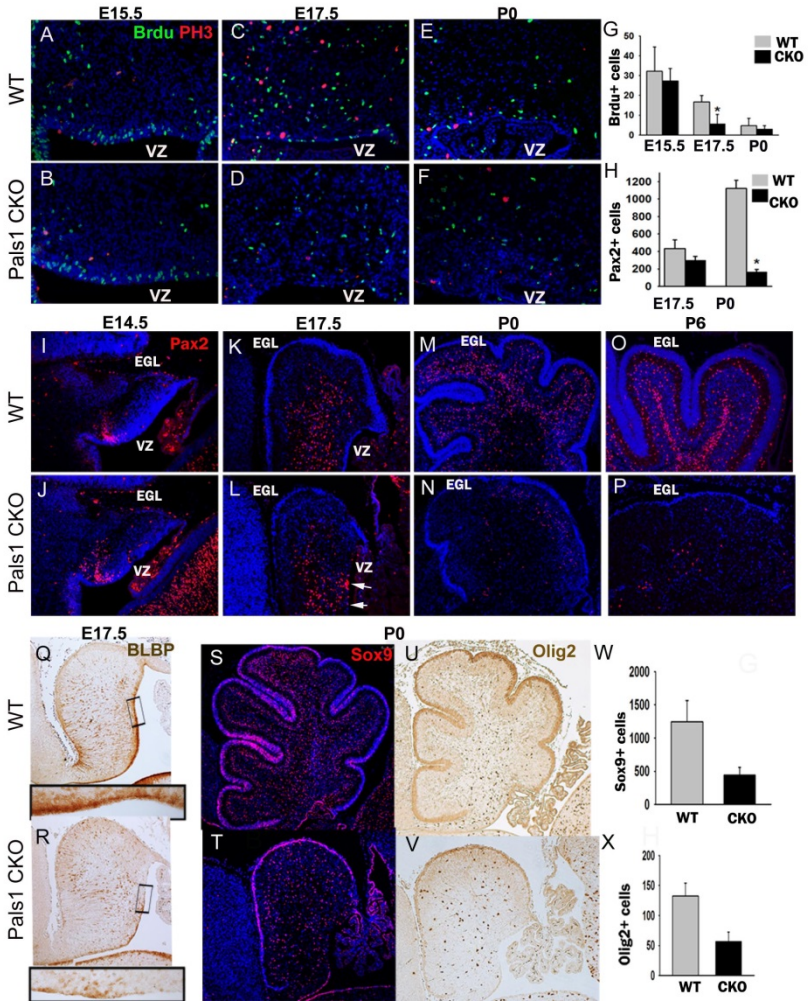


Figure 11. Pals1 depletion reduces proliferating VZ progenitors, interneurons and glia cells and their progenitors (**A-F)** Proliferative progenitors in the VZ were monitored with transient BrdU incorporation at E15.5, E17.5 and P0. (**G**) BrdU + cells are reduced at E15.5 and E17.5 in the Pals1 CKO, but the reduction is not statistically significant compared to WT only at E17.5 (p = 0.11). (**H-P)** GABAergic interneuron detected by Pax2 immunostaining in CKO are reduced but enriched at the apical side at E17.5 (arrows), and there is a significant decrease in Pax2 positive cells at P0 (P= 0.000074) (**H**). **(Q, R)** BLBP^{+} cells are reduced in the VZ at E17.5 in the Pals1 CKO compared to WT. Insets are enlarged images of VZ. **(S, T)** Sox 9^{+} cells are significantly reduced in the Pals1 CKO as compared to WT at P0. **(U, V)** Olig2^{+} cells are also reduced at P0. (**W, X)** Quantitative analysis of CKO and WT Sox 9 (p=0.0015) and Olig2 (p=0.0020) + cells performed by counting immunopositive cells at the midsagital section (n=3 for each genotype).

3.2.6 Pals1 is required for CGNP production and maintaining progenitor cells in EGL

To determine the cause of the significantly reduced CGN number in the Pals1 mutant cerebellum, we examined the production of CGNPs. The initial CGNP population is generated from the URL and migrates tangentially to the dorsal surface of the cerebellar plate underneath the pia from E13.5 to E18.5 (Wingate and Hatten, 1999). A second phase of massive amplification of CGNP through active proliferation occurs within the EGL during the early postnatal stage (peak at P6) (Roussel and Hatten, 2011). We first examined the production of CGNP during embryogenesis by labeling S-phase cells with BrdU. In brief, BrdU was injected into timed pregnant females or postnatal pups and 30 min later, cerebella of embryos or pups were harvested for immunohistochemistry staining (Fig. 12A – F). While indistinguishable at E15.5, a statistically significant diminution of BrdU positive cells in the EGL layer of the Pals1 mutant was observed at E17.5, the stage when URL shortening became apparent (Fig. 12C - D and G, Fig. 8L, M). Immunostaining for M-phase cells with Phospho-Histone H3 (PH3) and Phospho-Vimentin (4A4), and cycling cells with the pan-progenitor marker, Ki67, corroborates the findings with BrdU labeling (Fig. 12C, D). The reduced CGNP population was even more pronounced at later stages (Fig. 12E-G and data not shown).

To further understand the substantial reduction of CGNPs in the EGL, we examined cell death during late embryogenesis and at P0. At odds with the evidence of massive cell death observed in Pals1 CKO cerebral cortex (Kim et. al., 2010) no significant increase of dying cells was observed in the developing cerebellum (data not shown). Next, because the stunted URL is one of the most consistent and striking

features of the Pals1 mutant cerebellum, we sought to determine whether premature differentiation contributes to the reduced URL size by examining p27Kip1 (p27) and Tuj1 expression. Immunostaining for p27, which prevents cell cycle re-entry, allows us to determine precocious differentiation by labeling cells that have exited the cell cycle. Close examination revealed predominant p27 expression in the stunted URL of Pals1-deficient mice (Fig. 12I'), while, in the wild type, intense p27 expression was limited to the mid-zone (Fig. 12H', arrow). We also found that over 75% of total cells in the CKO are p27-expressing cells while only 35% express p27 in the wild type URL (Fig. 12H', I' boxed area, 12J). Thus, premature depletion of URL progenitor cells may contribute to the reduced CGNP population in the EGL by reducing the supply of URL-derived CGNPs.

We next examined the expression of the neural marker, Tuj1, at E17.5, to investigate whether excessive neuronal differentiation reduces the CGNP population. In wild type, the outer domain of EGL was occupied by proliferating CGNPs and Tuj1-expressing neurons were absent, whereas in the Pals1 mutant, Tuj1-expressing cells almost completely occupied the EGL (Fig. 12K and L). Intense Tuj1 positive cells at the outer domain of CKO EGL may indicate excessive CGNP differentiation at the EGL. Furthermore, comparison of the distribution of Pax6 positive cells in the CKO to WT revealed that many Pax6 positive cells are no longer maintained in the EGL, indicating their cell cycle exit and leave proliferating zone. Although the total number of Pax6 + cells at E17.5 was 30% lower in the CKO than in the WT, there was a threefold increase in the number of Pax6 + cells of CKO found in deeper layers compared to WT (Fig. 12O). In the WT, 80% of Pax6 + cells were found in the EGL, however, only 20% of

Pax6+ cells remained in the EGL of CKO (Fig. 12P, Q), demonstrating a Pals1 function in maintaining CGNP pool in the EGL. We consistently observed similar phenotypes one day later (data not shown).

To further investigate the population of cells exiting the cell cycle in the absence of Pals1 in the EGL, BrdU was administered at E16.5 and 24 hours later, embryos were harvested. First, we examined the BrdU+ cells among the Pax6+ cells that were not in the EGL and counted these as the population of cells that had exited the cell cycle (Fig. 12R, S). We found more than a threefold increase of cells exiting the cell cycle in the CKO than in WT (Fig. 12V). Next, we compared the number of BrdU+ cells that remained in the EGL but were postmitotic, as determined by the absence of Ki67 immunostaining (Fig. 12T, U). Again we consistently found that more BrdU+ cells are Ki67- in CKO than in WT (Fig. 12W). Next, to determine whether Pals1 deficiency induces changes in cell cycle length, we examined the proportion of cycling cells that were in S-phase by counting the BrdU + cells among Ki67 + cells after 30 minute pulse labeling. We failed to obtain statistically significant changes in the fraction of cells in the S-phase despite a significantly reduced number of proliferating cells in the CKO (Fig. 12X, Y, Z). This suggests that cell cycle length is not obviously altered by the loss of Pals1. Together, the failure of CGNPs to re-enter the cell cycle, which leads to premature differentiation, is a main contributor to the reduced CGNP in the EGL.

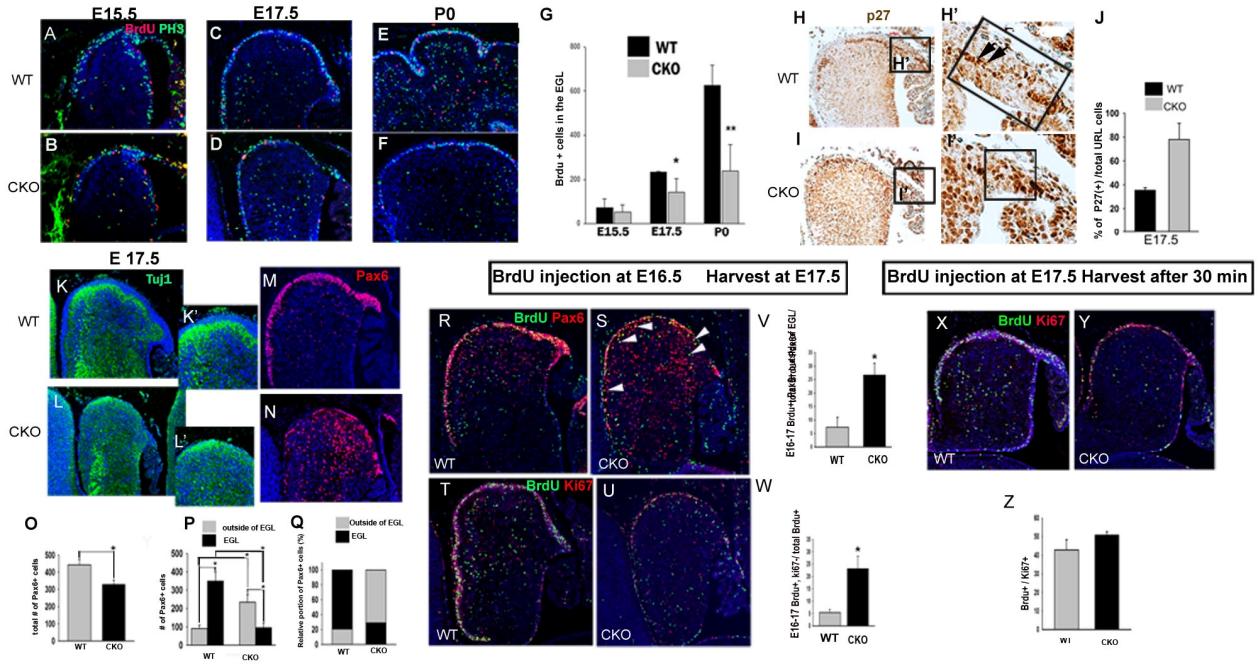


Figure 12. Loss of Pals1 reduces the proliferation of CGNP cells in the EGL

(**A-G).** Proliferative progenitors were labeled by BrdU incorporation at E15.5, E17.5, and P0 and stained for BrdU and with PH3 antibody. Although there was no statistically significant difference between WT and CKO at E15.5 **(A, B)**, BrdU positive CGNP cells in the EGL were significantly reduced at E17.5 **(C, D)** and P0 **(E, F)** in Pals1 CKO as compared to WT **(G)**. ** *P*= 0.000098, P<0.001. **(H, H', I, I')** Strongly p27 positive cells are lined up at the mid zone of the WT URL (arrow), but in the CKO, p27 positive cells are dispersed in a short and stunted URL. **(J)** More than 75% of CKO URL cells express p27; only 35% of WT express p27. (**K-L'),** Tuj1 positive cells (green) extend beneath the pia in the CKO, but are not in the outer EGL of WT. **(M-Q)** More Pax6 + cells dispersed in the outside of EGL in the CKO as compared to WT **(M, N)**. Fewer Pax6 + cells are found in the CKO **(O)** but the distribution of Pax6 + cells is greatly shifted to outside of EGL in the CKO compared to WT **(P, Q)**. **(R, S, V)** The fraction of BrdU labeled Pax6+ cells 24 hours before harvesting in the outside of EGL among total Pax6 + is significantly increased in the CKO**. (T, U, W)** Likewise, the proportion of Brdu+ Ki67- cells out of total BrdU+ cells in the EGL is increased in the CKO compared to WT. **(X, Y, Z)** The portion of S-phase cells marked by 30 minute BrdU labeling out of total progenitor cells (Ki67+) is slightly increased in the CKO but not statistically significant.

3.2.7 The expression of downstream effectors of Shh signaling is not obviously altered

To understand the mechanism for the loss of in Pals1-deficient CGNP in the EGL, we examined whether compromised mitogenic activity mediated by Shh contributes to the progenitor depletion. We therefore observed the expression of Shh downstream effectors, Gli1, Gli2, N-Myc, Cyclin D1 in the CGNP at E17.5, P0 and P6 (Fig.13). At E17.5, because of relative weak expression of those genes in both WT and CKO, we have utilized the real time PCR to compare the level of their expression. In contrast to obvious apparent reduction of Pals1 expression, the level of expression of Gli1 and 2, N-myc, Cyclin D1 is not distinctively decreased at E17.5. We collected and processed data from 3 mutants and 3 WT through normalizing the value to actin expression and calculated the relative proportion to the WT. Although variability among animals is apparent, there is no clear evidence of reduced level of their gene expression in the CKO as compared to WT (Fig. 13A). Furthermore, *in situ hybridization* analyses of Gli1, Gli2 and N-Myc expression show relatively intact their expression in the CGNP during the course of development in later stages (P0 and P6), suggesting that Pals1-deficient EGL cells possess unimpaired Shh signaling activation (Fig. 13B-M'). Together, our results suggest that Pals1 plays an important role in promoting the proliferation of EGL cells, which is distinct from activation of Shh signaling.

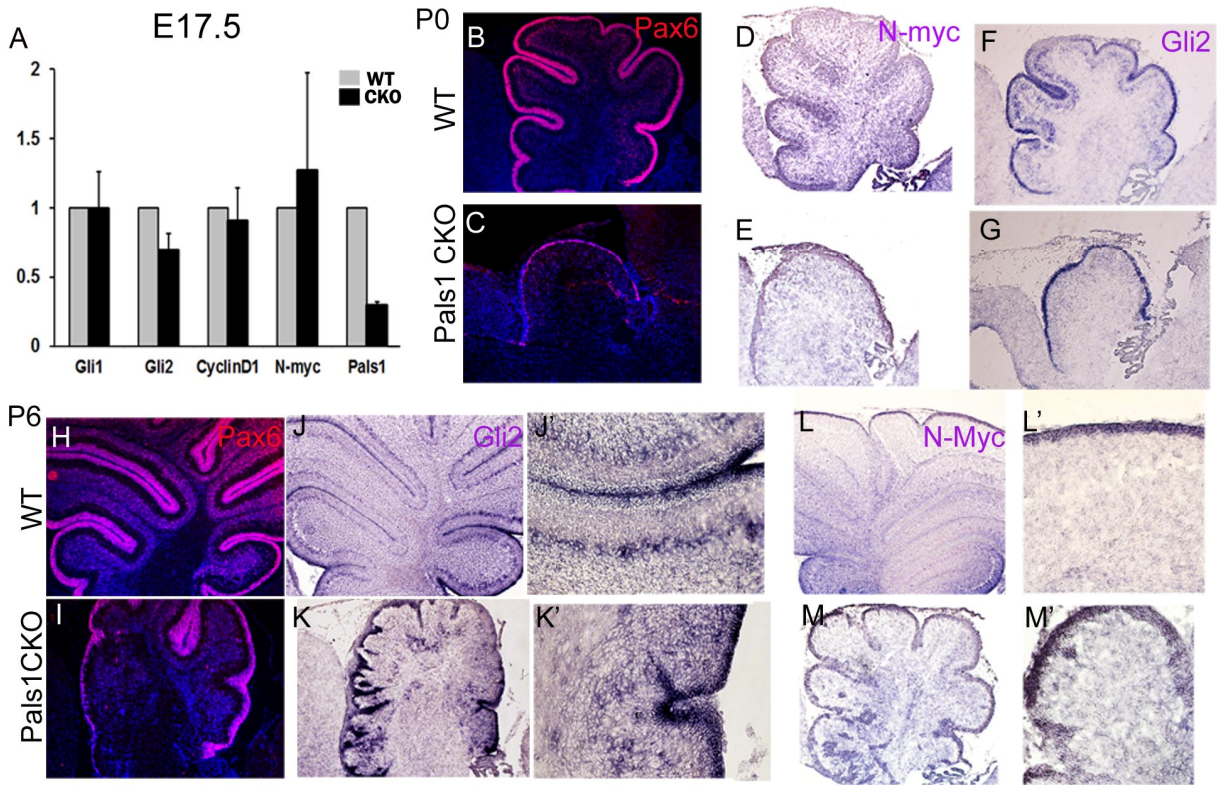


Figure 13. The expression of downstream effectors of Shh signaling are not obviously affected by Pals1 loss

(A) At E17.5, the level of Gli1, Gli2. Pals1, N-Myc and Cyclin D1 is analyzed by real time PCR. Except Pals1, there are no significant changes in gene expression caused by Pals1 deletion **(B-G)** N-Myc and Gli2 expression in the EGL cells at P0 examined by pairing Pax6 immunostaining **(B, C)** and N-Myc and Gli2 in situ hybridization **(D, E, F, G)** of similar sections. **(H-M')** Similarly, at P6, the intensity of Gli2 and N-Myc mRNA expression in the EGL of the Pals1 CKO **(K, K', M, M')** is not apparently changed from WT. **(J, J', L, L').**

3.2.8 Pals1 deficiency abrogates Shh signaling

To further explore the relationship between Shh signaling and Pals1 in CGNP proliferation, we have utilized the SmoM2 allele to achieve constitutively active Shh signaling in Pals1 deficient cells (Lau et al 2011). The SmoM2 allele carries a mutation (W535L) found in medulloblastoma patients, which causes activation of Shh signaling without ligand. As expected, highly activated Shh signaling in the SmoM2 with *hGFAP cre* mice results in hypertrophic cerebellum with overly abundant Pax6+ CGNP and CGN, and Pax2+ interneurons at P0 (Fig 14A, B, E, F). Unexpectedly, the SmoM2 allele crossing into the Pals1 CKO does not increase the number of Pax6+ and Pax2+ cells at P0 and double mutants are indistinguishable from Pals1 CKO in terms of general size and morphology (Fig. 14 A-Q). However, at a later stage, small regions of tissue containing a cluster of abnormal cells (Fig. 14 arrow) are apparent on the surface of the double mutant cerebellum, which lacked normal foliation, suggesting potential tumorigenic activity in the double mutant. Thus, although typical medulloblastoma cells, represented as small and round with a high nuclear to cytoplasmic ratio, are not obvious in the double mutants at P21, it is possible that tumors develop at later stages. However, severe hydrocephalus in the double mutant, similar to the single SmoM2 mutant, compromises viability beyond P21 and occludes us from testing the possibility. Further exploration of this small cell cluster revealed that these cells are mitotically active as they were labeled by Ki67. Furthermore, these cells can be marked by glia cell markers such as S100 and GFAP (Fig. 14Q, data not shown), suggesting glial characteristics. Collectively, Pals1 deficiency can block medulloblastoma formation in spite of a

tumorigenic mutation, providing evidence for the essential requirement of Pals1 function in uncontrolled proliferation of CGNP in Shh-mediated tumorigenesis.

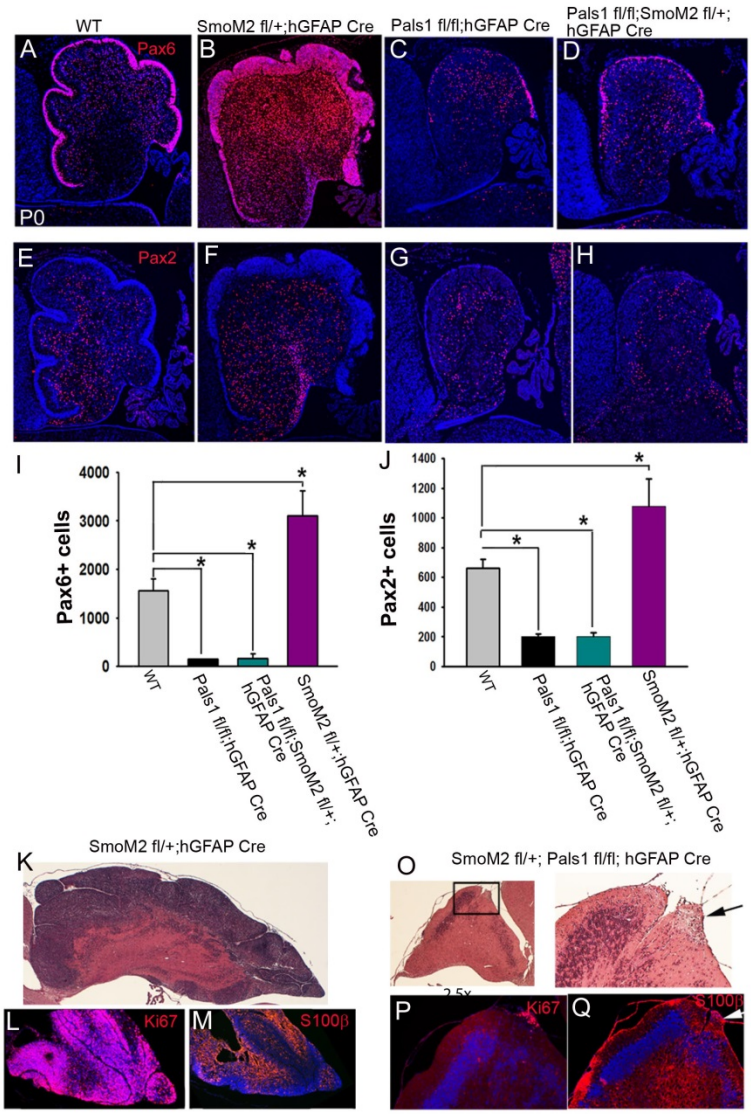


Figure 14. Pals1 deficiency blocks activated Smo-mediated tumorigenesis

(A, B, C, D, I) SmoM2 allele induces over production of Pax6+ cells and expanded EGL compared to WT **(A, B)**, which is no longer observed in the double mutant with Pals1 CKO **(D),** similar to that of Pals1 CKO **(C)**. **(E, F, G, H, I)** Pax2+ interneuron cells are also increased in the SmoM2 mutant, but this effect is abolished by Pals1 deletion at P0. **(K, L, M)** At P21, medulloblastoma is apparent in the SmoM2 mutant shown by expanded dark and high-nuclear tissue. Many tumor cells are Ki67+ **(L)** and S100□ expressing cells do not appear to overlap with Ki67 expressing regions **(M)**. **(O, P, Q)** In the double mutant, eosinophillic small clusters of cells are found on the surface of the cerebellum, which are Ki67+ **(P)** and S100□+ **(Q)**.

3.3 DISCUSSION

Our study demonstrated that asymmetrically distributed polarity complex proteins, specifically the apical complex protein Pals1, are required for maintaining the proliferative capability of progenitors in both cerebellar germinal zones by preventing premature differentiation. Remarkably, our genetic study revealed that Pals1 deficiency can completely overcome deregulated Shh signaling by activated Smo mutation, suggesting its potential function in mediating mitogenic signals or cellular adhesion required to maintain cycling cells in germinal zones.

3.3.1 EGL, URL and VZ progenitors require Pals1 function to remain in cycling pool

Owing to its well-defined mitogenic/morphogenetic signals for distinct germinal zones and its relatively lengthy developmental process, the cerebellum provides a unique opportunity to explore the contribution of epithelial polarity to neural development. The majority of cerebellar cells are generated from neuroepithelium-derived progenitors that are lineage restricted by specific basic helix-loop-helix transcription factors such as Atoh1 in the URL and Ptf1a in the VZ (Hashimoto and Hibi, 2012). Our study recognizes cell polarity as an intrinsic property that is important for maintaining all germinal zones including URL, VZ and EGL. It has been shown that certain subtypes of progenitors with distinct molecular signatures may regulate their proliferation capability by initiating either symmetric cell division (producing two progenitors) or asymmetric cell division (one progenitor and one intermediate progenitor or terminally differentiated cell) (Franco and Muller, 2013). For example, in the early

cortical neuroepithelium, early born neurons are generated through asymmetric cell division of Cux2 negative progenitors while Cux2 positive progenitors of late born neurons undergo self-renewing symmetric cell division (Franco et al., 2012). Furthermore, Lis1 deletion in cells undergoing symmetric cell division causes more profound defects than in later progenitors that divide asymmetrically (Pawlisz et al., 2008). Because our specific deletion of Pals1 occurs in the early cortical neuroepithelium, when symmetric cell division is prevalent, and causes near absence of cortical structure, we expect that Pals1 function is more critical for progenitor expansion than asymmetric division. Consistently, the cell division mode of CGNPs has been shown to be symmetric and clonally expanded progenitor cells exit the cell cycle after a few divisions and depart to IGL. The severe defects arising from CGNP depletion may support the idea that faithful symmetric cell division relies more on Pals1 function than does asymmetric cell division.

3.3.2 Pals1 loss forces cell cycle exit and blocks tumorigenesis

Our results demonstrated the striking effect of Pals1 deficiency on promoting a differentiation program even when mitogenic signaling was constitutively active. Although *in vivo* mechanisms that stimulate cell cycle exit of CGNPs during development are not yet fully established, factors like FGF, PKA, and Wnt are known to drive differentiation *in vitro* or in tumor cells. Previously**,** it has been shown that FGF signaling has a slight mitogenic effect on CGNPs (Wechsler-Reya and Scott, 1999), but when CGNPs are proliferating in the presence of Shh or deregulated Shh, FGF exerts an opposing effect and leads to differentiation (Fogarty et al. 2006; Emmenegger et al.,

2013). None of the identified factors, however, have been found to be required for CGNP differentiation *in vivo*, suggestion the existence of a more prominent factor or the cooperation of several factors. Remarkably, Pals1 deficiency enhances cell cycle exit during normal development and potentially in malignant cells, suggesting a fundamental requirement of Pals1 in proliferation. Since molecularly distinct mechanisms may regulate proliferation in embryonic versus malignant tumor cells in later stages, it will be important to determine the effect of removing Pals1 in mice with preexisting medulloblastoma. For this purpose, an inducible Pals1 conditional knockout in the Patched heterozygote mouse model , of which approximately 20% spontaneously develop medulloblastoma in later stages (Lau et al., 2012), can be used to determine whether Pals1 loss forces the differentiation of Shh-driven tumor cells. Interestingly, a recent study suggests that tumor-initiating cells in medulloblastoma are distinct Nestin positive progenitors in the EGL (Li et al., 2013). Those cells have reduced expression levels of DNA repair genes and are prone to develop genomic instability. It is possible this newly identified Nestin positive progenitor population may be depleted by Pals1 loss during development in the SmoM2 mice. In any case, it is extremely exciting that Pals1 loss can completely shut down uncontrolled proliferation elicited by aberrant Shh signaling. This may provide a new direction for understanding granule cell development and medulloblastoma treatment.

Our preliminary observation of cilia in *Pals1* mutant EGL cells did not reveal a striking loss or malformation of cilia phenotype (data not shown). Furthermore, Gli1 and 2 expression levels were still relatively intact in the Pals1 mutants (Figure 7) unlike the *Kif3a* mutant in which ciliogenesis is evidently disrupted, accompanied by strikingly

reduced Gli2 expression (Spasky, developmental biology). Similar to Pals1 deficiency, Kif3a deletion with *hGFAP Cre* completely blocks the SmoM2 mediated hyper-proliferation of EGL cells and medulloblastoma. However, Pals1 deletion-mediated arrest of SmoM2-induced deregulated proliferation appears to be distinct from primary cilia function. Interestingly, the presence of a small cluster of faint cells (eosinophillic) with Ki67+ above the molecular layer of the Pals1 and SmoM2 double mutant is distinct from the dense medulloblastoma cells. These cells may belong to Type II tumor cells observed in transgenic mice with excess Gli2 activation in which medulloblastoma is not formed unless cilia is removed. Since it is likely that Gli3 processing remained intact in the cilia, Gli3 repressor may prevent the medulloblastoma formation in spite of Gli2 hyper activation. Thus, one potential mechanism by which Pals1 deficiency inhibits SmoM2 mediated Shh signaling activation could be through promoting Gli3 processing to its repressor form. Together, future studies concerning cilia, Pals1 function, and Gli3 processing may reveal a new regulatory pathway that inhibits Shh signaling.

3.3.3 A distinct survival mechanism may exist in cerebellar cells.

VZ progenitors in the entire neuroepithelia were affected in the Numb/Numblike double mutant with Nestin Cre (Petersen et al., 2002), suggesting a general requirement of polarity complex proteins in maintaining progenitor pools. Consistent with this notion, we have shown their necessity for maintaining progenitor pools in the cerebral cortex (Kim et al., 2010) and, from this study, the cerebellum. Although the cortex and cerebellum progenitors show phenotype similarity, such as the depletion of progenitors due to defects in self-renewal in both germinal zones, the remarkable

difference is that dying cells are not noticeably increased in the cerebellum of the Pals1 mutant (Kim et al., 2010). In the cerebral cortex, prematurely generated neurons underwent rapid apoptotic cell death associated with impaired mTORC1 activity within a few days after cre recombination (Kim et al., 2010). In the Pals1-deficient retina, a moderate number of dying cells were found throughout developmental and adult stages (Cho et al., 2012). The late-onset retinal degenerative disease, Retinitis Pigmentosa (RP) 12, is caused by a mutation of the Crb1 gene in humans (Richard et al., 2006; van de Pavert et al., 2004). Crb1 mutation can also cause an earlier onset disease, the most severe form of retinal degeneration, Leber Congenital Amaurosis (LCA) 8 (Aleman et al., 2011). Our studies of Pals1 deletion in retinal progenitors have provided a model of LCA8 that reproduces its early degeneration and retinal lamination defects. In contrast, despite an extensive examination of embryonic and early postnatal stages (Fig. S2), we did not detect up-regulation of cell death in any cell type in the cerebellum. It is possible that the extremely slow kinetics of cerebellar cell death prevented us from observing an unambiguous and statistically significant increase in the earlier stages. A unique survival mechanism in cerebellar cells and their progenitors, however, may prevent an increase in cell death.

3.3.4 The role of Pals1 is to maintain apical junctions in the cerebellum.

Although we cannot rule out the possibility of Pals1 function in unknown signaling pathways, our current study suggests that Pals1-mediated maintenance of cell junction and cell polarity are the most consistent phenotypes observed in various neuroepithelia. In the developing and mature retinas of Pals1 mutants, apical junctions and tissue

polarity are severely disrupted. Furthermore, defects in the junction between Müller glia and photoreceptors are recognized as one of the initial steps in the pathogenesis of Crb1 mouse mutants. A previous study has also demonstrated that Pard3 serves a critical function in CGN migration by promoting cell adhesion through an interaction with the Junctional Adhesion Molecule (JAM)-C (Famulski et al., 2010). Defects in the localization of tight junction associated proteins such as Zo1 and apical complex proteins are therefore closely and constantly associated with the abnormal migration and the disorganization of lamina structure in various developmental contexts. The loss of adhesion between CGNPs causes them to prematurely detach from the germinal zone, which may serve as a potential mechanism for loss of proliferative capability. In *Drosophila* ovary, germline stem cells are lost if they fail to maintain attachment to their niche due to diminished junction proteins such as E-Cadherin (Song et al., 2002, science). Furthermore, Cdc42 deficiency causes adherens junction defects and leads to a marked increase of intermediate neurogenic progenitors by inducing detachment of apical progenitors in the developing cortex (Cappellio et al., 2006). In our study, upon loss of Pals1, a large portion of Pax6 positive cells were no longer maintained in the EGL and migrated out of the EGL, possibly due to the adhesion defects mediated by Pals1. Recent studies have shown that the homophilic interaction between extracellular domains of Crb proteins provide cell adhesion in the *Drosophila* wing (JCS 2014). It is possible that the Pals1-Crb adhesion system may play a critical role in CGNP attachment to the EGL layer by enabling interactions between CGNPs or between CGNP and pia meningeal cells until clonal expansion is completed.

Chapter Four:

The roles of Pals1 in cortical development and mitosis

4.1 Introduction

The cerebral cortex is the largest structure in the brain and is essential for cognition and higher mental capability. Abnormal development of the cerebral cortex causes neurodevelopmental disorders such as epilepsy, developmental delay and autism (Mochida & Walsh, 2001). Proper proliferation and differentiation of cortical progenitors, which depend on tightly regulated cellular and molecular events, is essential for normal cortical development. In addition, the balance between early and late born neuron generations, which is maintained through regulating progenitor cell division (symmetric versus asymmetric), is critical to maintain normal cortical development (Caviness et al., 1995). During neurogenesis, intrinsic cell mechanisms, such as polarity proteins and timing transcription factor expression, function as cell fate determinants for the transition from multipotent progenitors to layer specific neurons in the cortex (Alsio et al., 2013). In a fixed temporal order, multipotent progenitor cells produce different types of neurons in the developing cerebral cortex (Livesey & Sepko, 2001), and all pyramidal neurons are generated and formed in an inside out matter to develop the six cortical layers: layer 6 and the subplate neurons are generated first, followed by layers 5,4, and 2/3 in sequential order.

Although the role of Pals1 has been intensively studied in *Drosophila*, zebrafish and mammalian cells, the first study on the role of Pals1 in neocortical development was only recently reported with the generation of Pals1 CKO mice with Emx1-Cre. This study shows that the deletion of Pals1 in the cortex disrupts cell-cell adhesion and maintenance of polarity in NECs. This leads to the premature neurogenesis from early

neuroepithelial progenitors followed by apoptotic cell death, resulting in significantly undersized cortex at the adult stages. (Kim et al., 2010).

The major issues in our lab over the recent years has been characterizing the roles of Pals1 in another progenitor, radial glial progenitor (RGP)s, and proving if the reduced cortical size is due to the cell autonomous or non-autonomous defects by the ablation of Pals1 in RGPs. Furthermore, Pals1 function in mitotic progression and how Pals1 is differentially inherited or changes its subcellular localization during mitosis are important questions to identify the mechanism underlying Pals1 function in cortical development. Although polarity complex proteins are known for regulating the asymmetric localization of cell fate determinants and in turn determine cell fate, Pals1 localization studies through immunostaining was limited to only apical junction. Thus, it is critical to determine its subcellular distribution, how it affects the cell fate of progenitor cells and identify any downstream effectors.

Pttg1 gene is an oncogene discovered in rat pituitary tumor cells, and is the mammalian homolog of *Xenopus* securin that inhibits sister chromatid separation (Zou et al., 1999). This gene is highly expressed during M phase, and overexpressed in a variety of tumors, including pituitary, breast, thyroid, ovarian, uterine, colon, and lung (Kakar 1999; Zhang et al., 1999; Heaney et al. 2000; Shibata et al., 2002; Solbach et al. 2004; Chamaon et al., 2005, Tsai et al., 2005). Pttg1 null mice show testicular and splenic hypoplasia, aberrant cell cycle progression, and premature centromere segregation. Embryo fibroblasts lacking Pttg1 exhibited G2-M phase extension, showing aberrant cell cycle, multiple nuclei and increased aneuploidy (Wang et al., 2003).

Furthermore, enhanced Pttg1 levels are associated with tumor cell characterization, such as higher tumor grade, invasiveness and tumor vascularity (Heaney et al., 2000; Hlubek et al., 2006). In addition, the involvement of Pttg1 is also demonstrated in tumor transforming activity. Pttg1 is involved in the induction of chromosome instability binding to Separase to regulate sister chromosome separation (Waizenegger et al., 2002). It has also been known that Pttg1 interferes the formation of Ku heterodimer, inducing genetic instability through the inhibition of DNA damage repair (Kim et al., 2007). Pttg1 can function as a transcription factor to regulate several genes' expression, leading to the regulation of tumorigenesis and cancer development. Several mechanisms and interacting partners are shown in Figure 15 for the function of Pttg1.

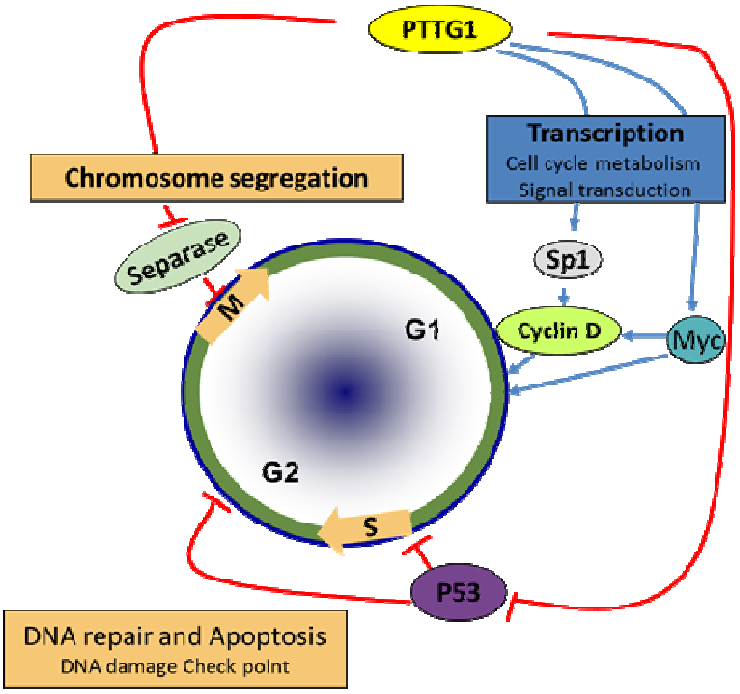


Figure 15. Schematic diagram for the role of Pttg1 in cell cycle and its interacting partners. Pttg1regulates sister chromatid separation and the transition from metaphase to anaphase by inhibition of Separase. Pttg1 is able to bind c-Myc promoter directly and regulate cell cycle per se or via Cyclin D. Interacting partners are CDK inhibitor p21, transcription factor SP1 and p53.

The study of the relationship between Pals1 and Pttg1 has not been elucidated yet. Intriguingly, gene expression profiling study of the light induced degeneration in the Crb1 mutant revealed that Pttg1 is the most consistently and profoundly affected gene upon light mediated damage. Furthermore, microarray analysis of Pals1 Emx1-Cre mutant mice noted that the Pttg1 gene is significantly downregulated, suggesting that Pttg1 may be functional as a critical downstream factor in Pals1 CKO. Our studies analyzing Pals1 deletion in RGPs establish the crucial function of Pals1in mitotic progression and faithful chromosome segregation by regulating Pttg1 expression potentially through Yap interaction.

4.2 Results

4.2.1 Pals1 is essential for histogenesis and RGP maintenance during cortical development.

Previous studies identified the significance of Pals1 for histogenesis of the cortex and NEC self-renewal using Emx1-Cre. Emx1-Cre mediated deletion of Pals1 showed almost complete ablation of the cortex, lacking all cortical neurons. However, this limits the study of the role of Pals1 in other aspects of cortical development since the apoptotic deletion of NECs leads to complete loss of medial cortex at postnatal stages in Pals1 CKO. We postulated that Pals1 is essential in the maintenance of RGP, which undergoes asymmetric cell division and generates the majority of the excitatory neurons in the cortex. Therefore we used hGFAP-Cre allele in which Cre expression is initiated at E12.5 in the RGP to delete Pals1 from the majority of the RGP pools. The Pals1 CKO; hGFAP-Cre mice were born in the expected Mendelian ratio. These mice developed severe seizures at around P12-20, and many of them died before weaning age. Similar to the Pals1;Emx1-Cre mice, Pals1;hGFAP-Cre CKO and Het mice showed remarkably undersized brain, compared to wild type littermates (Fig 16A). Unlike Pals1;Emx1-Cre mice, Pals1;hGFAP-Cre mice maintained general cortical layer structures and preserved Marginal Zone (MZ) (Fig 16B). Interestingly, malformed and severely undersized hippocampus with only rudimentary structure can be observed at P11, suggesting that Pals1 is also essential for the development of the hippocampus (Fig 16C). Together, the deletion of Pals1 demonstrates its essential role in normal lamina composition and layer structure in the cortex and hippocampus.

To define Pals1 function in cortical neuronal production and ultimate lamina structure, we utilized the layer specific markers to analyze the lamina composition at P11. The late born neuronal population (layer 2-4) is labeled by Cux1 immunostaining (Fig 16 D,E). The total number of Cux1 positive cells are significantly reduced in Pals1 CKO as compared to WT (Fig 1F), indicating that the paucity of late born neurons may due to the reduced size of the progenitor pool that generate late born neurons. When we observed earlier born neurons labeled with Foxp1 (layer 6 marker) (Fig 16G,H') and Ctip2 (layer 5 marker) (Fig 16 I,J'), it showed that the reduced number of earlier born neurons was not statistically significant between WT and Pals1 CKO, suggesting that earlier progenitor pools are maintained in Pals1 CKO. Although the number of early born neurons are maintained, at P11 these neurons are ectopically localized and dispersed by the deletion of Pals1. Some neurons are localized in layer 2-4 where late born neurons reside. This may imply that the ablation of Pals1 is required for the maintenance of cortical layer structure by regulating neuronal migration in early born neurons.

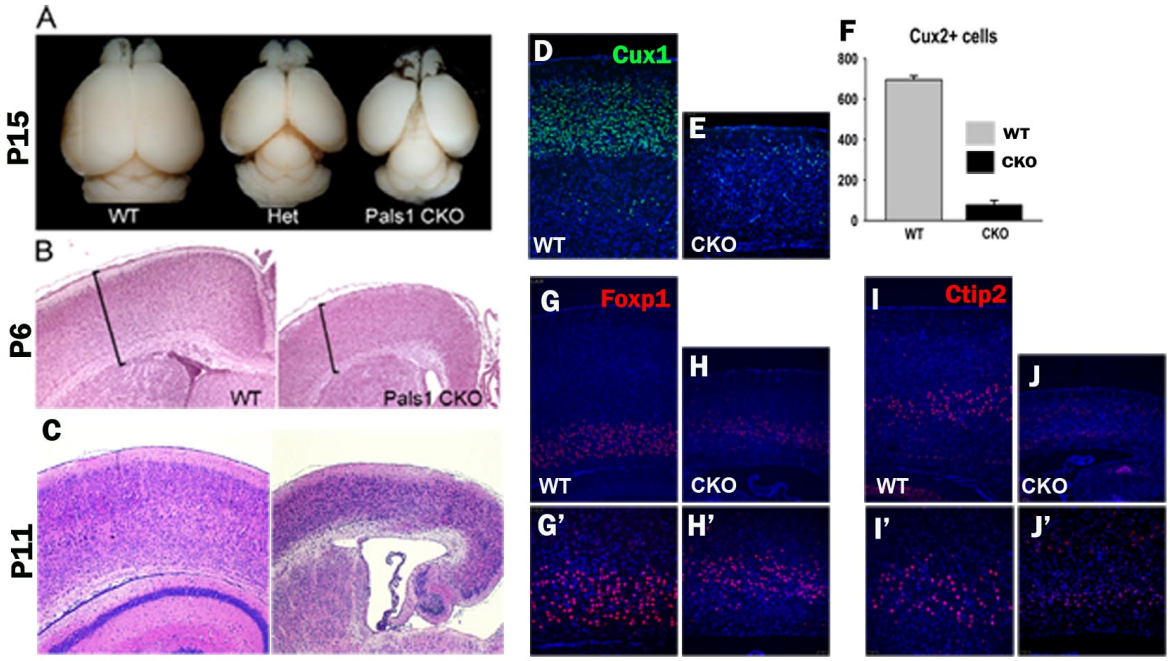


Figure 16. Pals1 ablation using hGFAP-Cre causes microcephaly by the reduction of RGP. (A) Pals1 deletion in the RGP by crossing with *hGFAP-Cre* induces small brain. (B,C) Histology of the whole brain at P6 and P11 shows reduced cortical size in Pals1 CKO mice, but cortical layers are relatively preserved. In the Pals1 CKO, hippocampi is reduced and malformed . (D,E) The severe reduction of late born neurons marked by Cux1 is observed at P11 in Pals1 CKO. (G,H,I,J) Early born neurons stained by Foxp1 (layer 6) and Ctip2 (layer 5) are not significantly reduced in Pals1 CKO.

4.2.2 Pals1 regulates RGP maintenance during cortical development.

To further evaluate the proliferation and maintenance defects leading to the paucity of late born neurons at later stages, the changes in the progenitor population during neurogenesis were investigated. First, we identified the S-phase cells during cell cycle and proliferating RGPs, by performing transient BrdU pulse-labeling and marker analysis with Pax6 for RGPs. The number of BrdU positive S-phase cells and RGPs are significantly reduced in Pals1 CKO at E14.5, compared to wild type animals (Fig 17A',A'',B',B'',E). Likewise, cells undergoing mitosis stained with phospho-histone3 (pH3), were significantly reduced in Pals1 CKO (Fig 17A',B',F). Taken together, it is clear that Pals1 is crucial for progenitor proliferation.

Since we observed a comparable number of early born neurons at later stages, we analyzed the population of early born neurons through staining with early born neuronal markers, Tbr1 and Ctip2 at E14.5 (Fig 17H',H'',I',I''). As shown in the previous study on Pals1;Emx1-Cre mice, early born neurons are significantly increased (Fig 17 J,K), suggesting excessive neurogenesis at the expense of RGP pools. To further exploit this, we stained with Tuj1 to mark neuronal cells and p27 and NeuN to mark differentiating progenitors that become neurons. As we expected, Tuj1 and p27 positive cells are increased in Pals1 CKO at E14.5 (Fig 17L-M'), suggesting that many progenitors exit the cell cycle precociously. However, there is no excessive early born neurons present in later states, the striking reduction of cortex at postnatal stages suggests that the precocious cell cycle exit observed in Pals1 CKO during neurogenesis subsequently leads to apoptotic cell death and eventual cortical size reduction.

Consistent with our expectation, precociously generated neurons undergo apoptotic cell death marked by CC3 staining (Fig2 L-M'). Most of the dying cells are newly generated neurons, whereas most progenitors marked with Pax6 do not overlap with CC3 (Fig 17 N-O'). This implies that the dying cells are not the progenitors but the excessive newly born neurons at early embryonic stages.

Intriguingly, basal progenitors are slightly increased at E14.5 (Fig 17 G,K), suggesting that more neurogenesis occurs via intermediate progenitors rather than RGP that directly differentiates into neurons through asymmetric cell division. Intermediate neurogenic progenitors, BPs, which are generated by symmetric RGP division, can undergo one or a few more rounds of cell division to generate neurons. Previous studies have illustrated that Par3 knockdown using shRNA inhibits asymmetric cell division of RGPs, thereby producing two BPs (Bultje et al., 2009). In addition, the deletion of Cdc42, which induces adherens junction defects and loosens the attachment of apical progenitors in the VZ, can also generate excessive basal progenitors; although, the length of cell cycle and basement membrane contract are normal (Cappello et al., 2006). Therefore, the increase of the BP population in Pals1 CKO may due to the failure to maintain RGP population by more asymmetric cell division of RGPs or loss of attachment of apical progenitors in VZ, both of which can generate more BPs.

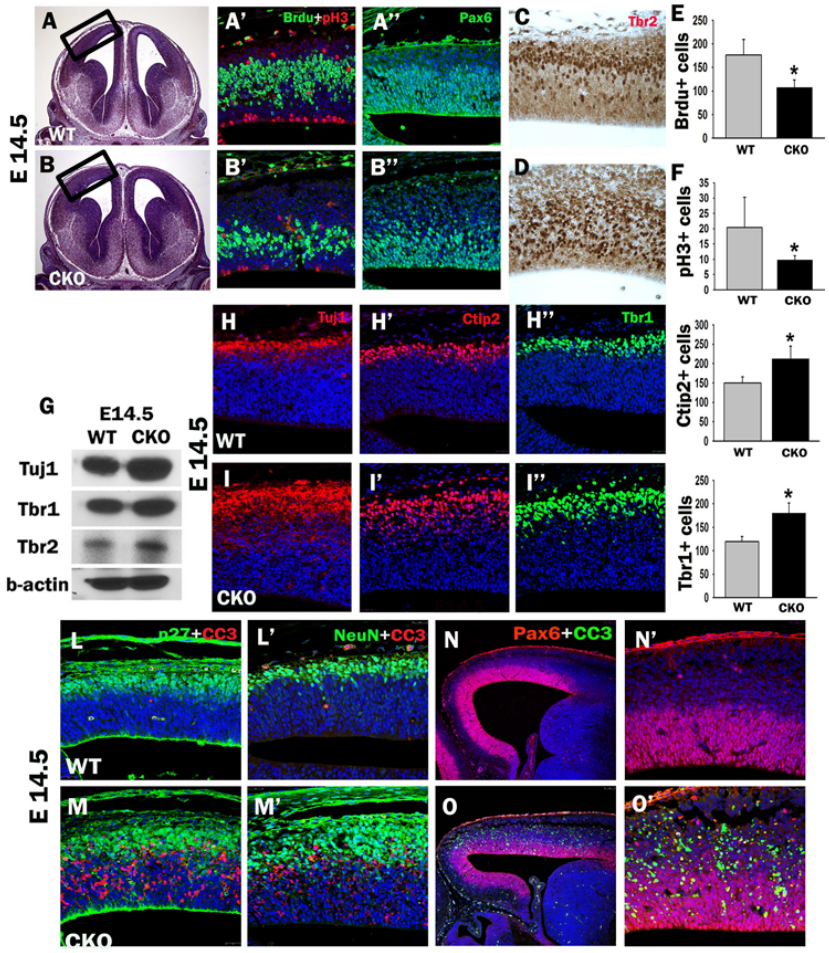


Figure 17. Loss of Pals1 shows substantially reduced RGP pool and apoptotic cell death by precocious differentiation. (A,B) Histology of Pals1 CKO shows reduced dorsal surface area, but interestingly increased cortical thickness at E14.5. Proliferating RGP pool labelled by Brdu incorporation (A',B') and staining with Pax6 (A'',B'') and pH3 (A',B') are significantly reduced in Pals1 CKO (E,F). (C,D) Basal progenitors are slightly increased in Pals1 CKO. Excessive neurogenesis was observed in Pals1 CKO analyzed with western blotting (G) and immunohistochemistry with Tuj1, Ctip2 and Tbr1 antibody (H-I''). The number of Tbr1 and Ctip2 positive cells are significantly increased in Pals1 CKO (J,K). Massive cell death was observed in Pals1 CKO by CC3 immunostaining with most of the dying cells being newly generated neurons (L-O') determined by overlapping expression of CC3 anp27 or NeuN.

4.2.3 Mitogenic signaling, such as Shh and Notch signaling, are not obviously defective in Pals1 CKO

To investigate the molecular mechanism underlying progenitor loss due to Pals1 deficiency, we examined alterations in the mitogenic signaling in the Pals1 mutant. First, we examined the Shh signaling as it functions as a strong mitogen for the neural progenitors, thereby maintaining proliferation, survival and differentiation during development of the neocortex (Komada, 2012). To examine the defect in Shh signaling, we performed *in situ* hybridization using downstream transcription factors: Gli1 and Gli2. Surprisingly, we found the expression of mRNA was not significantly different in Pals1 CKO compared to WT at E14.5 (data not shown).

Next, we examined Notch signaling, as this is another strong signaling pathway involved in many aspects of neocortex development, and therefore an ideal signaling pathway to be affected by Pals1 loss. *In situ* hybridization analyzed Notch1 mRNA expression, and similar to our findings with Shh, there was no significant difference between WT and Pals1 CKO. Since NICD is cleaved upon binding with Notch ligands to activate downstream factors, we also observed NICD protein expression. The intensity of NICD expression was not reduced in the Pals1 CKO (Fig18 A). Antibody staining with Notch downstream transcription factor, Hes1, and *in situ* hybridization using Hes5 ribo-probe also support that there was no significant difference in the protein and mRNA expression between WT and Pals1 CKO, respectively (Fig18 B). Taken together, this evidence suggests that the Notch signaling pathway is not impaired by the deletion of Pals1, leading to the idea that there are other mechanisms affected in Pals1 CKO.

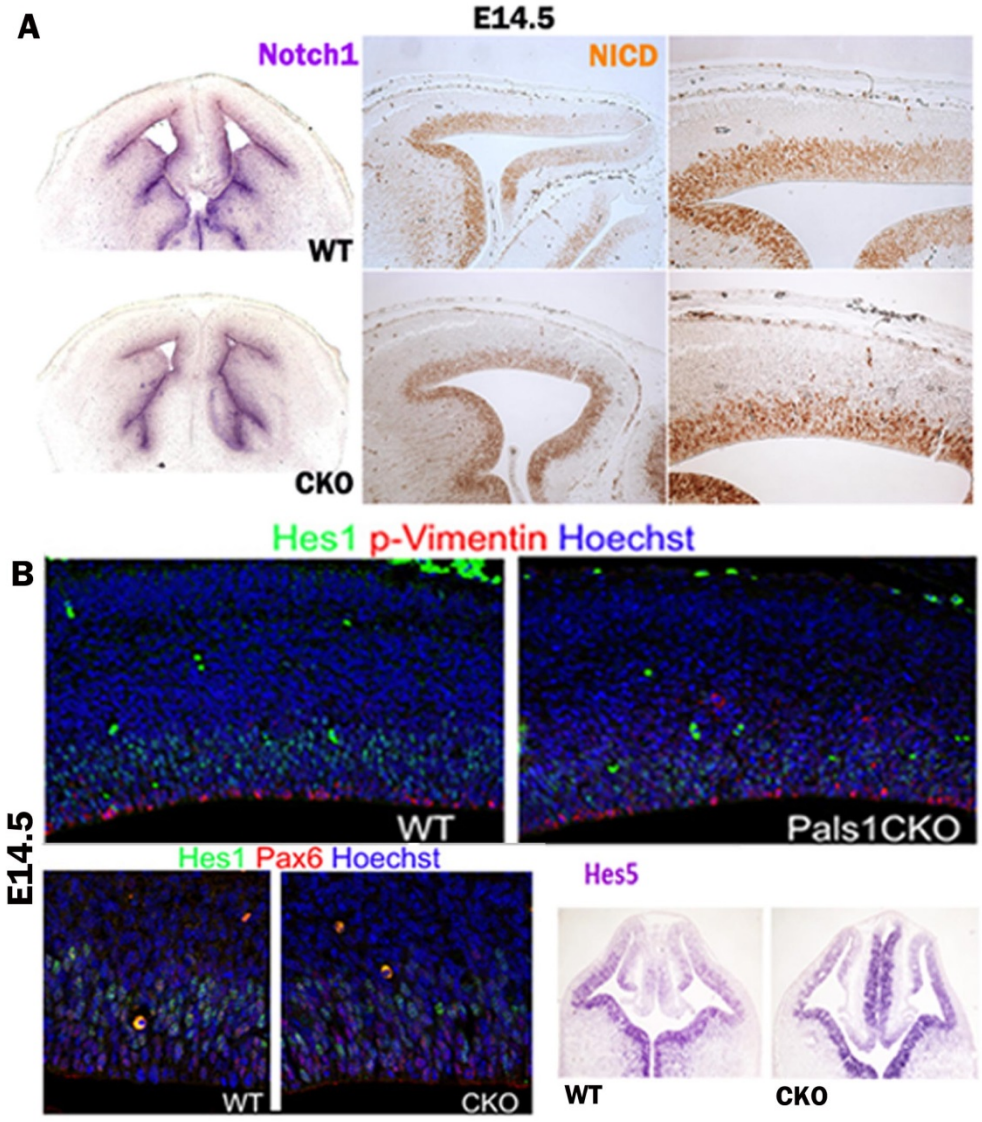


Figure 18. Notch signaling is not significantly defective in Pals1 CKO. (A) Notch1 mRNA and NICD protein expression is not significantly different between WT and CKO. (B) Hes1 antibody staining and Hes5 mRNA expression show that Notch downstream factors are not obviously changed in WT and CKO.

4.2.4 The role of Pals1 in apical complex localization and in the formation of cytoarchitecture of RGP

To gain insight into the role of Pals1 in the apical localization of the apical complex proteins, we observed protein expression at E14.5. In the Pals1 CKO, the localization of Pals1 at the apical junction is completely abolished, and other apical complex proteins such as Par3, aPKC, and Crb are diminished (Fig. 19 A), suggesting that Pals1 is critical component for the maintenance of apical complex proteins. Interestingly, Zo1 which is localized in tight junctions is reduced and disrupted in the Pals1 CKO (Fig. 19 C), whereas adherens junctions stained with β-catenin and N-cadherin are maintained (Fig19 B). Although we do not explain exactly how the tight junction associated molecule Zo1 is mislocalized, a previous study may provide a hint for the answer to this question that Pals1 regulates Zo1 expression. Pals1 is colocalized with Zo1 in MDCK cells, and treatment with siRNA delays the formation of tight junction without affecting adherens junctions when confluent cells are transferred to low calcium medium for dissociation of cell-cell contact (Straight et al., 2004). Another possibility is that the ablation of Pals1 may lose interaction with other apical complex proteins, such as other components of Crb or Par complex proteins, and ultimately lead to the failure of apical protein recruitment to tight junctions for their maintenance. It has been known that the disruption of either Crb or Par complex proteins interfere with recruitment of other proteins to the tight junction (Muller & Wieschaus, 1996). In addition, Par complex proteins themselves are well known to regulate the assembly of tight junctions through phosphorylation by aPKC (Yamanaka *et al.,* 2001 ; Suzuki *et al.,* 2002). The deletion of

Pals1 may disrupt the interaction between Crb and Par complex proteins, thus weaken the assembly and maintenance of tight junctions.

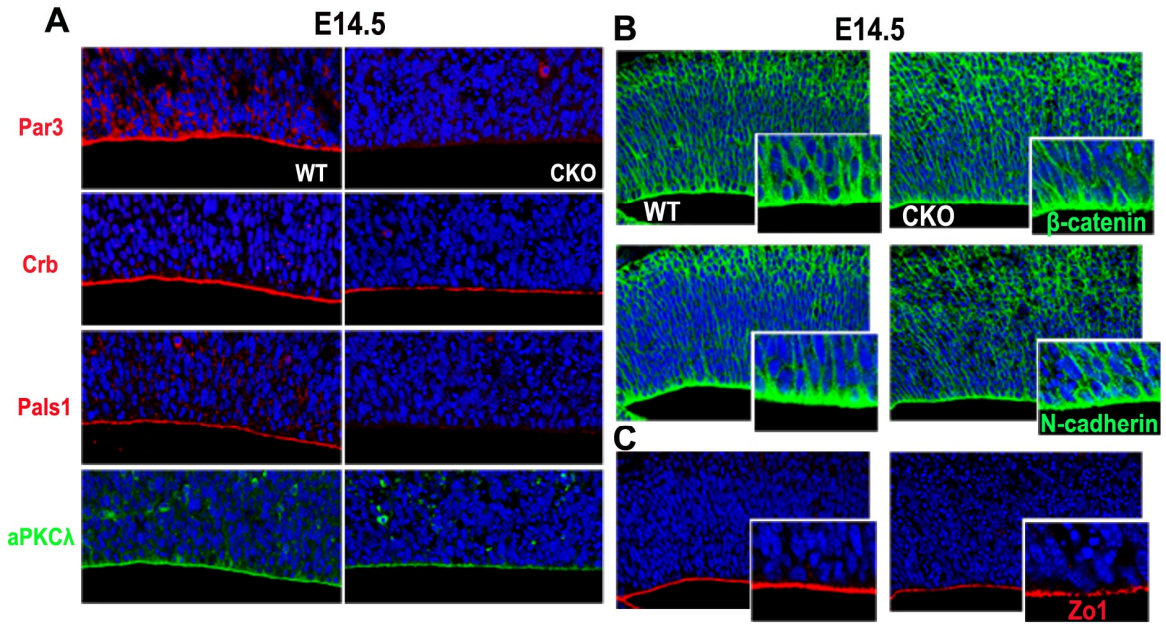


Figure 19. The expression of Crb, Par complex and tight junction molecules are defective in Pals1 CKO. (A) Crb complex proteins, Crb and Pals1, and Par complex proteins, Par3 and aPKCλ , expression is substantially reduced in Pals1 CKO. Furthermore, tight junction molecule, Zo1, shows defective expression in the absence of Pals1 (C), whereas adherens junction molecules, β-catenin and N-cadherin, are not changed in Pals1 CKO compared to WT(B).

Since Pals1 has a critical function in the maintenance of the apical region of the VZ, Pals1 deficient progenitors may show structural disruption of cell morphology. The EM study at E13.5 shows that the RGPs are less tightly bound to apical areas of the developing cortex, and the membrane structure of mitotic progenitors often shows abnormalities characterized by smaller membranes (Fig20 A-D). The mitotic cells in the apical area of the VZ are stained with Phospho-Vimentin and display irregular cell shape (Fig20 E,F), suggesting that Pals1 is involved in the formation of normal membrane structure in dividing cells.

When *in utero* electroporation was performed at E13.5 with a pCAG-GFP construct and analyzed at E14.5, Pals1 deficient RGPs showed disorganized localization in VZ. Furthermore, Pals1 deficient RGPs had irregular shapes and protruded from apical areas to the ventricle. This supports the idea that Pals1 is essential for the formation of normal membrane structure and maintenance of the apical region. The disruption of cell polarity is also demonstrated by the mislocalization of Crb protein marked by pan-Crb antibody (Fig20 G).

Taken together, Pals1 is essential for the maintenance of normal membrane structure, cell shape, polarity, and localization of RGPs in apical area of VZ.

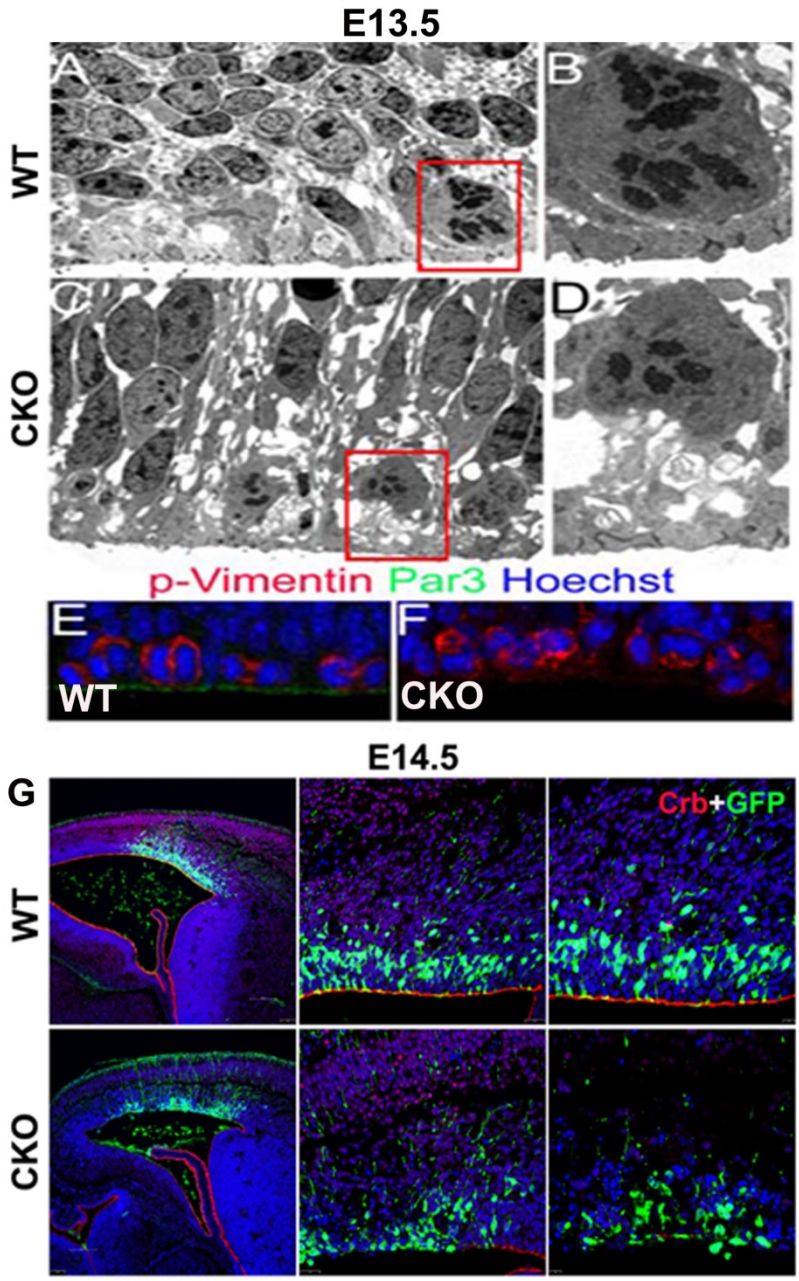


Figure 20. Pals1 is involved in cytoarchitecture of membrane structure of dividing RGPs. Membrane structure in apical area of VZ using EM (A-D) at E13.5 and p-Vimentin staining (E, F) at E13.5 confirms that Pals1 CKO shows abnormal membrane structure in dividing RGPs. (G) *In utero* electroporation with GFP shows that morphological shape of RGPs in the VZ are severely defective in Pas1 CKO.

4.2.5 Pals1 is a major regulator in mitosis

Pals1 expression, identified using antibody staining, shows that Pals1 is intensively localized in the membrane during M-phase, while it is less expressed during interphase in neuroblastoma (N2a) and NIH 3T3 cells (Fig21 A,B,D). The localization of Pals1 partially overlaps with Aurora B, marker for a cleavage furrow, in NIH 3T3 cells (data not shown) and at the apical surface of the VZ (Fig21 C), suggesting that Pals1 may be important for membrane partitioning or for anchoring the mitotic cells at the apical surface during cytokinesis. To prove this hypothesis, we performed electron microscopy and time-lapse imaging analysis. EM images show that a lagging chromosome is observed in Pals1 deficient RGPs in the dividing cells undergoing cytokinesis (Fig21 E). Furthermore, time-lapse imaging analysis after electroporation at E13.5 shows that Pals1 deficient RGPs are clustered and undergoing abnormal division when mice were analyzed at E14.5 (Fig22A,B). Many dying cells, shown as small particles in the image, are also observed.

Together, this evidence suggests that Pals1 function is required for normal mitotic progression and faithful chromosome segregation, which may be directly associated with cell fate changes and precocious cell cycle exit to generate neurons.

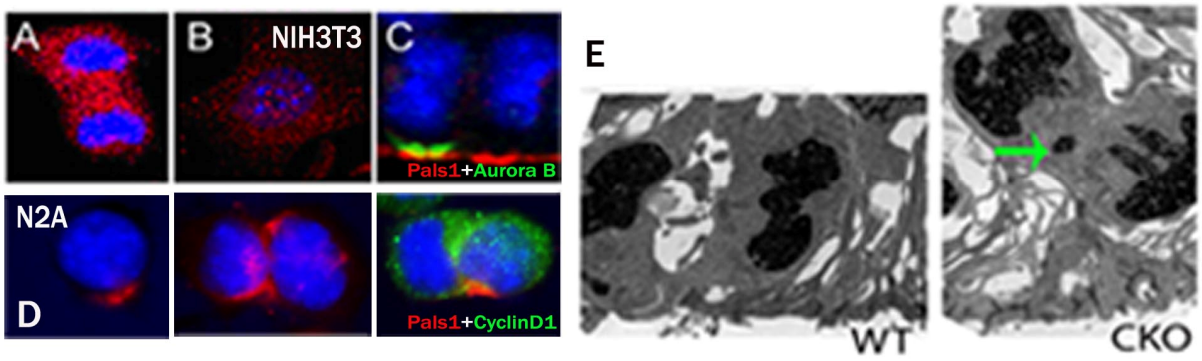


Figure 21. Pals1 is upregulated in cells undergoing mitosis, and a lagging chromosome is identified in Pals1 CKO. (A-D) Pals1 is weakly expressed during interphase (B, D left) but upregulated in dividing cells in NIH3T3 (A), VZ (C) and N2A (D middle and right). (E) EM study shows that chromosomal segregation is abnormal in RGPs of Pals1 CKO.

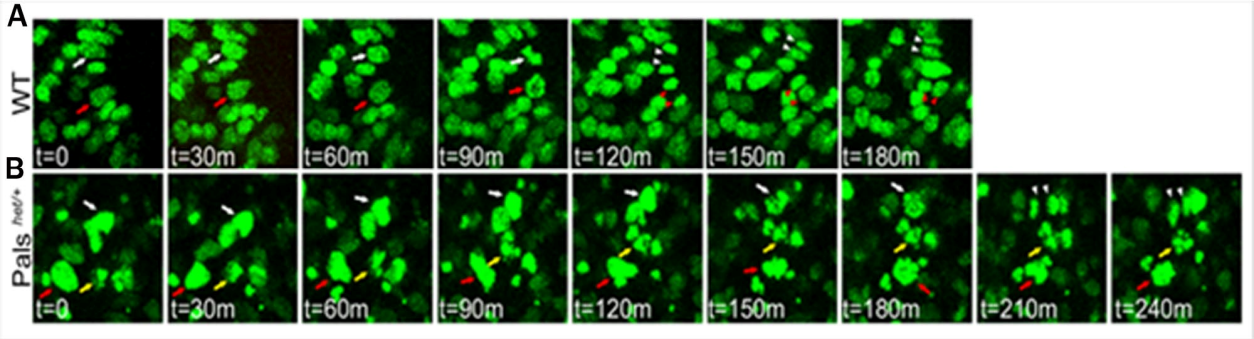


Figure 22. Time lapse confocal images in explant culture identifies that cell division is defective in Pals1 CKO. (A) In WT, dividing cells normally undergo mitosis (red and white arrow).. (B) Different size of daughter cells are generated by mitosis (white arrow), and cells are clustered and do not undergo mitosis (yellow and red arrow).

4.2.6 Pals1 subcellular localization

To study Pals1 function in cell fate determination and mitosis, the subcellular localization of Pals1 is investigated in great detail. A previous study noted that Pals1 is localized at the tight junction with other Crumbs complex partners (Roh et al., 2003). Through the physical interaction with Ezrin, Pals1 is co-localized to the apical membrane of gastric parietal cells (Cao et al., 2005). In our study, Pals1 usually shows high expression in Golgi apparatus marked by GM130, and its localization is almost identical with Par3 localization in NIH3T3 cells (data not shown). As I illustrated in chapter 1, Pals1 has two prospective NLS sequences and one NES sequence. This suggests that we cannot rule out the possibility that Pals1 has a function within the nucleus. For example, *Nagie oko*, the Pals1 ortholog in zebrafish, can be translocated and accumulated in the nucleus when it loses the predicted nuclear export signal; although, it was not identified how *Nagie oko* plays a role in the nucleus (Bit-Avragim et al., 2007). To examine Pals1 subcellular localization, we generated pCAG:Pals1-DsRed and pCAG:Pals1-GFP construct. The overexpression Pals1 with Pals1-GFP construct shows different results depending on cell types. Pals1 is mainly localized in the cytoplasm in HEK293 cells when it is overexpressed (Fig23 A). However, Pals1 is observed in the nuclei of MDCK and MCF7 cells, both of which are known for having epithelial structure (Fig23 B,C), suggesting that Pals1 nuclear localization may be present in epithelial cells. To support Pals1 expression in the nucleus, nuclear fractionation was performed with the cortex lysates at E14.5. Similar to the previous observation, Pals1 protein is identified in both nucleus and cytoplasm (Fig23 D).

A Pals1-GFP construct was electroporated *in utero* to cortical progenitors at E13.5 and harvested after 24 hours. Recapitulated Pals1 protein expression was observed in the cortex and aqueduct, which have epithelial structure, and the GFP expression mainly overlapped with Pals1 protein expression (Fig23 E,F). However, we failed to observe subcellular localization *in vivo*. This may be due to rapid protein degradation or that Pals1 is only localized to the apical tip of progenitors *in vivo*.

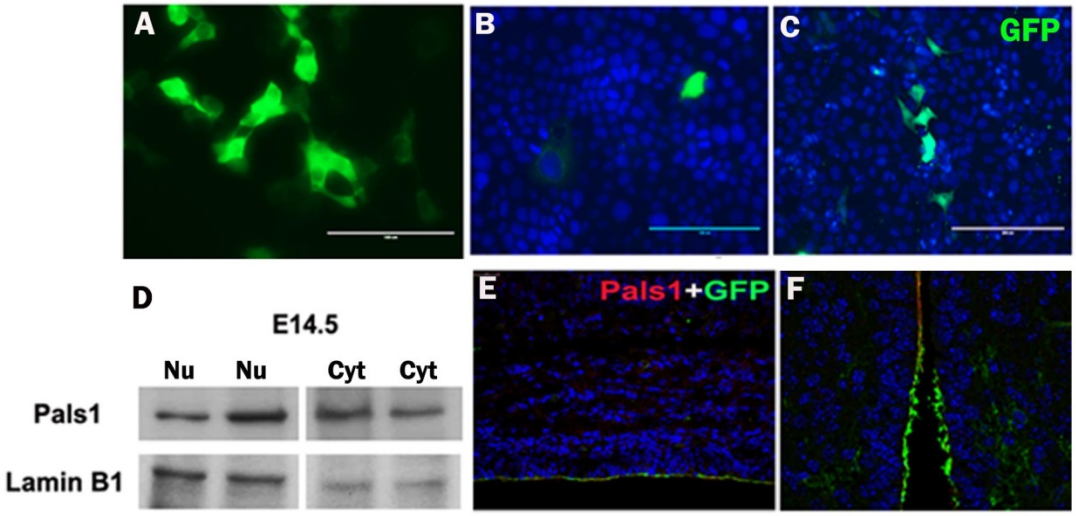


Figure 23. Pals1 subcellular localization in vitro and in vivo. Different cells are transfected with Pals1-GFP fusion construct. Pals1 is mainly localized in the cytoplasm in HEK293 (A), whereas Pals1 is localized in both the nucleus and cytoplasm of MDCK (B) and MCF7 cells (C). (D) Nuclear fractionation shows that Pals1 is also observed in the nucleus. In utero electroporation with Pals1-GFP fusion construct completely recapitulates Pals1 expression in the VZ of cortex (E) and aqueduct (F), which have epithelial structure, but it failed to observe other subcellular localization in the cell besides apical membrane.

4.2.7 Cell autonomous vs. non-autonomous defects in the absence of Pals1

To identify whether the defects in the cortex shown with the removal of Pals1 in RGPs are cell autonomous or non-autonomous defects, we co-electroporated with pCAG-Cre-GFP and pCAG-GFP constructs into homozygote floxed mice and analyzed at two different time stages: E15.5 and E17.5. The depletion of Pals by pCAG-Cre-GFP constructs not only shows complete loss of Pals1 and Crb proteins in the apical tip but also shows apoptotic cell death when mice are analyzed at E15.5 (Fig24 A); although, significant morphological changes in VZ are not observed. To further address the prolonged effect induced by Cre recombination in RGPs, we extended our analysis for two more days. Surprisingly, the electroporated mice at E13.5 recapitulated the severe phenotype that was shown in Pals1 CKO when they were analyzed at E17.5 (Fig24 B,C). The area targeted by Cre-GFP and GFP constructs shows loss of cells in VZ, and the apical surface of the VZ is significantly damaged by the deletion of Pals1. As shown in mice electroporated at E15.5, Pals1 expression is significantly reduced, and GFP positive cells undergoing apoptosis stained with anti-CC3 are also observed in VZ at E17.5. Furthermore, Pax6 positive RGPs are substantially reduced in homozygote floxed mice. Taken together, the deletion of Pals1 represents cell autonomous defects in RGPs.

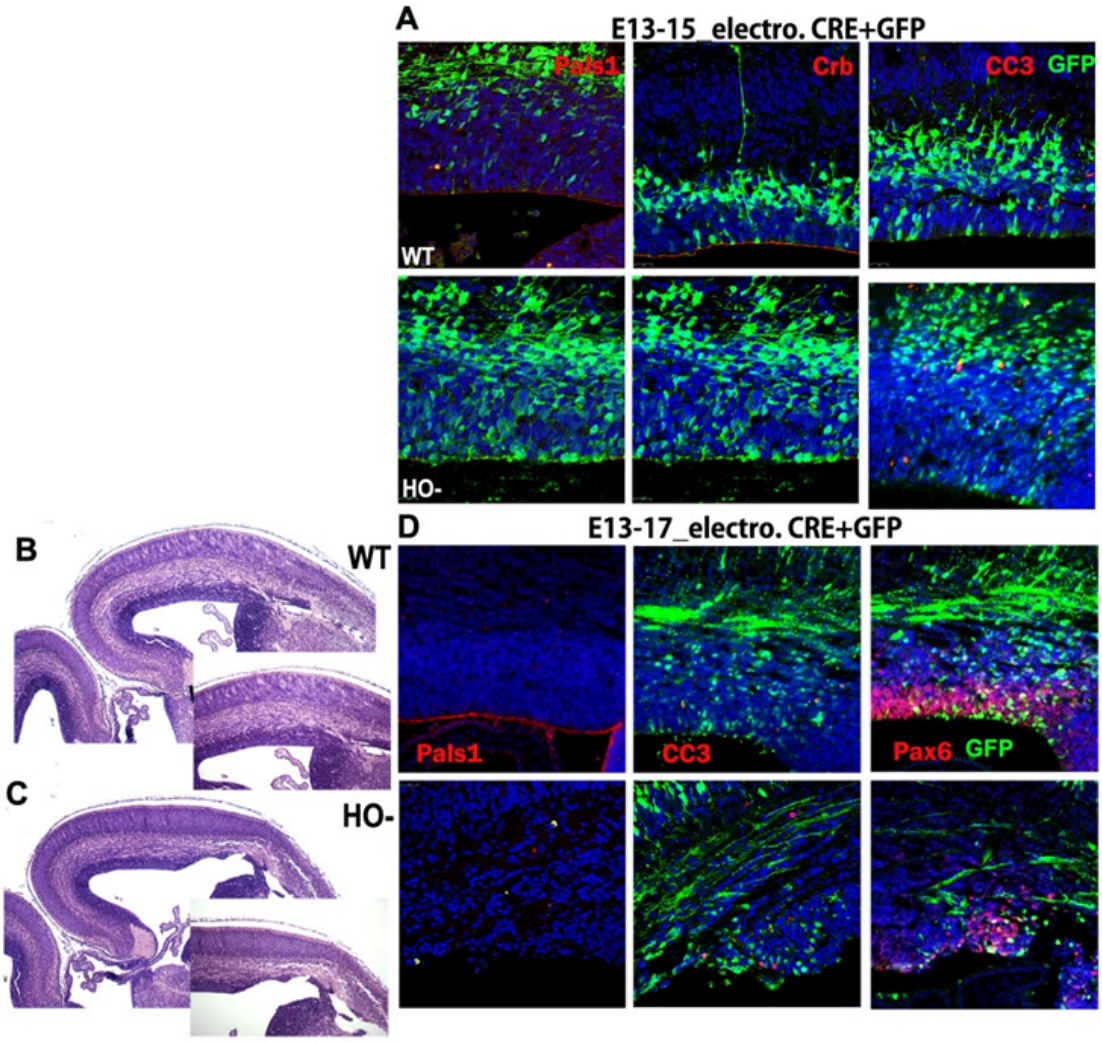


Figure 24. Pals1 induces cell autonomous defects in RGPs. (A) Co-electroporation with pCAG-Cre-GFP and pCAG-GFP constructs in homozygote floxed mice at E13.5 and analysis at E15.5 show that Pals1 deletion not only diminishes apical complex proteins, but also induces apoptotic cell death. (B,C) Four days after co-electroporation results in the depletion of progenitors, similar to phenotype shown in Pals1 CKO. (D) Pals1 is almost completely diminished in the electroporated area, and RGP pool marked by Pax6 is also reduced. of the maintenance of integrity of apical area is markedly compromised. Furthermore, more dying cells are identified at E17.5 in the Cre electroporated area.

4.2.8 Pttg1 functions as a downstream factor of Pals1

To identify major downstream factors regulated by the deletion of Pals1 in the developing cortex, we have performed microarray analysis with extracted RNA from WT and Pals1 CKO cortex. We identified several downstream candidate genes regulated by Pals1. Among these genes, Pttg1 was one of the most downregulated genes in Pals1 CKO. To confirm Pttg1 downregulation in Pals1 CKO, we first checked the protein expression of Pttg1 in Pals1 CKO (data not shown). *In situ* hybridization analysis with Pttg1 ribo-probe at E14.5 shows that Pttg1 mRNA expression is significantly reduced in Pals1 CKO, and its expression overlaps with Pals1 in the neocortex (Fig25 A-B').

To prove the interaction between Pals1 and Pttg1, we hypothesized that Pttg1 transcription will be regulated by Pals1 if Pttg1 acts as a downstream molecule. To answer this question, we *co*-electroporated with pCAG-Pals1 and pCAG-GFP in the cortex at E13.5 and analyzed at E14.5, followed by *in situ* hybridization with Pals1 and Pttg1 ribo-probes. We found that the GFP positive area where Pals1 is overexpressed (Fig25 E,E') shows both Pals1 and Pttg1 transcription level is highly upregulated (Fig25 F-I), suggesting that Pttg1 transcription is affected by Pals1 overexpression. These results provide evidence that Pttg1 is a downstream molecule of Pals1.

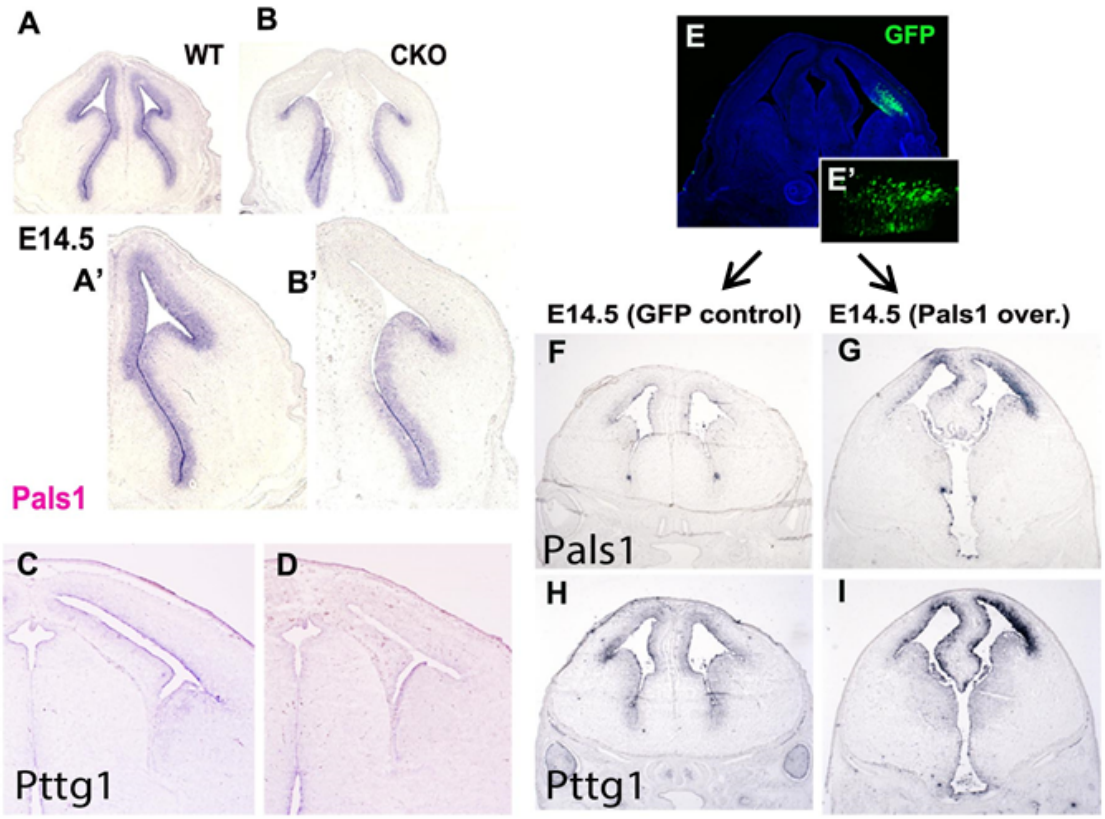


Figure 25. Pttg1 transcription is regulated by Pals. (A,A') In WT, Pals1 mRNA expression is observed in the progenitors of dorsal and ventral cortex germinal zones. However, Pals1 mRNA expression is absent in the dorsal cortex of Pals1 CKO (B,B'), where *hGFAP-Cre* is expressed. (C, D) Pttg1 expression is diminished in the Pals1 CKO(D) as compared to WT (C)(E) Illustration of GFP expression by in utero electroporation. Upregulated Pals1 and Pttg1 transcription in observed in the area, where Pals1 is overexpressed (G, I) compared to the area where GFP control plasmid is electroporated (F,H).

4.2.9 Overexpression of Pttg1 rescues Pals1 deleted phenotype, and Yap may act as a mediator between Pals1 and Pttg1

Based on the previous finding that Pttg1 is a major downstream factor of Pals1, we hypothesized that if Pttg1 is a major downstream factor of Pals1, overexpression of Pttg1 may rescue the phenotype of Pal1 CKO. To address this, we introduced Cre-GFP, pCAG-Pttg1, and pCAG-GFP plasmids in pregnant Pals1 floxed homozygote females using *in utero* electroporation. Strikingly, overexpression of Pttg1 almost completely rescued the phenotype of Pals1 CKO induced by Cre-GFP (Fig26 A), leading to the idea that Pttg1 is a major downstream effector of Pals1 function.

Although we identified that Pttg1 is a major downstream factor of Pals1, we do not know the underlying mechanism or molecules that may be involved in the interaction between Pals1 and Pttg1. Yes-associated protein (Yap) has been known as a transcription co-activator that plays an essential role in organ size control by the regulation of cell proliferation and inhibition of apoptosis. Yap is negatively regulated by the angiomotin (AMOT) family of proteins at tight junctions through direct interaction (Zhao et al., 2011). In addition, knocking down Crumbs3 or Pals1 in high density cells significantly increased nuclear Yap and Taz localization as well as reduced Yap phosphorylation (Varelas et al., 2010). These previous studies suggest that Yap interplays with polarity complex proteins, leading to the idea that Yap may be involved in Pals1-Pttg1 pathway. To test this idea, we first observed Yap protein expression in WT and Pals1 CKOs at E14.5. Surprisingly, Yap, which is mainly localized in the cytoplasm in WT, was translocated into the nucleus in Pals1 CKO (Fig26 C), which corresponds to

the previous observation that Pals1 knockdown in high density cells increased nuclear Yap. In addition, western blot analysis shows that Pttg1 is downregulated when Yap is overexpressed in N2a cells (Fig26 B), suggesting that Pttg1 may also be regulated via Yap. This leads to the idea that Yap may act as a mediator between Pals1 and Pttg1.

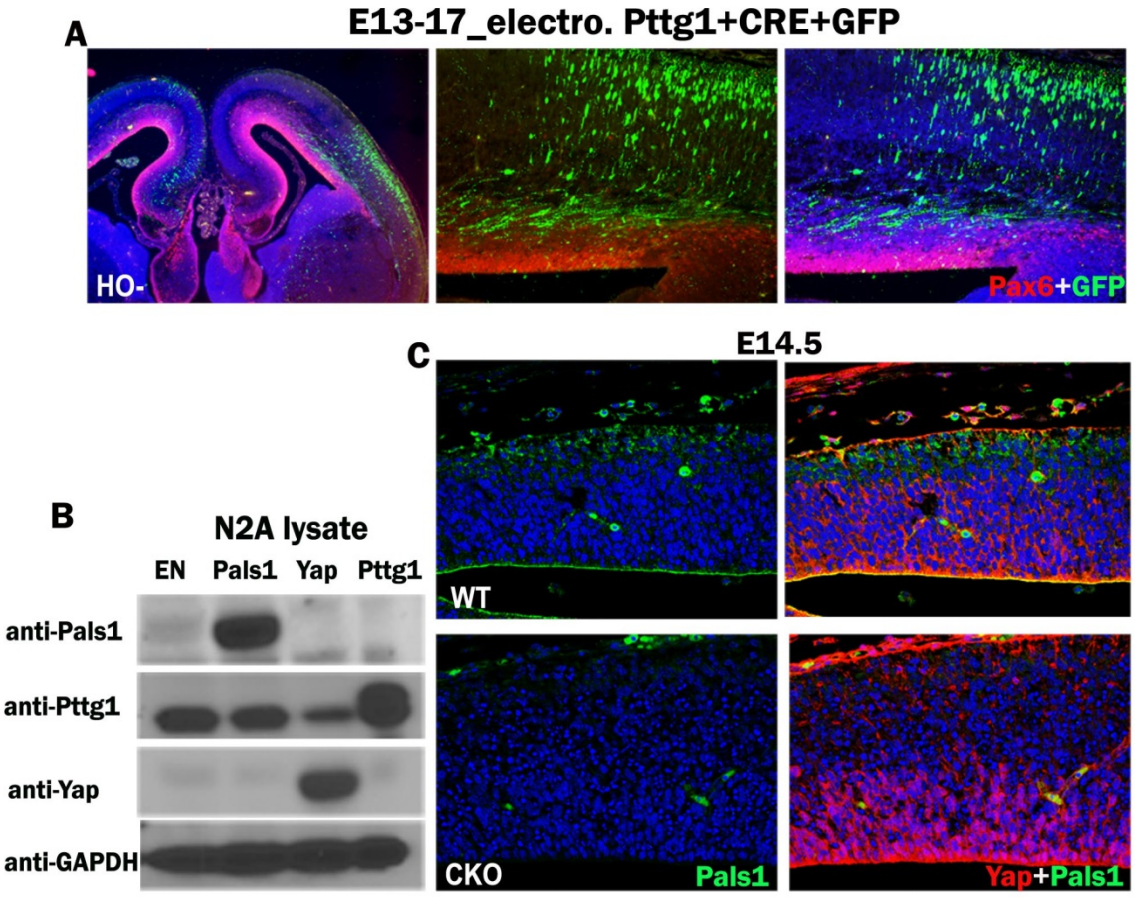


Figure 26. Pttg1 overexpression rescues Pals1 phenotype mediated by Cre recombination in homozygote floxed mice, and Yap may act as a mediator between Pals1 and Pttg1. (A) Depletion of the progenitor phenotype induced by Cre recombination in homozygote floxed Pals1 mice is rescued by Pttg1 overexpression *in vivo*. (B) Yap overexpression induces Pttg1 downregulation in N2A cells, and (C) Yap translocates to the nucleus in the Pals1 mutant.

4.3 Discussion

The primary focus of this chapter was to evaluate the role of Pals1 during neocortical development using Pals1 conditional knockout mice. For the first time, we showed that Pals1 is essential for cortical lamination and RGP maintenance during cortical development. Consistent with the previous study by Kim et al., the Pals1 CKO showed reduced cortical size, progenitor pool, and disrupted cell-cell junctions(Kim et al., 2010), though layer structures are maintained in Pals1;hGFAP-Cre mice.

Second, the most important novel finding with this study is that Pals1 is an essential molecule for mitotic progression. Several studies show that polarity proteins are important for segregation of cell fate determinants, but no study has shown that polarity proteins regulate cell cycle in dividing cells. EM and time-lapse image analysis support the idea that Pals1 is a major regulator that controls mitotic progression in dividing cells. Pals1 deleted cells do not undergo normal mitosis shown in Figure 21 and 22, and this leads to anaphase lag with sister chromatids are not normally segregated. It has been known that the generation of lagging chromosome induces aneuploidy related to genetic disorders (Holland & Cleveland, 2009).Our findings with the Pals1 mutant may be relevant to genetic disorders with abnormalities in the development of the cerebral cortex, which are often associated with severe mental and physical disabilities (Walsh, 1999; Francis, 2006). Among those, microcephaly is a neurodevelopmental disorder characterized by a small cerebral cortex at birth, and the consequences of microcephaly is strongly associated with neurological defects such as mental retardation and seizure (Mochida, 2001; Wood et al., 2005; Cox et al., 2006).

Primary microcephaly is induced by mutations of several centrosomal genes and is believed to arise from an enhanced asymmetric cell divisions that reduce neural progenitor pool for future brain growth (Lu & Jan, 2000). The key players identified thus far are Abnormal Spindle-like Microcephaly-associated protein (ASPM), Cyclin-dependent kinase 5 regulatory subunit associated protein 2 (CDKRAP2), Centromere protein J (CenPJ), and Microcephalin/MCPH1 (autosomal recessive primary microcephaly 1) (Cox et al., 2006; Fish et al., 2006; Paramasivam et al., 2007). These genes are highly expressed in neural progenitors during cortical development, and all of the proteins encoded by these genes are localized to the mitotic apparatus; some of which are known to be essential for the proper cell division of neural progenitors (Fish et al, 2006). The direct interaction between polarity proteins and microcephaly genes has not been addressed, but previous studies suggest that polarity proteins may interact with microcephaly genes via cytoskeleton-related proteins. For instance, Par complex interacts with 14.3.3. protein (Chen & Macara, 2006), Ymo1 with Crb complex (Laprise et al., 2006) and Myosin II for Scrib complex (Strand et al., 1995). These interactions are key to control cell shape and polarity through the modulation of actin cytoskeleton dynamic. A recent study has identified that Asp regulates its polarized distribution along the apico-basal axis in the cell through the interaction with myosin II. Mislocalized myosin II fails to perform proper interkinetic nuclear migration and the formation of proper tissue structure in the depletion of Asp. Thus, Asp is essential for the maintenance of tissue integrity via myosin II mediation to regulate neuroepithelium morphogenesis (Rujano et al., 2013). The known interactions between microcephaly genes, polarity genes and cytoskeleton-related genes leads to the idea that these genes

may all be inter-related to regulate progenitors, and the failure of regulation may induce microcephaly.

Third, another important novel finding is that Pttg1 acts as a downstream factor of Pals1. The loss of Pals1 blocks mammalian target of rapamycin (mTOR) pathway, which is essential for cell survival, and the Pals1 deficient phenotype is partially rescued by mTORC1 activation (Kim et al., 2010). Partial rescue in the Pals1 CKO proposes the possibility that there may be other major downstream molecules or pathways. Pttg1 is an important molecule involved in sister chromatid segregation and cell cycle progression (Yetemian & Craft; 2011). A previous study demonstrated that Pttg1 was downregulated in Crb1 knockout mice, suggesting the interaction with polarity complex proteins (Pavert et al., 2007). Microarray analysis with E11.5 brain tissues shows that Pttg1 is the most downregulated gene in Pals1 CKO. Furthermore, western blot analysis and *in situ* hybridization show that Pttg1 protein in E14.5 cortical lysates and mRNA expression in VZ were significantly reduced in Pals1 CKO. In addition, overexpression with Pals1 in E14.5 by *in utero* electroporation shows that Pttg1 transcription was regulated by Pals1 overexpression (Fig.25). Together, our results strongly suggest Pttg1's direct involvement with Pals1 function in mitotic progression. Our results suggest that the normal expression of Pttg1 is critical for the mitotic progression.

Conversely, overexpression of Pttg1 shows HEK293 and NIH3T3 cells are easily transformed to bigger tumors in nude mice and larger colonies in soft agar assay (Zhang et al., 1999; Pei & Melmed, 1997). Several mechanisms are proposed to identify the function of Pttg1 in cancer development. Pttg1 is able to interact with other

transcription factors such as Sp1, p53, and upstream stimulatory factor 1(USF1), suggesting that Pttg1 may regulate additional genes in cancer development by interacting with other transcription factors (Pei 2001; Bernal et al., 2002); Pttg1 not only binds to the c-Myc promoter to regulate transcription in NIH 3T3 cells (Pei 2001) but also binds to bFGF in NIH3T3, HEK 293, NT-2, JEG-3, and MCF-7 cells through the interaction with Pttg1 *binding factor (*PBF; Chien & Pei, 2000; McCabe et al., 2002). Pttg1 is able to interact with other transcription factors such as Sp1, P53, and upstream stimulatory factor 1(USF1), suggesting that Pttg1 may regulate additional genes in cancer development interacting with other transcription factors (Pei 2001; Bernal et al., 2002; Tong et al., 2007) Because Pals1 deletion prevents Shh signaling mediated tumorigenesis of CGNP as shown in the previous chapter, it is possible that the reduction of Pttg1 may play a role in blocking medulloblastoma. Consistent with this, I also observed Pttg1 reduction in Pals1 deficient cerebellum. It will be important to further explore the Pttg1 involvement in tumourogenesis of medulloblastoma in the future.

Lastly, we found that Yap might act as a mediator between Pals1 and Pttg1. Protein-protein interaction refers to physical contact as a result of biochemical events, which are integral in the organism to form macromolecules to be functional as proteins are rarely act alone. In our study, Yap translocated to the nucleus in Pals1 CKO, and upregulation of Yap induced Pttg1 downregulation in N2a cells. Under this perspective, we may cautiously address the interaction between polarity complex proteins and Hippo signaling pathway. Recent studies have addressed the interaction between several tight junction proteins such as Pals1, Patj, and Lin7C and Yap. These proteins were co-

purified with Yap and their interaction was mediated by Amot family proteins (Zhao et al., 2011). In addition, Merlin, a known tumor suppressor, interacts with Crb complex proteins via direct interaction with Angiomotins (Yi et al. 2011). Thus, it stands to reason that Merlin may activate the Hippo signaling pathway interacting with junctional complex proteins (Yi & Kissil, 2013). In our study, Pals1 has a direct interaction with Yap1 in HEK293 cells (Zhao et al., 2011), and Pals1 deletion induces nuclear translocalization of Yap, whereas overexpression of Yap induces Pttg1 downregulation. Therefore, Pals1 may inhibit Yap translocation to nucleus in the cortical progenitors by direct physical interaction thereby inhibiting Yap activity in the nucleus. Since Pals1 has two NLS and one NES sequence and Yap is a well-known transcription co-factor, it is also possible that their physical interaction may also exist in the nucleus and be important for Pttg1 gene expression. However in this scenario, although excess Yap is translocated to nucleus, it cannot induce Pttg1 transcription without Pals1. Further studies will be required to determine how Pals1 interacts with Yap, and how their interaction regulates the Pttg1 expression level in cortical development.

Chapter Five:

Summary and Future Directions

5. Summary and Future directions

5.1 Summary

The goal my thesis is to understand the cellular and molecular mechanisms underlying cell fate decision between progenitors or progenitor and neuronal cell, as well as identification of unknown molecular partners of Pals1.

In the first part of my research, I started out introducing the roles of Pals1 during cerebellum development, specifically the expression of Pals1 in progenitors, and the effects of Pals1 deletion during the developmental process through use of conditionally deleted Pals1 mice. I uncovered that Pals1 is highly expressed in different types of progenitors, and Pals1 acts as an essential molecule that regulates progenitor pool by the control of cell polarity and mitotic progression. The depletion of Pals1 induces a reduction in the progenitor pool and premature cell cycle exit from the proliferation niche, leading to the significantly reduced cerebellum size in the adult. In addition, relatively intact Shh and Notch signaling downstream molecules are found in Pals1 CKO, supporting that Pals1 acts as an independent factor involving cell fate decision compared to previously known signaling pathways. Finally, I addressed that the loss of Pals1 can block the proliferation of cerebellar granule cell precursors carrying the mutation that causes medulloblastoma through uncontrolled Shh signaling activation.

In the second part of my research, I revealed that Pals1 is essential in proper cell division of RGP during neocortical development. The depletion of Pals1 induced the disruption of cell-cell adhesion and junction defects in VZ. In addition, premature

neurogenesis occurred, and these neurons were removed by apoptosis, leading to the significant reduction of late born neurons.

Third, Pals1 was essential for mitotic progression. The failure of proper chromosome segregation induced lagging chromosome in the Pals1 deficient mitotic cells, confirmed by EM studies. Furthermore, time lapse image also showed that cells underwent aberrant mitosis when they divide in the absence of Pals1.

Lastly, I found Pttg1 as a major downstream molecule that is affected by Pals1 deficiency as Pttg1 transcription was significantly downregulated in Pals1 CKO, and its transcription was regulated by Pals1. Furthermore, I discovered Yap1 as a potential mediator between Pals1 and Pttg1 by demonstrating the translocalization of Yap in Pals1 CKO and downregulation of Pttg1 expression in Yap overexpressed N2a cells. Although I uncovered several critical novel findings for the role of Pals1 in brain development, further functional and molecular studies still needs to be elucidated to expand our study.

5.2 Future directions

The future directions I foresee fall into several steps below:

A. Mechanism study of Pals1-induced Pttg1 expression & novel function of Pals1 in the nucleus
B. Identification of novel binding partners
C. Generation of Pttg1 knockin mouse and rescue experiment

More details will be addressed below.

5.2.1 Mechanism study of Pals1-induced Pttg1 expression & novel function of Pals1 in the nucleus

One question still remaining to be elucidated is how Pals1 regulates Pttg1. Although my thesis presented the idea that Pals1 can control Pttg1 expression at the transcriptional level, it still remains to identify how Pals1 can induce the Pttg1 mRNA expression. To explore further on this, a luciferase assay will be a useful method to monitor Pals1 effects on Pttg1 gene activation. Previous studies identified that serial deletions of the Pttg1 promoter showed that -313bp of the 5'-flanking area, which has several transcription factor binding sites, was essential for promoter activity (Wang and Melmed, 2000). Luciferase activity by overexpression and knockdown of Pals1 using recombinant DNA and shRNA can provide the evidence if Pals1 regulates Pttg1 transcription or not, although further studies are required if upregulated or downregulated Pttg1 promoter activity is directly coming from Pals1 or binding with other factors. Furthermore, domain deleted Pals1 constructs can be transfected or electroporated along with the Pttg1 promoter to assess Pttg1 gene expression, which may provide us information about which domain in Pals1 is critical for the Pttg1 regulation.

Although previous studies failed to address the function of Nagie oko in the nucleus, this study suggested an important point that Pals1 can be translocated in the nucleus. In my thesis, Pals1 was not only identified in the cytoplasm but also highly expressed in the nucleus at E14.5 cortex. This may suggest that Pals1 involved in

transcriptional regulation as a cofactor or transcription factor. By the generation of Pals1 mutants in NES sequence or deletion of L27, which is mediating junctional association through interacting with Patj, I may obtain the restricted localization of Pals1 in the nucleus. Once such constructs are generated, first, I will address the function of Pals1 in the nucleus by rescuing the Pals1 mutant phenotype. If the nucleus function is important in mitotic progression or regulation of Pttg1 expression, the restoration of normal mitosis or Pttg1 expression will be obtained. To further explore Pals1 function in nucleus as cofactor or transcription factor for transcriptional regulation of Pttg1, chromatin immunoprecipitation (ChIP) can be performed with the Pttg1 promoter.

5.2.2 Identification of novel binding partners

The previous studies of Pals1 have identified several binding partners such as Crb, Patj, Yap, and so on. However, there might be several other novel binding partners specifically in the brain. I have generated 1xflag-Pals1 fusion construct and validated with western blot in HEK293 cells and brain lysates after coelectroporation with a GFP construct to identify targeted region. I will pull down the proteins with flag antibody binding (M2) beads (Sigma) after *in utero* electroporation. Using this eluted proteins, mass spectrometry can be performed to identify potential novel binding partners.

5.2.3 Generation of Pttg1 knockin mouse and rescue experiment

Although overexpression of Pttg1 *in utero* by electroporation rescued the phenotype of Pals1 CKO induced by Cre-GFP fusion construct, temporal survey within a limited timeframe hinders more detailed studies during the lifespan. By designing a targeting vector, I will generate a knockin allele to overexpress Pttg1 in mice using Rosa

26 locus with CAG promoter. The stop codon with flanking LoxP sequences will be inserted in front of Pttg1 coding sequence so that its expression would be Cre or CreERT2 dependent. By generating double mutant mice with Pals1 and Pttg1, I will study in depth that Pttg1 rescues the phenotype of Pals1 in other aspects. It would be interesting to determine whether Pttg1 overexpression can rescue the deformed cerebellum where I observed the severe defects in progenitor proliferation in the EGL as well as VZ and URL. However, constitutively activated Pttg1 may cause cancer development, thus it would be important to regulate Pttg1 expression in the limited number of cells /tissue or restricted timing.

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