The tumour suppressor ASPP2 plays a novel role in the maintenance of epithelial cell polarity

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ASPP2 has been identified as a haploinsufficient tumour suppressor in mice, and an activator of the apoptotic function of the p53 family. Yeast two-hybrid experiments have also shown that ASPP2 interacts with a large number of proteins involved in other major signalling pathways. The mechanism(s) of action of ASPP2 are therefore complex, and likely to involve more than just the stimulation of the apoptotic programme.

Since a study previously conducted in our laboratory revealed that the deletion of *ASPP2 in vivo* leads to severe hydrocephalus in the J129/C57BL6 background (Vives et al., 2006), it can be hypothesised that ASPP2 safeguards the normal development of the mammalian central nervous system (CNS), in addition to its role as a tumour suppressor. Deletion of ASPP2 leads to the development of hydrocephalus, most probably by affecting tight junctions (TJs) in the choroid plexus, thereby impairing its blood-cerebrospinal fluid (CSF) barrier function. TJ defects are likely to be the underlying cause of the loss of cell polarity observed in the neuroepithelium of several areas of the CNS. As cell polarity plays a key role in multiple aspects of CNS development, ASPP2 appears to be required for the proper lamination of the cerebral cortex and retina.

In addition to a loss of polarity, *ASPP2* mutant neural stem cells (NSCs) exhibit increased proliferation accompanied by a shortening of the cell cycle, and form neuroblastic rosettes which resemble primitive neuroectodermal tumours. Lack of differentiation in the neo and postnatal cerebral cortex is also a feature of *ASPP2* mutant mice. The ability of ASPP2 to inhibit the transcription of Notch target genes provides a novel mechanism by which ASPP2 may inhibit cell growth and promote differentiation. Since several tumour suppressors have been identified to be major players in cell polarity, and a loss of polarisation is a hallmark of cancer, the ability of ASPP2 to maintain cell polarity may further contribute towards its tumour suppressive function.

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Abbreviations

AJs	Adherens junctions
AJC	Apical junctional complex
CNS	Central nervous system
CP	Choroid plexus
CP	Cortical plate
CSF	Cerebrospinal fluid
GCL	Ganglionic cell layer
GNP	Granule neuron precursors
H&E	Hematoxylin and eosin
IHC	Immunohistochemiistry
INL	Inner nuclear layer
INM	Interkinetic nuclear migration
IPL	Inner plexiform layer
IZ	Intermediate zone
NICD	Notch intracellular domain
NSCs	Neural stem cells
ONL	Outer nuclear layer
OPL	Outer plexiform layer
P-H3	Pospho histone3
PKEC	Primary kidney epithelial cells
PP1	Protein posphatase 1
RGCs	Retinal ganglionic cells

SVZ Subventricular zone

TEM Transmission electron microscopy

TJs Tight junctions

VZ Ventricular zone

1. Introduction

1.1 The p53 family

The tumour suppressor p53, first identified in 1979 (Lane and Crawford, 1979; Linzer et al., 1979), plays a pivotal role in controlling cell growth through its ability to induce cell cycle arrest or apoptosis in response to multiple stress signals (Levine, 1997). These functions are mediated by its ability to activate the transcription of target genes like *p21* (cell cycle arrest) (el-Deiry et al., 1994) or *Puma* (apoptosis) (Nakano and Vousden, 2001).

Two further p53-related genes, p63 and p73, were identified over a decade after p53's discovery (Kaghad et al., 1997; Osada et al., 1998; Trink et al., 1998; Yang et al., 1998). Although p63 and p73 are similarly able to cause cell cycle arrest or apoptosis they are not functionally redundant with p53, as indicated by knock-out mice studies (Yang et al., 1999a; Yang et al., 2000), and each protein in the p53 family has a unique function.

1.1.1 Isoforms of the p53 family members

The p53, p63 and p73 genes are located on chromosomes 17 (17p13.1), 3 (3q27-29) and 1 (1q36) respectively, and exist as several splice variants (Figure 1.1) (Murray-Zmijewski et al., 2006). All three genes encode two primary transcripts that are controlled by separate promoters (P1 and P2). Transcripts generated from the P1 promoter produce proteins that contain the transactivation domain (TAD), DNA-binding domain (DBD) and oligomerisation domain (OD) (TAp53, TAp63 and TAp73). In contrast, transcripts generated from the P2 promoter do not have the TAD (Δ133p53, ΔNp63 and ΔNp73) and have a dominant negative effect, as they are able to occupy promoter binding sites without activating transcription. For both p53 and p73, additional ΔN variants can be generated by

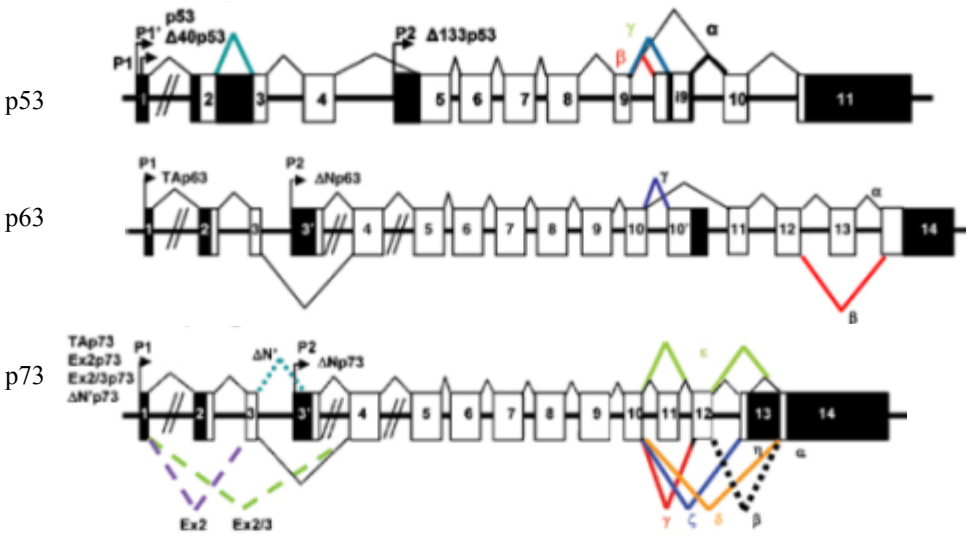


Figure 1.1: The p53 family. Schematic diagram of the gene structures of human p53, p63 and p73. Alternative splicing (α, β, γ, δ, ε, ζ) and alternative promoters (P1, P2 and P1') are indicated.

alternative splicing at the N-terminus or alternative initiation of translation (Δ40p53, Δex2p73 and Δex2/3p73). Further complexity is added by the fact that the P1 and P2 transcripts can be spliced at the C-terminus. For p53 and p63, six isoforms have been described: three TA variants and three ΔN variants. For p73, a total of eight different isoforms from the P1 and P2 promoters have been identified to date.

1.1.2 The p53 family is a key mediator of the apoptotic response

Apoptosis is the process of programmed cell death, which plays a critical role in the maintenance of tissue homeostasis and control of cell number during development. The integrity of apoptotic mechanism is, therefore, critical in normal tissues and the failure of this process is implicated in many pathological conditions such as cancer, anomalies during development, neurodegeneration and autoimmune disease.

Two main apoptototic pathways have been identified in mammals: the extrinsic or receptor-mediated pathway; and the intrinsic or mitochondrial-mediated pathway. Both

pathways activate caspases (Cysteine ASPartyl ProteASEs), which are the final executors of the apoptotic process. The extrinsic pathway involves the activation of a death receptor, which belongs to the tumour necrosis factor family, through the formation of the death-inducing-signalling-complex (DISC) and subsequent activation of Caspase-8 and Caspase-3 (Ashkenazi and Dixit, 1998). The intrinsic pathway is triggered in response to DNA damage, and is associated with mitochondrial depolarisation and a release of cytochrome c from the mitochondrial intermembrane space into the cytoplasm. Cytochrome c, apoptotic protease-activating factor 1 (APAF-1) and procaspase-9 then form a complex termed the apoptosome, in which caspase-9 is activated and promotes activation of caspase-3, caspase-6 and caspase-7 (Nicholson and Thornberry, 2003). The release of cytochrome c from the mitochondrion is regulated, in part, by Bcl2 family members, with anti-apoptotic (Bcl2/ Bcl-XL/Mcl1) and pro-apoptotic (Bax, Bak, tBid, NOXA and PUMA) members inhibiting or promoting its release respectively (Cory and Adams, 2002).

1.1.2.1 p53

The level of p53 within the cell is usually kept low under physiological conditions, mainly by MDM2-catalysed poly-ubiquitylation and subsequent degradation by the proteasome. Upon DNA damage, stress and chronic mitogenic stimulation, the p53 level increases, followed by its transient stabilisation and activation. Several post-translational modifications like acetylation, phosphorylation, methylation, neddylation and sumoylation are involved in the activation and stabilisation of p53 (Bode and Dong, 2004; Toledo and Wahl, 2006). A small subset of these modifications are specifically linked to p53's apoptotic functions, as they increase its ability to transactivate pro-apototic target

genes. p53 target genes include components of both the extrinsic or intrinsic apoptotic pathways (Haupt et al., 2003). For instance, p53 can induce the expression of the death receptor Fas (Muller et al., 1998). Moreover, the expression of several members of the Bcl-2 family of proteins like NOXA and PUMA can be regulated by p53 (Oda et al., 2000; Yu et al., 2001). Several studies indicate that p53 can also induce apoptosis by transcription-independent mechanisms (Caelles et al., 1994; Gao and Tsuchida, 1999). Indeed, p53 can translocate to the mitochondria and directly activate components of the apoptotic machinery (Ding and Fisher, 1998; Ding et al., 2000).

1.1.2.2 p63 and p73

Similar to p53, cellular protein levels of p63 and p73 increase upon treatment with DNA damaging agents (Bergamaschi et al., 2004; Katoh et al., 2000; Okada et al., 2002). p73 is also activated in response to the oncogenes E1A and Myc (Zaika et al., 2001). Once activated, p63 and p73 can regulate induction of apoptosis primarily by their ability to transcriptionally activate pro-apoptotic p53 target genes (Gressner et al., 2005; Muller et al., 2005). While no transcription independent mechanism for p63 induced-apoptosis has been identified to date, recent evidence suggest that p73 can induce apoptosis by the mitochondrial pathways (Melino et al., 2004; Sayan et al., 2008).

1.1.3 The p53 family induces cell cycle arrest

All of the three p53 family members can induce cell cycle arrest at G1 and G2 following DNA damage. This event is driven by activation of the transcription of the cyclin-dependent kinase (CDK) inhibitor p21WAF1/CIP1, which is mediated by p53, p63

and p73 (Costanzo et al., 2002; Dohn et al., 2001; el-Deiry et al., 1994). CDKs and their corresponding cyclin proteins are key regulators of cell cycle progression. p21WAF1/CIP1 inhibits the activity of several CDKs, including cyclinE-CDK2 and cyclinA-CDK2, and has been shown to be needed for the activation of cyclinD-CDK4/6 (Sugimoto et al., 2002).

1.1.4 The p53 family and tumour suppression

Aberrant cell survival, which occurs as a consequence of failure of the apoptotic programme, plays a vital role in tumour development. Although recent studies are providing new mechanistic insights into the role of p53, its ability to induce apoptosis represents the major pathway by which p53 acts as a tumour suppressor. Emerging evidence suggest that p63 and p73 also exert their functions as tumour suppressors by mediating the apoptotic response in various cellular contexts.

1.1.4.1 p53

p53 function is often lost in cancer by various mechanisms involving mutations within the TP53 gene, or in regulators of p53 like the ubiquitin ligase MDM2 (Vousden and Lu, 2002). TP53 is mutated in half of human tumours, with 95% of mutations occurring in the central region of the gene, which is responsible for sequence-specific DNA binding. The early onset of tumours in p53 knock-out mice further supports a central role for p53 in tumour suppression (Donehower et al., 1992). Germline p53 mutations are also found in Li-Fraumeni syndrome, a tumour-prone disorder predisposing affected individuals to specific tumour types (Malkin et al., 1992).

Evidence derived from the study of tumours expressing mutated p53 shows that p53 mainly exerts its tumour suppressor function by inducing apoptosis (Ryan and Vousden, 1998). Recently, a new mechanism by which p53 can function as a tumour suppressor came to light. Loss of p53 has been shown to favour the expansion of cancer stem cells (CSCs), a small population of stem cells identified in human cancers which are believed to drive and maintain tumour growth (Huntly and Gilliland, 2005). CSCs, like normal stem cells, have two main modes of cell division: symmetric and asymmetric. Symmetric divisions generate two cells that are destined to acquire the same fate, while asymmetric divisions generate one daughter cell with a stem cell fate and another that differentiates (Morrison and Kimble, 2006). Stem cells undergo symmetric division in order to expand the stem cell pool in the early stages of development, and later switch to asymmetric division to produce differentiated cells. A failure of asymmetric division has been linked to the development of tumours in several *in vivo* models. An analysis of primary cells derived from a p53-/- mammary gland revealed that loss of p53 leads to a switching of cell division from asymmetric to symmetric, causing an expansion of the mammary CSC population, and therefore driving tumourigenesis (Cicalese et al., 2009) .

1.1.4.2 p63 and p73

Although rarely mutated, p63 and p73 are abnormally expressed in human cancers (Muller et al., 2006). ΔNp63 and ΔNp73, which exert a dominant negative function, have been found to be upregulated in a multitude of different cancers. For instance, ΔNp63 was upregulated in head and neck squamous cell carcinoma (Dominguez et al., 2006; Wager et al., 2006; Weber et al., 2002), while ΔNp73 was overexpressed in gliomas as well as

colon carcinoma (Dominguez et al., 2006; Wager et al., 2006). In contrast, TAp63 and TAp73 are lost or downregulated in human tumours (Corn et al., 1999; Urist et al., 2002).

Research on the predisposition of p63 and p73-deficient mice to tumour development has generated conflicting results. Two independent studies have shown that p73 knock-out mice and p63 heterozygous mice do not develop spontaneous tumours (Keyes et al., 2006; Yang et al., 2000). However, analysis of tumour onset in aging p63 and p73-deficient mice suggested that p63 and p73 heterozygous mice develop spontaneous tumours, similar to p53 heterozygous mice (Flores et al., 2005). In addition, double mutants heterozygous mice (p53 +/-; p73 +/- and p53 +/-; p63 +/-) displayed a higher tumour incidence and increase in metastasis, compared to the p53 +/- mice therefore suggesting that p63 and p73 cooperates with p53 in tumour suppression. Furthermore, mice deficient for the TA isoforms of p73 are predisposed to spontaneous as well as carcinogenic induced tumourogenesis, indicating that TA p73, like p53, acts as a tumour suppressor (Tomasini et al., 2008).

1.1.5 The p53 family in normal development

Although the p53 family plays a pivotal role in the stress response through its ability to induce cell cycle arrest or apoptosis, recent evidence suggests that in physiological conditions the p53 family members are also necessary for normal cell function. p53 controls the expression of genes involved in a vast number of cellular processes like cell adhesion and motility, angiogenesis and translation (Riley et al., 2008), suggesting that its biological functions in the absence of stress go beyond its ability to induce apoptosis

1.1.5.1 p53

The observation that p53 negatively regulates the proliferation and survival of both adult and embryonic neural stem cells points at a role for p53 in controlling stem cell function (Armesilla-Diaz et al., 2009; Meletis et al., 2006). Many studies have demonstrated that expression of p53 in undifferentiated cells can promote differentiation (Almog and Rotter, 1997; Lin et al., 2005), suggesting that p53 not only controls cell renewal, but also cell fate. One mechanism by which p53 can affect cell fate is the regulation of asymmetric cell division of stem cells (Cicalese et al., 2009).

Studies conducted in p53-deficient mice have shown that p53 is required for normal embryonic development (Armstrong et al., 1995; Sah et al., 1995). A significant proportion of female p53 knock-out mice die during embryogenesis because of developmental abnormalities. 23% of p53 knock-out embryos exhibit craniofacial malformation and exencephaly, which results from failure of the neural tube to close properly. The observation that this phenotype is common to other proteins involved in the intrinsic apoptotic pathway, such as Apaf-1 (Cecconi et al., 1998; Yoshida et al., 1998) and Caspase-3 (Kuida et al., 1996), lead to the theory that exencephaly results from an absence of cell death in the developing neural tube. Interestingly, no significant difference in the rate of apoptotic death was observed when p53-null embryos were compared to wild-type littermates. Moreover, recent evidence derived from the treatment of embryos in culture with inhibitors of apoptosis suggests that the entire sequence of neural tube closure events occurs in the absence of cell death (Massa et al., 2009). Therefore, other cellular functions of p53 might explain the neuronal phenotype observed in p53-null embryos. In addition to its role in neurulation, p53 plays an important pro-apoptotic role in sympathetic neurons of the superior cervical ganglion (SCG) (Slack et al., 1996).

1.1.5.2 p63 and p73

Genetic experiments on mice have shown that p63 and p73 have a more profound effect on normal development than p53, and p63 deletion has the most striking effect. In mice lacking p63, all squamous epithelia and their derivatives are missing, limb formation is impaired and the mice die shortly after birth (Mills et al., 1999; Yang et al., 1999a). Complementation studies in p63-deficient mice have shown that both TAp63 and DNp63 are required for normal epithelial development (Candi et al., 2006). ΔNp63 controls the expansion of the progenitor pool, while TAp63 controls their differentiation. Consistent with the role of p63 in epithelial morphogenesis and limb development, p63 germ line mutations are found in humans and cause six autosomal dominant developmental diseases that recapitulate, less severely, the phenotype of p63-deficient mice (Celli et al., 1999). Although p73-deficient mice are more viable than p63 knock-out animals, they exhibit profound defects including hippocampal dysgenesis, hydrocephalus, chronic infections and inflammation, and abnormalities in the pheromone sensory pathway (Yang et al., 2000).

Evidence coming from cell culture systems, as well as genetically modified mice, has shown that both p63 and p73 play a role in the development of the central nervous system (CNS). TAp63 is an important pro-apoptotic factor in developing neurons, and p63-deficient mice display a defect in sympathetic neuron death (Jacobs et al., 2005). ΔNp73, the main isoform expressed in developing neurons, plays a pro-survival role by opposing the apoptotic functions of TAp63 and p53 (Pozniak et al., 2000). In addition, ΔNp73 isoforms exerts similar function in cortical neurons and their deletion causes a gradual loss of cortical neurons after birth in mice (Pozniak et al., 2002).

p73 has also been implicated in the differentiation of neuroblastoma cells. TAp73 levels rise in neuroblastoma cells that are induced to differentiate by retinoic acid

treatment, and TAp73 expression induces neuronal differentiation (De Laurenzi et al., 2000). A recent study suggests that TAp73 may support differentiation by antagonising Notch signalling in neuroblastoma cells and primary neurons (Hooper et al., 2006).

The role of p63 and p73 in the regulation of neural precursor cell survival has recently been investigated by Dugani and colleagues (Dugani et al., 2009). This study revealed that knock down of p63 causes apoptosis of cortical precursor cells without affecting their proliferation. The apoptosis induced by p63 knock down could be rescued by overexpression of DNp63, but not TAp63 therefore suggesting that the former promotes the survival of cortical precursor cells. Interestingly, in this study ΔNp73 did exhibit similar function in cortical precursor cells.

1.2 The ASPPs: new co-activators of the p53 family

The factors that help the p53 family members decide how to respond to particular cellular stresses are still largely unknown. Various post-translational modifications of p53 seem to influence its ability to decide between cell cycle arrest and apoptosis. Moreover, interaction with p63 and p73 selectively enhances the activation of apoptotic genes mediated by p53 (Flores et al., 2002). The ASPPs provide another level of regulation for the selectivity of the p53 family response.

1.2.1 Identification of the ASPP family

The ASPP (Apoptosis Stimulating Protein of p53) family is constituted by 3 members: ASPP1, ASPP2 and iASPP. (Figure 1.2). The inhibitory form, iASPP, is the most

conserved member and is the only one expressed in *C. elegans* (Bergamaschi et al., 2003b). Mammals have evolved two additional members: ASPP1 and ASPP2. The ASPP family members are characterised by a highly conserved carboxyl-terminal end, which is composed of 3 domains: ankyrin repeat, an SH3 domain and a proline-rich region. Thus, ASPP also stands for Ankyrin repeats, SH3 domain, Proline-rich region Protein. The amino-terminal end is only conserved in ASPP1 and ASPP2.

ASSP2 was the first member of the family to be identified. In a yeast two-hybrid screen of a transformed B cell cDNA library, using mouse p53 with a truncated amino-terminus (residues 73-390) as bait, two proteins were identified: 53BP1 and 53BP2 (Iwabuchi et al., 1994). The two proteins were 1027 and 529 amino acids long and had no sequence similarity. Only 53BP2 contained the ASPP signature, while 53BP1 was unrelated to the ASPP family. 53BP2 was also identified as a Bcl-2 interacting protein in a yeast two-hybrid screen of a human Epstein-Barr virus-transformed B cell cDNA library, using Bcl-2 as bait (residue 1-188). Its cDNA was cloned and found to encode a protein of 1005 amino acids, which was originally believed to correspond to full-length ASPP2 (Naumovski and Cleary, 1996). The full-length protein was, in fact, only recently identified as being 1128 amino acids long (Samuels-Lev et al., 2001). ASPP2 exits as two splice variants derived from alternative splicing of exon3, called 53BP2S (Bbp) or 53BP2L (ASPP2) which are 1005 and 10028 amino acids long respectively (Takahashi et al., 2004).

ASPP1 and iASPP were identified in a human genome screen using the Ankiryn repeat, proline-rich region and SH3 domain as a signature. iASPP was originally reported to be an inhibitor of RElA/p65 (RAI), and 351 amino acids in length (Yang et al., 1999b). Subsequent studies have identified full length RAI to be 828 amino acids long, and an

inhibitor of p53 in *C. elegans* (Bergamaschi et al., 2003b). Since *C. elegans* does not express RelA/p65, the protein was renamed iASPP for its inhibitory function.

1.2.2 Functions of the ASPP family

The most well known function of the ASPP family of proteins is its ability to regulate apoptosis mediated by the p53 family (Bergamaschi et al., 2004). ASPP1 and ASPP2 enhance the ability of p53 to specifically stimulate the expression of pro-apoptotic target genes, but not genes involved in cell cycle arrest (Bergamaschi et al., 2004; Samuels-Lev et al., 2001).

The crystal structure of the complex between the C-terminus of ASPP2 and the DNA core binding domain of p53 reveals that the third and fourth ankyrin repeats, and the SH3 domain of ASPP2, interacts with p53 (Gorina and Pavletich, 1996). Although not directly involved in p53 binding, the N- terminal domain of ASPP2 is required for maximal transactivational activity. 53BP2 (Bpb) is, in fact, less active than the corresponding full length proteins in stimulating p53-dependent apoptosis (Samuels-Lev et al., 2001).

Besides its role in controlling the activity of ASPP1 and ASPP2, the N-terminus also seems to play a role in determining their cellular localisation. While full length ASPP1 and ASPP2 are predominantly cytoplasmic, removal of the N-terminal half of ASPP2 causes its C-terminus, which contains a nuclear localisation signal (Sachdev et al., 1998), to localise to the nucleus (Samuels-Lev et al., 2001). A recent study has shown that the N-terminus of ASPP2 has an ubiquitin-like structure which can mediate its interaction with other components of the apoptotic network, therefore regulating the pro-apoptotic activity of ASPP1 and ASPP2 (Tidow et al., 2007).

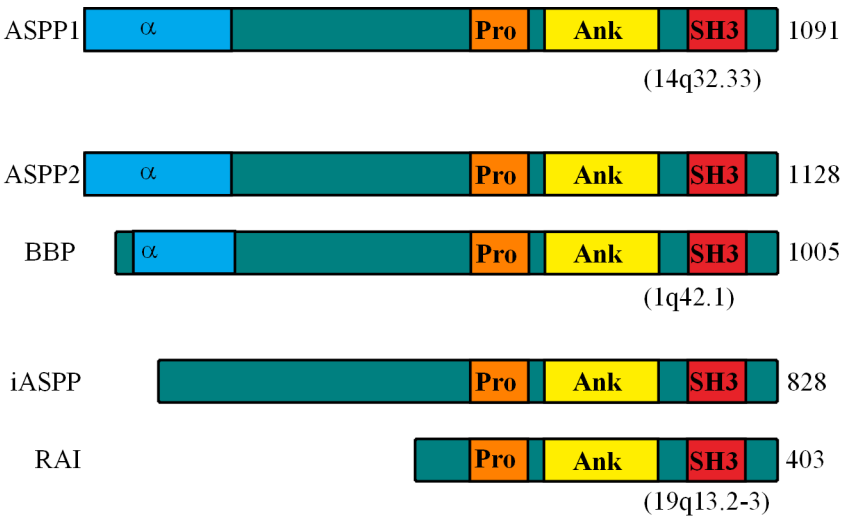


Figure 1.2: The human ASPP family. Schematic representation of the protein modular structure of the ASPP family members. The amino acid lengths of each ASPP member and splice variants are indicated on the right. The chromosomal locus for each gene is indicated in brackets. α: helical domain; Pro: proline rich region; Ank: ankyrin repeats; SH3: SH3 domain.

The N-terminus is not conserved in iASPP, which is the inhibitory member of the ASPP family. The short form of iASPP (RAI) corresponds to the C-terminal part of full-length iASPP as well as Ce-iASPP (*C. elegans* iASPP), and has an inhibitory effect on p53-mediated apoptosis (Bergamaschi et al., 2003b). Full-length iASPP has a similar effect on p53-dependent apoptosis, even if is less effective that the short form RAI (Slee et al., 2004). This may be explained by the fact that full-length iASPP is mainly cytoplasmic, while RAI is almost exclusively nuclear.

The crystal structure of the C-terminus of iASPP with p53's DNA binding domain revealed a similar interaction to the one described for ASPP2, suggesting that iASPP might simply function as a competitive inhibitor of ASPP1/2 (Robinson et al., 2008). A more recent characterisation of the p53-ASPP complex identified a new region of p53, outside the DNA binding domain, that interacts exclusively with iASPP, thus providing a

new mechanism by which iASPP can interfere with the apoptotic function of p53 (Ahn et al., 2009).

The ankyrin repeats and SH3 domain also mediate interactions between the ASPPs and binding partners other than members of the p53 family. In the last ten years, several new ASPP interacting proteins have been identified including protein phosphatase 1 (Helps et al., 1995), p53 (Gorina and Pavletich, 1996), RelA/p65 (Yang et al., 1999b), adenomatous polyposis coli-like (Nakagawa et al., 2000), YES-associated protein 1 (Espanel and Sudol, 2001), amyloid-precursor protein-binding protein 1 (Chen et al., 2003a), the hepatitis-C core protein (Cao et al., 2004), SAM68 (Thornton et al., 2006) and the DEAD protein Ddx42p (Uhlmann-Schiffler et al., 2009). A study recently carried out in *Drosophila* has also identified ASPP2 as a partner of dCsk (C terminal Src kinase) (Langton et al., 2007).

1.2.3 Regulation of ASPP expression

Like other tumour suppressor genes, ASPP1 and ASPP2 can be epigenetically silenced by aberrant methylation of their promoter. A recent study showed that ASPP1 and ASPP2 genes are frequently down-regulated by DNA methylation in hepatitis B virus (HBV) - positive hepatocellular carcinoma (Zhao et al., 2010) ASPP1 expression is also reduced in acute lymphoblastic leukaemia by a similar mechanism (Agirre et al., 2006). In addition to gene silencing by methylation, ASPP1 and ASPP2 levels can also be modulated by E2F, a downstream target of the tumour suppressor retinoblastoma (Rb). ASPP1 and ASPP2 have been identified as transcriptional targets of E2F and an increase in E2F-1 is able to induce their expression (Fogal et al., 2005). Moreover, treatment of cells with chemotherapeutic agents able to inhibit the proteasome increase ASPP2 protein levels (Zhu et al., 2005).

1.2.4 The ASPP family in tumour suppression

Considering the key role of the ASPP family members in regulating the apoptotic process, it is logical to expect that these proteins also have an important role in tumourigenesis. A study conducted in our laboratory on ASPP2 knock-out mice has suggested that ASPP2 plays a central role in tumour suppression (Vives et al., 2006). ASPP2 knock-out mice exhibit postnatal lethality. In addition, ASPP2 heterozygous mice have 45% tumour incidence in their life span, while that of wild-type mice is 15%. Compared to wild-type mice, the heterozygous mice develop twice as many spontaneous lymphomas, rhabdomyosarcomas and squamous cell carcinomas. The ASPP2 heterozygous mice also have an increased susceptibility to the onset of tumour development in response to ionising radiation. Consistent with the role of ASPP2 in regulating p53-mediated apoptosis, a combination of p53 and ASPP2 heterozygosity accelerates the onset of tumour development. A recent study using an ASPP2 knock-out generated in another laboratory also confirmed that ASPP2 is a tumour suppressor *in vivo*, since ASPP2 heterozygous mice showed increased incidence of spontaneous, as well as induced, tumours (Kampa et al., 2009).

A recent study conducted in *Drosophila* (Langton et al., 2007) has shown a novel function for dASPP in modulating the activity of dCSK (C-terminal Src kinase), which maintains Src in an active state by inhibitory phosphorylation. dASPP mutants had enlarged wings and eye, suggesting a role for dASPP in controlling cell growth and possibly acting as a tumour suppressor.

The expression of the ASPP family members is also altered in a variety of human cancers. The expression of ASPP1 and ASPP2 is frequently down-regulated in human breast tumours (Samuels-Lev et al., 2001) and tumour cell lines (Liu et al., 2005)

expressing wild-type p53, suggesting that there may be a selective advantage for loss of ASPP1 and ASPP2 expression. Reduced levels of ASPP2 have also been detected in human lung cancer cell lines (Mori et al., 2000), and ASPP1 down-regulation was observed in one non-small cell lung carcinoma cell line and two mesothelioma cell lines (Mori et al., 2004). Furthermore, in leukaemic cell lines, a concomitant downregulation of ASPP1 with an upregulation of iASPP has been observed, suggesting that abnormal ASPP expression could be an important step in the formation of human haematopoietic neoplasms and might be a useful molecular marker for the diagnosis of leukaemia (Liu et al., 2004). Significantly higher levels of iASPP were detected in human breast carcinomas, (Bergamaschi et al., 2003a) leukaemic cell lines and acute leukaemias (AL) (Zhang et al., 2005). Other studies have linked reduced ASPP2 levels with poor prognosis and metastasis. Lowered ASPP2 expression was linked to poor clinical outcome in diffuse large B-cell lymphoma (Lossos et al., 2002), and with poor distant recurrence-free survival in breast cancer patients (Cobleigh et al., 2005). Reduced expression of ASPP2 has also been observed in a microarray study of metastatic breast cancer samples compared to non-metastatic samples (Sgroi et al., 1999), suggesting that ASPP2 might be associated with breast cancer progression.

1.2.5 The ASPP family in normal development

The ASPPs have been identified as binding partners of several other proteins in addition to p53, p63 and p73, and play a role in normal development. Studies conducted in our laboratory have shown that the tumour suppressor ASPP2 is a key regulator of mouse CNS development. ASPP2 transcripts are strongly expressed in the brain, spinal cord and optic cup from E9.5 onwards (V. Vives, personal communication). In a mixed

129/C57BL6 background, a majority of ASPP2-null mice died around birth. The ones that survived were runty with a dome-shaped head, and died around weaning of hydrocephalus (V. Vives *et al*., 2006). Moreover, about 20% of 129SvxC57BL/6J ASPP2-null embryos exhibited neural tube closure defects (NTD) at E9.5 (V. Vives, personal communication). Further evidence of potential involvement of ASPP2 in neural development comes from the identification of ASPP2 as a negative regulator of APP-BP1, a binding partner of the amyloid precursor protein and activator of the ubiquitin-like protein NEDD8 (neural precursor cell expressed, developmentally down-regulated 8) (Chen et al., 2003b).

Studies with ASPP1 and iASPP-deficient mice have shown that the function of the three ASPP family members is not redundant during development, and that each model has a unique phenotype. iASPP knock-out mice generated in our laboratory display cardiomyopathy and skin abnormalities (M. Notari, personal communication). A gene trap expression screen identified ASPP1 as an endothelial specific gene functioning during mouse embryogenesis (Hirashima et al., 2004). Subsequent studies have shown that ASPP1 is required for the initial assembly and function of the lymphatic vasculature, independent of p53 (Hirashima et al., 2008).

1.3 Cell polarity

Polarity is a feature of epithelial cells that is defined by an apical surface facing the external environment, which is separated from the basolateral surface by the apical junctional complex (AJC) constituted by adherens (AJ) and tight (TJ) junctions. The integrity of the AJC is critical for the establishment and maintenance of cell polarity in epithelial tissues. In addition to the AJC, three groups of proteins play a central role in cell polarity: PAR (PARtioning defective), Crumbs (CRB) which are localised apically, and Scribble (SCRIB) which is localised basolaterally (Figure 1.3). Cell polarity mechanisms have an intimate relationship with the AJC, and the activity of polarity complexes is required for its maintenance. Cell polarity controls cell differentiation, proliferation and morphogenesis in both unicellular and multicellular organisms.

1.3.1 The AJC: adherens and tight junctions

AJ and TJ provide adhesive contacts between neighbouring cells in epithelial tissues. Classic cadherins are the major transmembrane proteins of the AJ (Figure 1.4). The cadherin family comprises E, N, P and R-cadherin, which are all single-pass transmembrane proteins characterised by the presence of five extracellular cadherin (EC) repeat domains. The interaction between the EC domains is responsible for the initiation of weak cell-cell adhesion and subsequent AJ formation (Halbleib and Nelson, 2006). The cytoplasmic tail of E-cadherin has two well-defined catenin binding domains (CBD), one juxtamembrane domain (JMD) that binds p120, and a second domain in the C-terminus that interacts with β-catenin (Aberle et al., 1994; Yap et al., 1998). Associations of p120 with the JMD of cadherins stabilise cadherin at the plasma membrane, increasing the

adhesiveness between cells (Thoreson et al., 2000). The binding between β-catenin and cadherin is crucial for full adhesive function. β-catenin protects the cytoplasmic domain of cadherin from proteolysis *in vitro* and is also required for the exit of cadherin from the endoplasmic reticulum (Chen et al., 1999; Huber et al., 2001). β-catenin also interacts with α-catenin which, thanks to its actin binding domain, is able to switch between the AJ and the actin cytoskeleton (Drees et al., 2005; Rimm et al., 1995) and is crucial for actin polymerisation near AJs *in vivo* (Vasioukhin et al., 2000).

In addition to controlling cell-cell adhesion, TJs have two main functions: a fence function which prevents mixing between the apical and basolateral domain; and a gate function which controls the paracellular passage of ions and solutes between cells. TJs are multiprotein complexes composed of three major types of integral membrane protein: the IgG-like family of junctional adhesion molecules (JAMs), and the claudin and occludin families (Figure 1.5) (Aijaz et al., 2006). JAMs can be engaged in homophilic and heterophilic adhesion but do not induce the formation of tight junctional strands when expressed in fibroblasts (Ebnet et al., 2004). Occludins are not essential for TJ formation, but have been implicated in regulating their permeability properties (Balda et al., 2000). In contrast, claudins play a crucial role in TJ assembly. No direct interactions have been found between occludins, claudins, and JAMs, however, an important group of TJ scaffolding molecules able to connect these major components are the zonula occludens proteins ZO-1, ZO-2, and ZO-3. These proteins belong to the membrane-associated guanylate kinase-like homologue family and are characterised by three N-terminal PDZ domains, and an SH3 domain followed by a GUK domain. These proteins can interact directly with occludin and claudins via their PDZ domains, whereas their C-termini can associate with actin, thus providing a direct link with the cytoskeleton (Schneeberger and

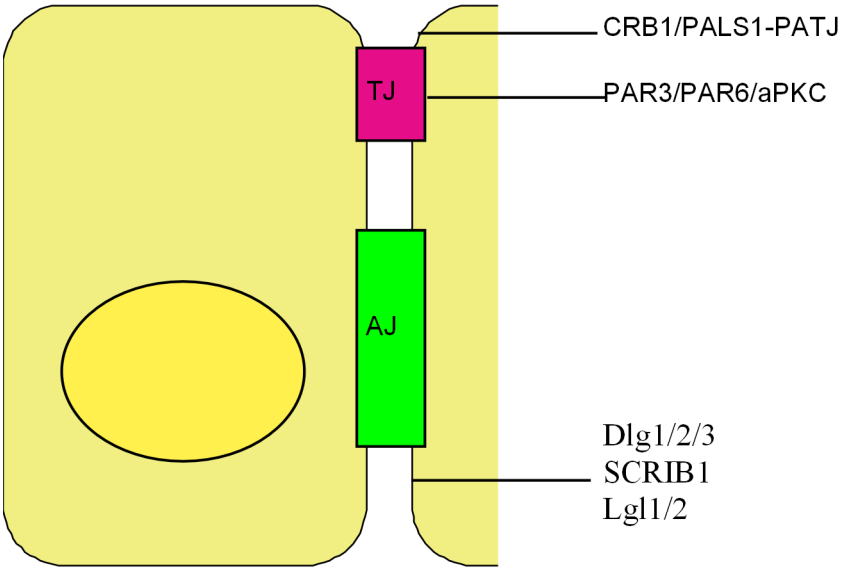


Figure 1.3: Distribution of polarity proteins in mammalian epithelial cells. The PAR and CRUMBS polarity complexes localises apically and colocalise with TJs, while the Scribble complex is localised basolaterally. αPKC: atypical protein kinase C; CRB1: Crumbs homologue-1; Dlg1/2/3: Discs large homologue-1/2/3; Lgl1/2: Lethal giant larvae homologue-1/2; PALS1: protein associated with LIN7; PATJ: PALS1-associated tight-junction protein; SCRIB1: Scribble homologue 1; TJ: tight junctions; AJ: adherens junctions.

Lynch, 2004). Moreover, ZO-1 is also able to interact with α-catenin, thereby connecting AJs and TJs (Itoh et al., 1997).

1.3.2 The PAR complex

The PAR proteins (name derived from "partitioning defective") were first identified in a *C. elegans* screen for mutants that were defective in the anterior posterior partitioning of proteins in the early embryo (Kemphues et al., 1988). The six PAR proteins identified have since been found to be conserved from *C. elegans* to *X. laevis* to mammals. The Par complex includes two of these proteins, PAR3 and PAR6, the serine/threonine kinase

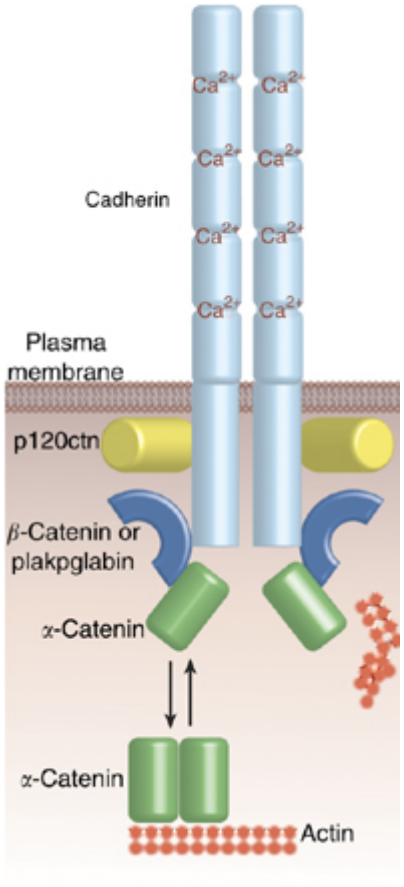


Figure 1.4: Core components and organisation of adherens junctions. Cadherins mediate Ca^{2+}-dependent intercellular adhesion via the extracellular repeat domains. Cadherins form a basic complex with the catenins, α-, β-, and p120 catenin (p120ctn). Both p120ctn and β-catenin bind directly to the cytoplasmic tail of cadherin, whereas α-catenin connects via β-catenin. Plakoglobin, also known γ-catenin, is a close relative of β-catenin. α-catenin is able to bind β-catenin or actin and connects AJs with the cytoskeleton. Reproduced, with permission, from Niessen, 2007. © 2007, Nature Publishing Group.

aPKC (atypical protein kinase C) and small GTPase-like Cdc42 and Rac1 (Joberty et al., 2000; Johnson, 1999; Lin et al., 2000).

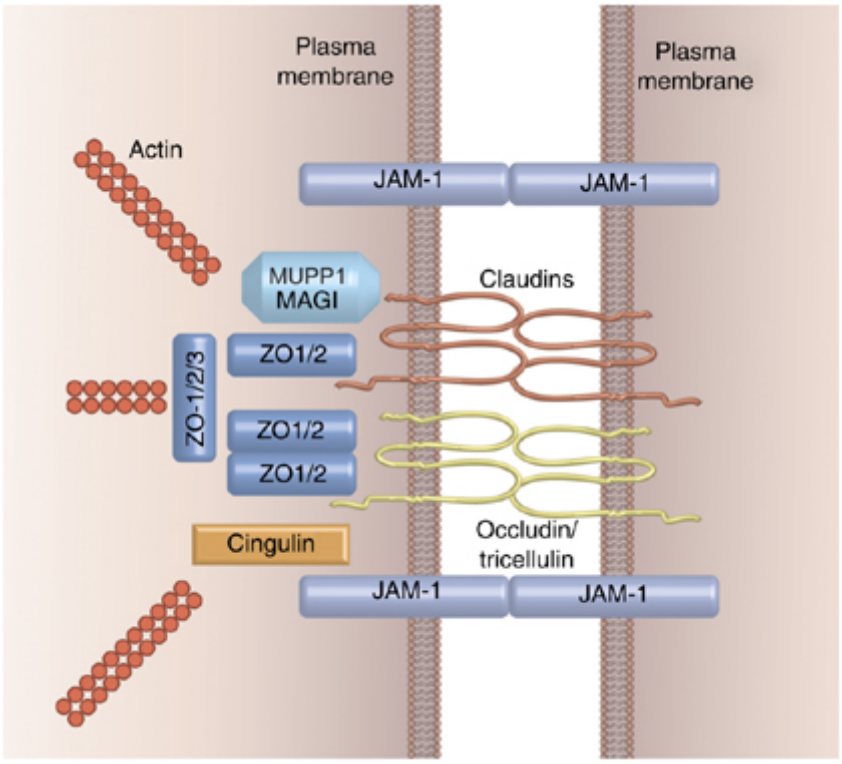


Figure 1.5: Structural organisation of tight junctions. TJs are composed of three main transmembrane proteins: junctional adhesion molecules (JAMs), occludin and claudin. ZO-1 and ZO-2 are important for clustering of claudins and occludin, resulting in the formation of tight junctional strands. ZO-1 and ZO-2 together with other scaffold proteins like ZO3 , MUPP1, MAG1 and cingulin connect claudin and occludin to the actin cytoskeleton. Reproduced, with permission, from Niessen, 2007. © 2007, Nature Publishing Group.

1.3.2.1 aPKC

In mammals, two aPKC isoforms of 75 KDa have been identified: aPKCë/é and aPKCæ (Selbie et al., 1993). Unlike conventional PKCs, aPKCλ/ι and aPKCζ have a PB1 domain at the N-terminus which is known to interact with PAR6 (Figure 1.6). The catalytic domain present in the C-terminal region phosphorylates several proteins such as PAR3 (Nagai-Tamai et al., 2002) and Lgl (Plant et al., 2003). The phosphorylation of PAR3 at S827 by aPKC leads to its accumulation at TJs (Nagai-Tamai et al., 2002).

Both isoforms are expressed in the lungs and brain, while aPKCæ is also expressed in kidney and testis. aPKCs localise with other member of the PAR complex at TJs in MDCK epithelial cells (Izumi et al., 1998). The kinase activity of PKCs is required for TJ assembly, as expression of aPKC kinase-dead mutants blocks their formation and causes mislocalisation of PAR3 and PAR6 (Suzuki et al., 2001). Several studies have shown that the interaction between PAR6 and aPKC is constitutive in epithelial cells (Noda et al., 2001). aPKC activity is repressed by its association with PAR6, and this inhibition is partially released by Cdc42, which binds to the CRIB domain of PAR6 (Garrard et al., 2003; Yamanaka et al., 2001). Cdc42 is also fundamental for the correct localisation of the PAR6/aPKC complex in MDCK cells (Martin-Belmonte et al., 2007).

1.3.2.2 PAR6

Three PAR6 proteins of similar molecular weight (37 KDa) have been identified in mammals: Par6A/C, PAR6B and PAR6D/G. All three proteins contain conserved domains that mediate their interaction with other members of the complex (Figure 1.6). A Phox/Bem1 domain (PB1), located in the N-terminus, binds to other PB1-domain–containing proteins like aPKC. The adjacent Cdc42/Rac interaction binding (CRIB) motifs binds to the Cdc42 or Rac GTPases only when in their active, GTP-bound state (Bose and Wrana, 2006). Finally, the PDZ domain binds to PAR3 and other polarity proteins like Lgl and CRB (Lemmers et al., 2004; Plant et al., 2003). The three PAR6 proteins have different tissue expression patterns and subcellular localisations. PAR6G is widely expressed, while PAR6A and B show more restricted expression patterns. In MDCK cells, PAR6A is present in both TJs and the cytosol, whereas PAR6B is localised to the cytosol and PAR6G predominately to the TJs (Gao and Macara, 2004). In

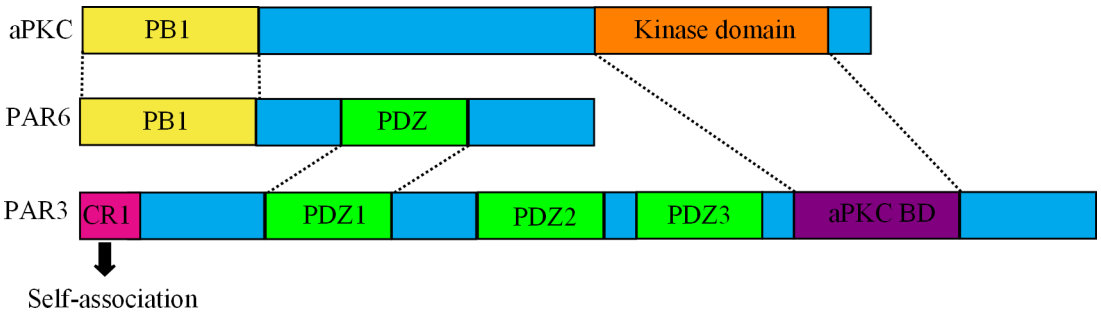


Figure 1.6: The PAR complex. Domain organisation of PAR6, PAR3 and aPKC. The amino-terminal conserved region of PAR3 (CR1) is required for oligomerisation of PAR3. Connecting lines indicate regions of the protein that interact with one another. aPKC BD: aPKC binding domain.

mammalian epithelial cells, overexpression of PAR6B delays the formation of TJs, and when PAR6B is expressed in cells already polarised, the TJs are disrupted. (Gao et al., 2002a; Hurd et al., 2003). Since PAR6 does not have any enzymatic activity, its function consists of bringing together several proteins at specific cellular locations, enabling the interaction of aPKC with its downstream effectors such as PAR3 and Lgl (Yamanaka et al., 2003).

1.3.2.3 PAR3

Two PAR3 genes, PAR3A and PAR3B, have been identified in mammals. PAR3A exists as three different isoforms with different molecular weights (180KDa, 150KDa and 100 KDa respectively), all of which contain three PDZ domains (Figure 1.6). Only the shortest form lacks the aPKC binding domain. The first PDZ domain of PAR3A interacts with PAR6 (Lin et al., 2000). Although it has a similar structure to PAR3A, PAR3B does not bind aPKC and its interaction with PAR6 is still not understood (Gao et al., 2002b).

PAR3A shows high levels of expression in the heart, kidney and brain, while PAR3B is predominantly expressed in kidney, lung and skeletal muscle (Lin et al., 2000). Both PAR3A and PAR3B can colocalise with ZO-1 at TJs (Izumi et al., 1998; Kohjima et al., 2002). PAR3A has been extensively studied in epithelial cells, forming a dimer via its N-terminal region, and this association is required for correct PAR3 localisation during epithelial polarisation (Mizuno et al., 2003). PAR3A is recruited to the junctional complex through direct interaction with JAM (junction adhesion molecule) at an early stage in the junction formation process (Itoh et al., 2001). PAR3 then acts as a scaffold in the recruitment of proteins involved in junction formation, like PAR6 and aPKC. Depletion or overexpression of PAR3A in epithelial cells subsequently causes disruption of TJs and mislocalisation of PAR6 and aPKC (Izumi et al., 1998; Joberty et al., 2000; Mizuno et al., 2003). Interestingly, PAR3 can regulate TJ assembly by controlling the activity or localisation of the Rac exchange factor Tiam1, independently of PAR6 and aPKC (Chen and Macara, 2005).

1.3.3 The CRB complex

The Crumbs complex was originally identified in epithelia in *D. melanogaster*, and subsequently in vertebrates. Three genes encoding transmembrane proteins have been identified in mammals, called CRB1, 2 and 3. PALS1 is a scaffold protein that mediates the interaction between CRB and the third member of the family, PATJ (Assemat et al., 2008). The Crumbs complex is located at the apical/lateral boundary in both flies and mammalian epithelia (Macara, 2004). All the three components of the Crumbs complex play a role in the formation of TJs. Knock-down of PALS1 in MDCK cells leads to TJ and polarity defects (Straight et al., 2004), while overexpression or down-regulation of PATJ

disrupts the localisation of TJ markers such as ZO-1, ZO-3 and occludin (Michel et al., 2005; Shin et al., 2005).

1.3.4 The SCRIB complex

The Scribble complex comprises three proteins: Scribble, Discs Large (Dlg) and Lethal giant larva (Lgl). There are five close mammalian homologues of *Drosophila* Dlg (Dlg1-5), two of Lgl (Lgl1-2) and only a single Scribble homologue in higher vertebrates (Assemat et al., 2008). Scribble and Lgl co-localise at the lateral membranes of Drosophila and mammalian epithelial cells (Bilder and Perrimon, 2000; Navarro et al., 2005; Reuver and Garner, 1998). Similarly, Lgl is localised basolaterally in epithelial cells (Musch et al., 2002). SCRIB seems to play a role in TJ assembly, since its suppression in MDCK cells leads to a delay in their formation and defects in epithelial morphology (Qin et al., 2005). Interestingly, Lgl1 and Dlg5 interact with the plasma membrane protein t-SNARE syntaxin-4, and may contribute to cell polarity by regulating targeted membrane delivery of adhesion molecules like N-cadherin and β-catenin (Musch et al., 2002; Nechiporuk et al., 2007).

1.3.5 Functional interaction between polarity complexes

The formation of a polarised epithelial cell layer requires the coordination of the activity of the three polarity complexes (Figure 1.7). A connection between these three complexes was first shown using genetic studies in flies and biochemical studies in mammalian cell cultures. In order to establish and maintain cell polarity, it is essential that these different complexes are asymmetrically distributed across the cell, with the CRB and

PAR complexes restricted to the apical region of the lateral membrane, and the SCRIB complex concentrated on the lateral membrane. Studies on *Drosophila* epithelia have shown that SCRIB restricts the PAR complex to the apical membrane, where the PAR complex recruits the CRB complex and the two act to exclude SCRIB from the apical membrane (Bilder et al., 2003; Tanentzapf and Tepass, 2003). This spatial segregation relies on the biochemical interaction of the SCRIB and PAR complexes through Lgl. For example, in the *Drosophila* ectoderm, phosphorylation by aPKC is required to prevent the localisation of Lgl to the apical cortex (Hutterer et al., 2004). Similarly, in mammalian cells, the phosphorylation of Lgl by aPKC leads to its dissociation from the PAR6/aPKC dimer followed by its distribution to the lateral membrane (Plant et al., 2003). aPKC is then able to phosphorylate PAR3, allowing the formation of the active PAR complex (Hirose et al., 2002; Izumi et al., 1998). aPKC also phosphorylates another member of the PAR family, the serine-threonine kinase PAR-1, which is asymmetrically localised in the lateral domain of polarised epithelial cells (Figure 1.8) (Bohm et al., 1997). The phosphorylation of PAR1 by aPKC in the apical domain permits the binding of PAR5, which induces the release of PAR1 into the cytoplasm (Hurov et al., 2004; Suzuki et al., 2001). This chain of events keeps PAR1 from the apical surface in mammalian epithelial cells. In *Drosophila* epithelia, the presence of Par1 at the lateral domain can phosphorylate any PAR3 molecules that diffuse into its territory (Benton and St Johnston, 2003). The binding of phosphorylated PAR3 with PAR5 triggers the release of PAR3 from the cell cortex, keeping PAR3 from the lateral domain. CRB is also a substrate for aPKC and its phosphorylation is important for apical-basal polarisation in *Drosophila* (Sotillos et al., 2004). Furthermore, CRB3 binds to PAR3 to promote the differentiation of premature

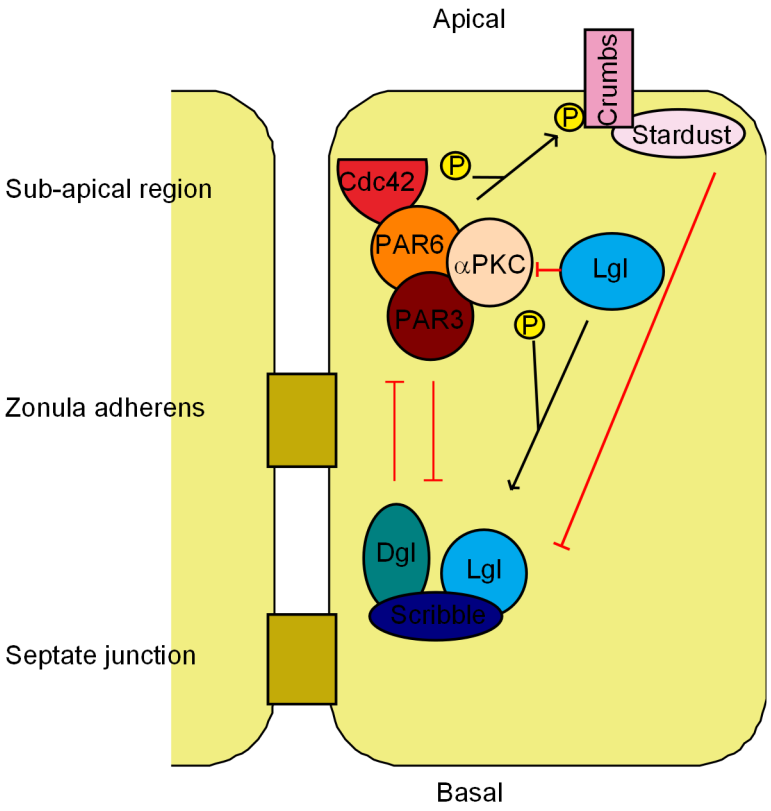


Figure 1.7: Interplay between the three polarity complexes. A diagram of the *Drosophila* ectoderm, showing the interactions involving the PAR and SCRIB complexes. Red lines indicate functional antagonism between complexes as defined by genetic and biochemical studies. The PAR complex acts to repress the activity of the SCRIB complex and promote the activity of the CRB complex. aPKC phosphorylates and excludes Lgl from the apical cortex.

junctional structures into mature epithelial structures in mammalian cells (Lemmers et al., 2004).

1.3.6 Polarity proteins and components of the apical junctional complex in proliferation and tumour suppression

Drosophila lethal (2) giant larvae (lgl) was the first tumour suppressor gene to be described in the scientific literature. *Lgl* mutant flies display overgrowth in their imaginal discs and the brain, accompanied by a loss of polarity and the formation of tumour-like lesions (Schneiderman and Gateff, 1967). A few years later, in 1972, a genetic screen assaying imaginal disc morphology identified a similar mutation called *disc-large (dlg)* (Stewart et al., 1972). More recently, a third mutation was identified in a similar screen and called *scribble* (Bilder et al., 2000). Concordant with their role as fly tumour suppressors, Dlg, Lgl and Scrib have been found to be either down-regulated or completely lost in human tumours (Cavatorta et al., 2004; Gardiol et al., 2006; Kuphal et al., 2006; Nakagawa et al., 2004). Activation of aPKC has been implicated in human cancer. PKCλ is overexpressed in ovarian and non-small cell lung cancer, while aPKCζ is hyper-activated in squamous cell carcinoma of the head and neck (Cohen et al., 2006; Eder et al., 2005; Regala et al., 2005). aPKCæ is also crucial for the proliferation of human glioblastoma cell lines (Donson et al., 2000).

The observation that these polarity proteins are also able to affect cell proliferation has aroused interest in whether growth control and cellular architecture are linked. Loss of cell polarity and tissue architecture are hallmarks of cancer. Several lines of evidence indicate that a loss of cell polarity could have a causative role in tumour initiation, and several well-known oncogenes directly target basic cell polarity mechanisms. Examples can be found in the functions of viral oncogenes like E6, which targets Dlg and Scrib for proteolytic degradation (Gardiol et al., 1999; Nakagawa and Huibregtse, 2000). Furthermore, ErbB2, a receptor tyrosine kinase abnormally activated in a variety of

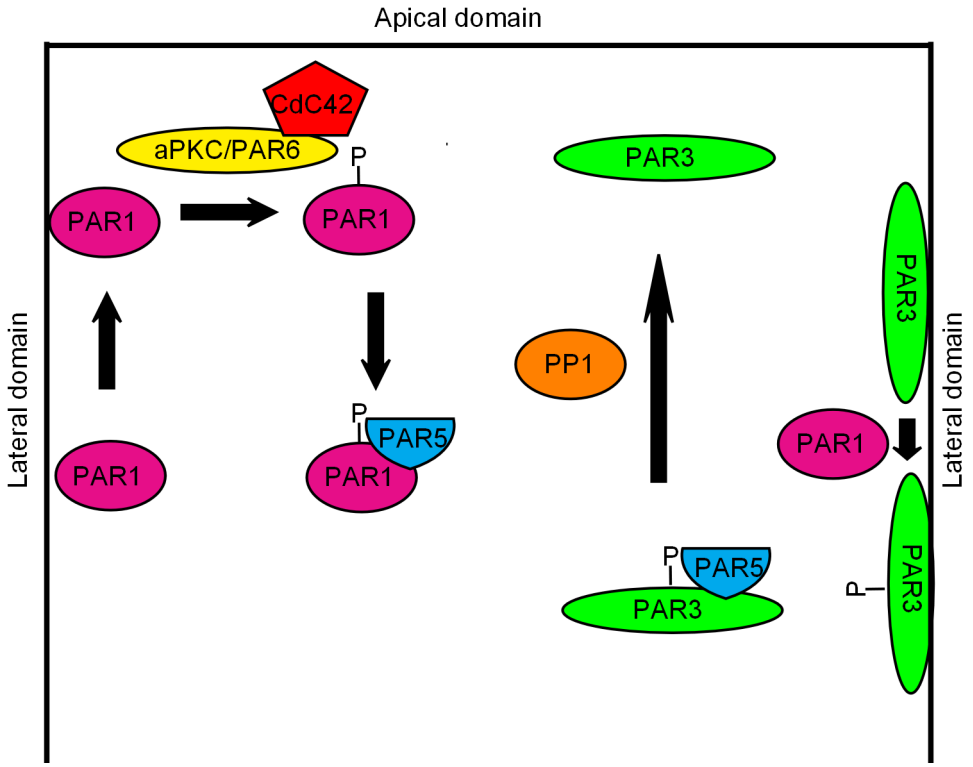


Figure 1.8: PAR3 localisation is controlled by phosphorylation. On the left, PAR1 that has diffused into the apical domain is phosphorylated by aPKC, which inhibits the PAR1 kinase activity and induces binding of PAR5, which in turn triggers release into the cytoplasm. Similarly, PAR3 that diffuses into the basolateral domain is phosphorylated by PAR1, which induces binding of PAR5 and release into the cytoplasm. PAR3 dephosphorylation is mediated by Protein Phosphatase 1 (PP1).

cancers, interacts with PAR6/aPKC inducing their dissociation from PAR3 and therefore disrupting the Par complex (Aranda et al., 2006). In addition to oncogenes, several tumour suppressors are also involved in the regulation of cell polarity. For example, PTEN is targeted to the apical membrane domain during epithelial cell polarisation, where it generates phosphatidylinositol-4,5-bisphosphate (PIP2), which facilitates the recruitment of the Par complex (Martin-Belmonte et al., 2007).

The mechanism by which a loss of cell polarity may lead to cellular overgrowth is still not understood. One of the most accredited models suggests that epithelial polarity loss could lead to the spatial disorganisation of signalling pathways that control cell proliferation. β-catenin, a junctional protein also able to control proliferation via Wnt signalling, has been proposed as a candidate. The loss of E-cadherin, often observed in cancer (Birchmeier and Behrens, 1994), disrupts cell-cell adhesion and could free β-catenin into the nucleus, activating the Wnt signalling pathway. Several studies have revealed that forced overexpression of cadherins antagonises Wnt/β-catenin signalling (Fagotto et al., 1996; Orsulic et al., 1999; Sanson et al., 1996). In addition to β-catenin, an increasing number of junctional proteins have been shown to control intracellular signalling, providing new mechanistic insight in the proliferation-polarity link. Studies with transgenic mice have shown that α-catenin, in addition to its role in cell polarity, also controls cell proliferation, most likely via the Hedgehog signalling pathway (Lien et al., 2006). Moreover, the TJ component ZO-1 inhibits cell proliferation by cytoplasmic sequestration of the Y-box transcription factor ZONAB, which interacts with the cell cycle regulator CDK4 and controls expression of cell cycle regulators such as cyclin D1 and PCNA (Balda et al., 2003). Reduced expression of ZO-1 correlates with increased proliferation of normal and transformed epithelial cells. For instance, ZO-1 is down-regulated in breast cancer tissue and in primary and metastatic pancreatic cancers (Hoover et al., 1998; Kleeff et al., 2001).

Studies in *Drosophila* have shown that polarity proteins regulate the asymmetric cell division of stem cells, and perturbation of this process results in an accumulation of overproliferating cells, suggesting another way in which cell polarity and growth control can be coupled. Mutations in the mammalian counterparts of Dlg, Lgl or Scribble do not

affect asymmetric division, or show defects in asymmetric cell division but do not develop cancer , leaving open the question of whether a failure in asymmetric division can result in increased proliferation and tumour formation in mammals .

Finally, polarity proteins have been shown to directly regulate cell proliferation. The loss of Scribble, Dlg and Lgl in *Drosophila* ovaries results in ectopic expression of cyclin E and precocious S-phase and mitosis (Zhao et al., 2008). Moreover, the E2F1 cell cycle transcription factor is upregulated in Lgl mutant clones in the developing *Drosophila* eye (Humbert et al., 2008). Increased aPKC protein levels are associated with increased cyclin E protein expression and proliferation in *Drosophila* epithelia, and also in ovarian cancers (Eder et al., 2005; Rolls et al., 2003).

Regardless of the mechanism involved, several studies have highlighted the reciprocal relationship between cell architecture and proliferation control signals in mammalian tissues. For instance, 3D culture systems have demonstrated the remarkable impact of cell architecture on a variety of cancer-related phenomena. Cells derived from malignant breast tumours are unable to form polarised alveoli in 3D cultures and instead overproliferate in a disorganised multilayered fashion. If integrin pathways are inactivated in malignant cells, polarity is restored and the malignant growth phenotype is also reverted (Wang et al., 1998; Weaver et al., 1997). The maintenance of a polarised cell phenotype can therefore act as a tumour suppressive mechanism.

1.4 The development of the cerebral cortex

Two main types of neural precursor cells have been identified in the developing CNS: neural stem and progenitor cells. Neural stem cells (NSCs) have the ability to either expand, generating two cells which are biologically identical stem cells, or to self-renew, generating one cell that is a biologically identical stem cell and one differentiated cell. Neural progenitors generate two cells with a more restricted potential, such as more differentiated progenitors or post-mitotic cells. NSCs in the developing CNS form a polarised epithelium known as the ventricular zone (VZ), the apical domain of which delimits the lumen of the neural tube. As development proceeds, NSCs switch from proliferative to self-renewal divisions, generating post-mitotic neurons which then migrate to their final resting place. Two main pathways are known to play a pivotal role in controlling the proliferation and differentiation of NSCs: Notch and β-catenin/Wnt signalling.

1.4.1 Classification of neural precursor cells

The first group of NSCs to appear in the developing CNS are neuroepithelial cells, which form a single layered neuroepithelium in the neural plate and neural tube before the onset of neurogenesis. Neuroepithelial cells show typical epithelial features and are highly polarised along the apical-basal axis. TJs and AJs are present at the most apical end of the lateral plasma membrane (Aaku-Saraste et al., 1996; Manabe et al., 2002; Zhadanov et al., 1999). Before the onset of neurogenesis, neuroepithelial cells divide symmetrically, generating two daughter stem cells. With the onset of neurogenesis, they start to divide asymmetrically, generating distinct types of secondary neural stem or progenitor cells

(radial glial cells also called apical progenitors and basal progenitors) and neurons (Figure 1.9). Radial glial cells (RGCs) appear in the CNS between embryonic day 10 (E10) and E12. By mid-neurogenesis, RGCs constitute the majority of stem cells in the CNS region (Hartfuss et al., 2001; Noctor et al., 2002). RGCs in the developing cortex are characterised by the expression of the transcription factor Pax6, which is absent in the RGCs of the ganglionic eminence (GE) (Gotz et al., 1998). They exhibit residual neuroepithelial and astroglial properties, the neuroepithelial properties of which are the expression of neuroepithelial markers such nestin and the maintenance of apical-basal polarity. In contrast to neuroepithelial cells, RGCs express several astroglial markers like glial fibrillary acid protein (GFAP) and brain lipidic binding protein (BLBP) (Campbell and Gotz, 2002). RGCs undergo stem cell-like asymmetric division to produce a neuron and a further RGC (Noctor et al., 2001). RGCs can, therefore, be regarded as NSCs. With the onset of neurogenesis the cortical neuroepithelium, which consists of a single germinal layer (the VZ), becomes stratified. A further type of progenitor cell appears in the neocortex, the basal or intermediate progenitors (BP). BPs delaminate from the apical surface of the neuroepithelium to form a second germinal layer, the subventricular zone (SVZ), and retract their apical and basal processes before mitosis. BP divisions are asymmetric and self-consuming, each generating two neurons (Haubensak et al., 2004). BPs are also molecularly distinct from RGCs by the expression of the transcription factor Tbr-2 (Englund et al., 2005).

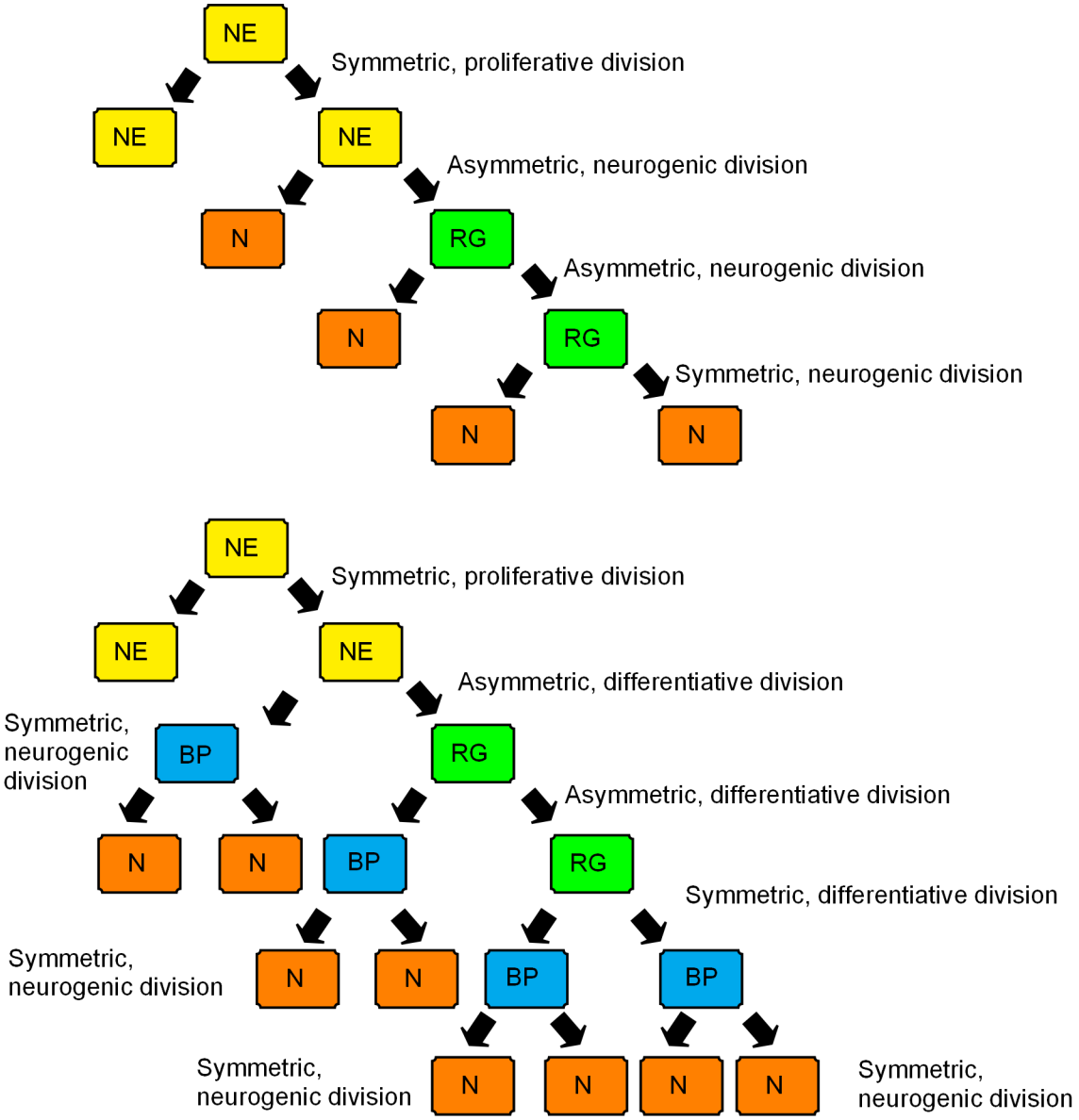


Figure 1.9: Neural precursor cells in the developing central nervous system. Schematic representation of the relationship between neuroepithelial cells (NE), radial glial cells (RGCs) and neurons (N) with or without basal progenitors (BP). The mode of cell division is also indicated.

1.4.2 Interkinetic nuclear migration (INM): a distinctive feature of neuroepithelial and radial glial cells

The nuclei of neuroepithelial and radial glial cells migrate in an apical basal manner, in phase with the cell cycle, in a process called interkinetic nuclear migration (INM) (Sauer and Chittenden, 1959). Cell divisions are confined to the apical location, while S-phase occurs in more basal areas. In neuroepithelial cells, INM spans the entire apical-basal axis of the cells, whereas it is confined within the VZ in RGCs (Figure 1.10). Although the process of INM was first described many decades ago in 1935, the mechanisms underlying INM are poorly understood. Studies using inhibitors like cytochalasin B, which blocks actin polymerisation, have suggested the importance of actin and the microtubule cytoskeleton in the process of INM (Tsai et al., 2005; Webster and Langman, 1978). LIS1, a regulator of the cytoskeletal motor proteins dynein and dynactin, is also essential for normal INM (Tsai et al., 2005). Centrosomal proteins, like Cep120, have similarly been implicated (Xie et al., 2007), and the transcription factor Pax6 has been shown to regulate INM by stabilising the centrosome (Tamai et al., 2007). While the function of INM is not yet fully understood, some have proposed that it facilitates the invagination of the neuroepithelium during neural plate development (Messier, 1978), while others have speculated that it may promote cell diversity during CNS development (Baye and Link, 2008).

1.4.3 Radial migration ensures lamination in the developing cerebral cortex

The construction of highly laminated structures in the CNS, like the cerebral cortex, spinal cord and thalamus, is achieved through the radial migration of newborn neurons along radial glial fibers. The hypothesis that radial glia provide a scaffold for directed

migration in the brain originates from analysis of the alignment of post-mitotic neurons with radial glial fibers during the development of the cerebral and cerebellar cortex (Rakic, 1971a, b, 1972). These observations are further supported by several *in vitro* (Anton et al., 1996; Edmondson and Hatten, 1987) and *in vivo* studies (de Carlos et al., 1996; Misson et al., 1991), leading to the formulation of a model in which neuronal precursors migrate along radial glial fibers in most areas of the CNS. The morphological features of RGCs are vital for the process of radial migration. Consistent with this, molecular abnormalities affecting the development of radial glial cells lead to abnormal neuronal migration (Caric et al., 1997; Gotz et al., 1998; Halfter et al., 2002).

Radial migration has been preferentially studied during the development of the cerebral cortex. The earliest born neurons appear around E10.5 in the mouse and form a layered structure termed the preplate, which is later split into the more superficial marginal zone and the deeply located subplate (Figure 1.11). The cortical plate, which will give rise to the multilayered neocortex, begins to develop in between these two layers. The development of the cerebral cortex progresses with successive waves of migration which position neurons within different layers in the cortical plate. The lamination of the cerebral cortex occurs in an inside-out manner, where the earliest wave of neuronal migration forms the deepest layer of the cortical plate (V-VI), while the last waves of neurons will be localised in the more peripheral layers II and III (Marin and Rubenstein, 2003). Recently, tremendous advances have been made in the identification of laminar markers through large-scale *in situ* hybridisation projects, and gene expression studies comparing microdissected regions of neocortex. Examples of layer-specific genes include, among many others: Cux1, Cux2 and Lhx2, markers of layers II/III to IV (Bulchand et al., 2003; Nieto et al., 2004); SMI32 a marker of layer III and V (Voelker et al., 2004); Brn2 (also

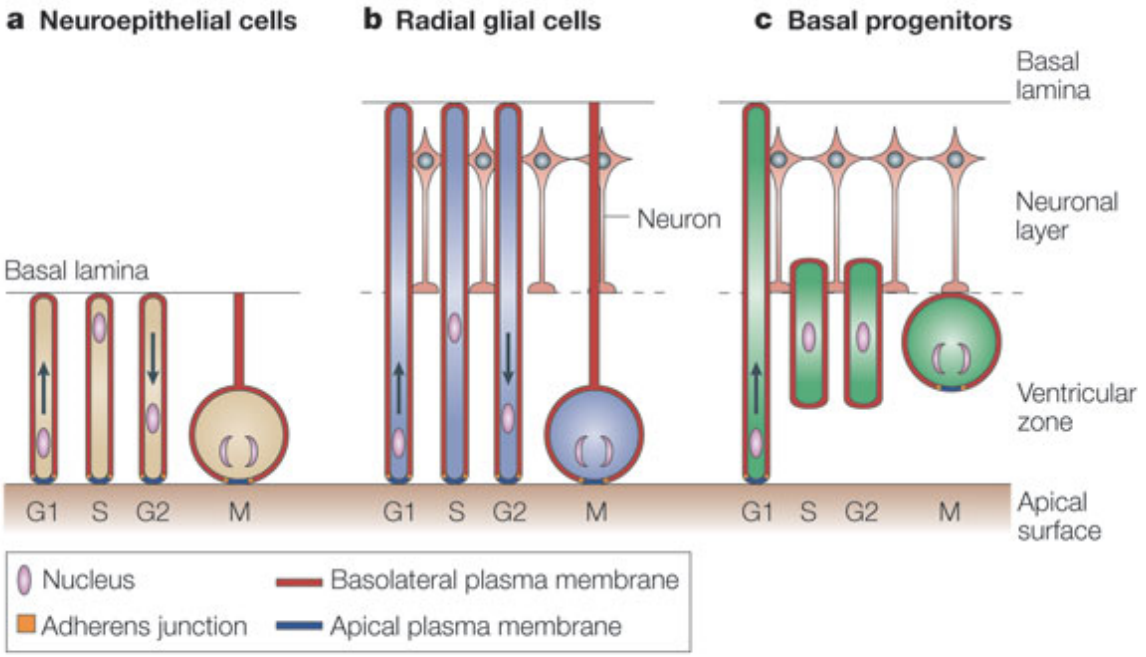


Figure 1.10: Morphological features and properties of neuroepithelial cells, radial glial cells and basal progenitors. (A) Neuroepithelial cells are polarised and they span the entire wall of the neural tube. The nuclei of neuroepithelial cells migrate to the basal side during G1, undergo S phase at the basal side and migrates back back to the apical side, where mitosis occurs, during G2. In neuroepithelial cells, interkinetic nuclear migration spans the entire axis of the cell. (B) Radial glial maintain the same elongated morphology of neuroepithelial cells. In radial glial cells, Interkinetic nuclear migration is confined to the portion of the cells between the apical side and the basal boundary of ventricular or subventricular zone (dashed line). (C) Basal progenitors retract from the apical surface concomitantly with the migartion of the nucleus to the boundary of ventricular zone or subventricular zone during G1. Both neuroepithelial cells and radial glia cells maintain the basal process during mitosis. Reproduced, with permission from Gotz and Huttner, 2005. © 2005, Nature Publishing Group.

known as Pou3f2), a marker of layer II/III and V (McEvilly et al., 2002); and Foxp2, a marker of layer VI (Ferland et al., 2003).

1.4.4 Molecular mechanisms regulating neuronal migration

Neuronal migration requires the regulation of repetitive steps. First the cell extends a leading neurite preceded by a growth cone, which explores the microenviroment. The growth cone is a very dynamic structure, exhibiting extension and retraction of filipodia and lamellopodia. Next, the nucleus translocates into the leading neurite. The last stage of migration is represented by the retraction of the trailing process.

The actin cytoskeleton is heavily involved in the regulation of neuronal migration, and disruption of actin with cytochalasin can inhibit neuronal migration (Rivas and Hatten, 1995). Mutations that destabilise actin lead to severe abnormalities in the CNS. For instance, the actin crosslinking protein filamin1 is mutated in periventricular heteropia, in which neurons completely fail to migrate out of the VZ and form clusters along the ventricular surface (Hong et al., 2000).

Microtubules provide stability to the growing neurite and are essential for the association of the centrosome with the nucleus during nucleokinesis. Mutations in two non-classic microtubule associated proteins, Lis1 and DCX, which control microtubule stability, cause severe brain development disorders characterised by a smooth brain surface and abnormal cortical layering known as lyssencephaly (Lambert de Rouvroit and Goffinet, 2001; Reiner et al., 1993). In addition, a large number of kinases like Src, PI3K and Cdk5 have been shown to mediate neuronal migration by phosphorylating key actin regulatory and microtubule-associated proteins (Kawauchi et al., 2006; Konno et al., 2005).

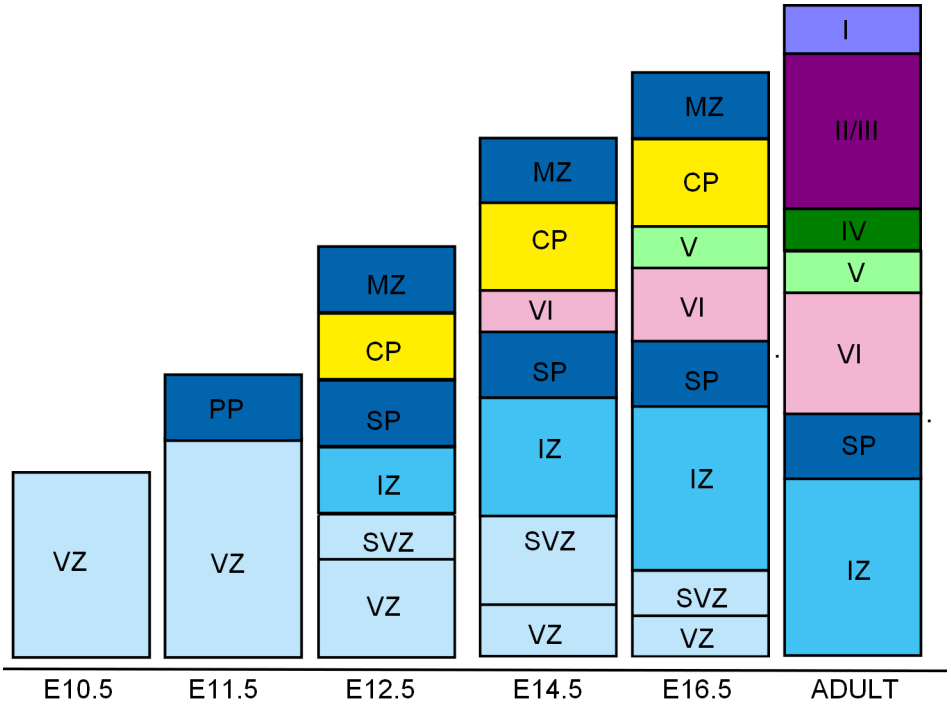


Figure 1.11: Schematic representation of the structure of the developing mouse cortex from embryonic to adult life. The earliest born neurons form the preplate (PP), which is later split into the more superficial marginal zone (MZ) and the deeply located subplate (SP). The cortical plate (CP), which will give rise to the multilayered neocortex, develops in between these two layers, such that later born neurons arriving at the cortical plate migrate past earlier born neurons. Different classes of projection neuron are born in overlapping temporal waves. E: embryonic day; IZ: intermediate zone; SVZ: subventricular zone; VZ: ventricular zone; WM: white matter.

Neuronal migration also involves cell non-autonomous mechanisms related to the microenviroment in which the neurons migrate. A large number of molecules, ranging from chemotropic molecules like netrins to neurotrophic factors like brain derived neurotrophic factor (BDNF), have been shown to function as extracellular signals for migrating neurons (Marin and Rubenstein, 2003).

1.4.5 Role of polarity and apical junction complex components in neural development

Polarity proteins play a pivotal role in controlling asymmetric cell division, a process that has been deeply analysed in *Drosophila* neuroblasts. Each asymmetric division produces one large daughter cell, which retains neuroblastic identity, and a smaller daughter cell called a ganglionic mother cell, which divides terminally to generate two post-mitotic neurons/glia. The acquisition of differential cell fate requires the segregation of cell fate determinants at one pole of the mother cell, and a mitotic spindle orientated orthogonally to the axis of polarisation. This ensures that only one daughter cell will receive the cell fate determinants that drive differentiation. The Par complex is localised apically in the neuroepithelia where it colocalises with Inscuteable (Insc), establishing apical-basal polarity. During mitosis, the Insc/Par complex localises to the apical cortex as a crescent and recruits another evolutionarily conserved protein complex, comprised of Partner of Inscuteable (Pins) and the heterotrimeric G protein subunit Gái. The primary roles of the two protein complexes are distinct: the Pins/Gái complex is mainly involved in spindle orientation whereas the Par complex appears to play a primary role in the basal localisation of cell-fate determinants (Izumi et al., 2004; Schober et al., 1999; Wodarz et al., 1999). The apical Par complex directs the basal localisation and segregation of cell-fate determinants, such as Prospero (Pros) and Numb and their adaptor proteins Miranda (Mira) and Partner of Numb (Pon), through two cortically localised tumour suppressors: Discs large (Dlg) and Lethal (2) giant larvae (Lgl) (Betschinger et al., 2003; Ohshiro et al., 2000; Peng et al., 2000). Phosphorylation of Lgl by apically localised aPKC leads to Lgl inactivation and prevents its association with the apical cortex, while non-phosphorylated Lgl in the basal cortex is in an active state and recruits cell-fate

determinants (Betschinger et al., 2003). Although a similar pathway is conserved in mammalian cells, the role played by PAR3 or Lgl in asymmetric division remains unclear. A recent study suggests that PAR3 regulates asymmetric cell division of radial glial progenitors in the developing mouse cortex. In line with the *Drosophila* model, mammalian PAR3 is asymmetrically distributed in dividing radial glial cells and can determine cell fate by acting through Numb and its homologue Numb-like to control Notch signalling activity (Bultje et al., 2009). Lgl1-null mice display defects in asymmetric cell division and differentiation in the developing brain (Klezovitch et al., 2004). Lgl1 progenitors fail to asymmetrically localise Numb and therefore down-regulate Notch activity, suggesting that mammalian Lgl1 functions like its *Drosophila* counterpart in controlling Notch activity by asymmetrically localising Numb in neural progenitors.

Cell polarity and AJC proteins are also heavily implicated in the process of neuronal migration. In most migrating neurons, the movement of the centrosome into the leading neurite precedes nucleokinesis, indicating that the centrosome establishes the polarity that is important for directional movement. In migrating granule neurons of the cerebellum, PAR6/aPKC localises to the centrosome and signals forward movement prior to translocation of the cell soma (Solecki et al., 2004). Over-expression of PAR6 in fact inhibits glial-guided neuronal migration. PAR6/aPKC has also been implicated in the orientated migration of astrocytes in cell culture. aPKC can phosphorylate and thereby inactivate GSK3b, leading to stabilisation of the microtubule binding protein APC at the leading edge of the astrocyte (Etienne-Manneville and Hall, 2001, 2003; Etienne-Manneville et al., 2005). In addition, Lgl and Dlg participate in astrocyte-directed migration, mainly through their ability to control the microtubule network (Etienne-Manneville et al., 2005). Components of the AJC are also indirectly involved in the

process of cell migration, as they control cell-cell adhesion between radial glial cells and therefore preserve the integrity of the scaffold used by migrating neurons. Deletion of the adherens junction components N-cadherin and β-catenin leads to impaired neuronal migration in the developing cortex and retina. (Kadowaki et al., 2007; Machon et al., 2003; Masai et al., 2003). Moreover, disruption of cell polarity following deletion of Cdc42, which is the primary activator of the PAR complex, causes defects in INM accompanied by a loss of adherens junctions (Cappello et al., 2006).

1.4.6 Overview of the Notch pathway

The notched-wing phenotype associated with the haploinsufficiency of the Notch locus was first observed in *Drosophila* in 1919 (Mohr, 1919). Notch received its notoriety as a result of classic embryonic analyses of lethal loss-of-function mutations, which were conducted by Poulson (Poulson, 1937). These mutations produced a "neurogenic" phenotype, where cells destined to become the epidermis switched fate and instead gave rise to neural tissue. The cloning of the Notch locus in 1985 revealed that Notch is a 300 KDa, single-pass transmembrane receptor with 36 EGF-like repeats in its extracellular domain (Wharton et al., 1985). EGF repeats 11 and 12 are sufficient to mediate the interaction between Notch and its ligands: members of the Delta and jagged gene families. The ligands for Notch are also transmembrane proteins and, therefore, cell-cell contact is an important prerequisite for signalling events (Lissemore and Starmer, 1999). Upon binding with its ligands, Notch first undergoes cleavage by transmembrane proteases of the ADAM/TACE family, and two further intra-membranous cleavages by the g secretase complex (Figure 1.12) (De Strooper et al., 1999; Mumm et al., 2000). These events lead

to the release of the intracellular domain of Notch (NICD) from the membrane and its translocation to the nucleus, where it converts the CBF1 (C-promoter binding factor-1) repressor complex to an activator complex (Struhl and Adachi, 1998). In the absence of Notch signalling, CBF1 represses the transcription of Notch target genes by recruiting co-repressor proteins and, subsequently, the histone deacetylase complex (HDAC), which converts the local chromatin to a transcriptionally silent form (Lai, 2002). In the presence of active Notch signalling, the NICD binds to CBF1, displacing the co-repressors. The transcriptional co-activator protein Mastermind is then recruited and binds to CBF1 and NICD, forming a ternary complex (Fryer et al., 2002). Finally, transcription is activated following the recruitment of the general transcription factors PCAF/GCN5 and CBP/p300 (Wallberg et al., 2002). Notch target genes include the *Hes* and *Herp* genes, which are basic helix-loop-helix transcriptional regulators that antagonise proneural genes like Mash1 and neurogenins (Kageyama et al., 2008). Recent evidence suggests that genes other than *Hes* and *Herp* are effectors of Notch signalling, which can also act independently of CBF1 (Anthony et al., 2005; Martinez Arias et al., 2002).

1.4.6.1 Notch signalling inhibits neuronal differentiation

Many components of the Notch pathway have been mutated in mammals, providing an important resource for understanding the fundamental aspects of Notch function. One of the first Notch pathway genes to be mutated in mice was *Notch1*. A detailed analysis of CNS development revealed an upregulation of differentiation markers in *Notch1 -/-* mutants, suggesting that Notch activity is required for progenitor maintenance (Conlon et al., 1995; Swiatek et al., 1994). Interestingly, the expression of *Hes5* was found to be reduced in *Notch 1 -/-* mutants, while *Hes1* expression did not seem to be affected. Similar

results were obtained in *CBF1* mutants, suggesting that *Hes1* is likely to be regulated by other signalling cascades (de la Pompa et al., 1997). This notion is consistent with the finding that *Hes1* is up-regulated in post-natal cerebellar granules treated with the mitogen Sonic hedgehodge (Solecki et al., 2001). Following the original *Notch1* deletion studies, mutants for *Notch2*, *Notch3* and *Notch4* have also been generated. *Notch2* mutants die at embryonic day 11 without showing altered expression of *Hes5* in the CNS (Hamada et al., 1999). In contrast, deletion of *Notch3* and *Notch 4* does not cause any significant phenotype. Mutations of *Hes1* and *Hes5* have also revealed that Notch signalling inhibits neuronal differentiation in the developing CNS. *Hes1 -/-* mutant embryos show severe defects in neural development accompanied by precocious neurogenesis (Ishibashi et al., 1995). Although some precocious differentiation was observed in *Hes5 -/-* mutants, these animals were largely normal, suggesting that *Hes1* is able to almost completely compensate for the lack of *Hes5* function. In line with a partially redundant function of Hes1 and Hes5 in neural progenitors, *Hes1* and *Hes5* double mutants show a far more severe phenotype than *Hes1* and *Hes5* single mutants (Ohtsuka et al., 1999). Interestingly a constitutively activated form of Notch1 is able to inhibit neuronal differentiation in *Hes1 Hes5* double mutants, raising the question of whether other targets can mediate Notch signalling in the CNS (Ohtsuka et al., 1999). *Hey1* and *Hey2* are also expressed in neural progenitors, and double mutants for these genes display thinner neural tubes (Fischer et al., 2004; Sakamoto et al., 2003). In addition, several reports have identified ErbB2 as a Notch target that has a role during mammalian neural progenitor maintenance (Patten et al., 2003; Schmid et al., 2003). Although Notch ligand mutations have been generated in mice, only a few studies have analysed their impact on the development of the CNS. In addition to decreased *Hes5* expression and increased neuronal markers, *Delta-like 1*

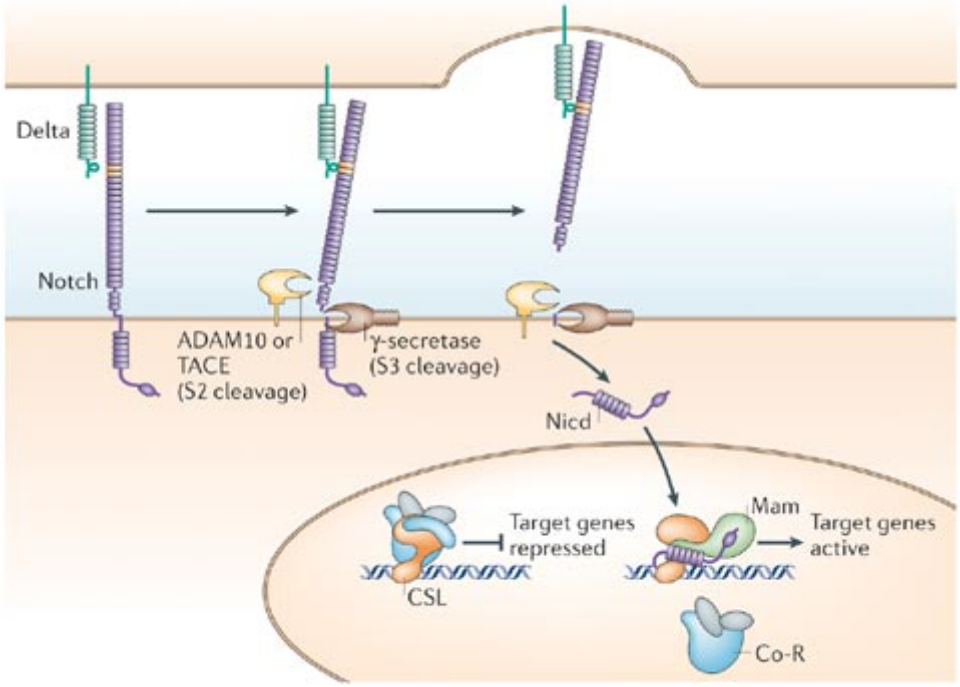


Figure 1.12: Components of the Notch pathway. Notch receptors binding to the delta ligand expressed on neighbouring cells leads to two proteolotic cleavages. The S2 cleavage, mediated by the ADAM10 or TACE metalloprotease, generates a substrate for S3 cleavage by the γ -secretase complex. Thus, the Notch intracellular domain (NICD) is released and translocates into the nucleus where it dislodges co-repressor (Co-R) associated with the DNA-binding CSL transcription factor. NICD and CSL form a ternary complex with mastermind (Mam) that recruits transcription factor activating target gene expression. Reproduced, with permission, from Bray, 2006© 2006, Nature Publishing Group.

mutants also show a reduction in the radial glial marker RC2 (Grandbarbe et al., 2003; Yun et al., 2002). This observation suggests that Notch signalling might also regulate the diversification of the precursor pool into distinct subtypes, shedding new light on to the role of Notch signalling during CNS development.

1.4.6.2 Numb is a modulator of Notch signalling

Numb, originally identified in *Drosophila* as a cell fate determinant, is evolutionarly conserved and its mammalian homologues are encoded by two genes (mNumb and mNumb-like). *Drosophila* Numb (dNumb) is a membrane-associated protein that segregates asymmetrically in dividing cells, and is believed to determine distinct cell fates by inhibiting Notch activity, thereby promoting the endocytosis of the receptor. Although some studies suggest that this function of Numb is conserved in mammals, evidence obtained from the analysis of mouse mutants does not fully support this view. One study showed that deletion of Numb leads to precocious neuronal differentiation in the forebrain, indicating that Numb is required for the maintenance of the progenitor pool and therefore acts as a positive regulator of Notch signalling. To eliminate the possible redundancy between Numb and Numb-like, two different groups have analysed the effect of a conditional deletion of Numb in a Numb-like null background, and have reached different conclusions. One group, using nestin-Cre or D6-Cre to delete Numb, found precocious widespread neuronal differentiation in the cortex. In contrast, the other group using Emx-1 Cre observed an expansion of the progenitor pool, accompanied by the presence of rosette-like structures. The different timing or efficiency of deletions obtained by the different Cre drivers used in these two studies may partially explain the opposing phenotypes obtained. Mutation in Lgl1, which is essential for the segregation of Numb in *Drosophila* neuroblasts, has a phenotype similar to the Emx-1 mediated Numb deletion. This result suggests that mammalian Lgl and Numb functions similarly to their *Drosophila* counterparts in neural progenitors.

1.4.6.3 Notch signalling and the cell cycle

Although Notch signalling has traditionally been implicated in the control of cell fate, several studies suggest that the Notch pathway can also affect cell proliferation. At the larval stage in *Drosophila*, Notch expression correlates with mitotically active tissues in the imaginal discs and CNS, raising the possibility that Notch may play a role in the cell cycle (Markopoulou and Artavanis-Tsakonas, 1989). Subsequent studies in *Drosophila* have shown that activation of Notch signalling by expression of a constitutively active form of the receptor, or by mutation of proteins involved in its recycling to the cell surface, leads to an increase in cell proliferation in both cell-autonomous and non-autonomous manners (Go et al., 1998; Moberg et al., 2005; Thompson et al., 2005). A growing body of evidence suggests that mammalian Notch plays a role in directly controlling cell proliferation, in addition to modulating cell fate decisions. In granule neuron precursors (GNPs) of the cerebellar cortex, activation of Notch 2 promotes cell proliferation (Solecki et al., 2001). Consistent with this observation, Notch 2 expression has been found to be upregulated in medulloblastoma, a CNS cancer thought to originate from GNPs (Fan et al., 2004). Moreover, over-expression of the Notch1 intracellular domain in committed glial precursors directly leads to proliferation and expansion of the precursor pool (Chambers et al., 2001). This result raises the possibility that Notch may act as an oncogenic signal in CNS tumours originating from glial cells. Notch 1 has in fact been found to be over-expressed in astrocytic gliomas and glioma cell lines, and targeting of Notch1 by siRNAi in these cell lines has been associated with inhibition of cell growth (Xu et al.). Although the mechanisms by which Notch signalling targets the cell cycle machinery have not yet been determined, several studies suggest that the activation of cyclin D1 transcription plays a major role in this process. Cyclin D1 has been shown to

play a critical role in the control of the G1/S transition of the cell cycle, and is able to directly induce cellular proliferation. Two independent studies have revealed that the intracellular domain of Notch is able to induce transformation in rat embryonic kidney cells (REKs), by activating the transcription of cyclin D1. Both groups identified a CBF1 binding site in the cyclin D1 promoter, suggesting that activation of cyclin D1 transcription by Notch is CBF1 dependent (Ronchini and Capobianco, 2001; Stahl et al., 2006). Moreover, microarray profiling screening of breast cancer cell lines transfected with siRNAi against the Notch ligand JAG1 also identified cyclin D1 as a direct target of Notch1 and Notch3 (Cohen et al., 2009).

Further evidence derived from various cell lines has revealed that Notch 1 induces the transcription of S phase-kinase associate protein 2 (SKP2), the F-box subunit of the ubiquitin ligase complex SCF SKP2 that targets proteins like the cyclin dependant kinase inhibitors p21 Cip1 and p27 Kip1 for degradation. Up-regulation of SKP2 by Notch leads to increased degradation of p21 Cip1 and p27 Kip1and premature entry into S phase (Sarmento et al., 2005). In contrast, Notch activation has been found to lead to cell cycle arrest via p21 Cip1 and p27 Kip1in keratinocytes and small cell lung cancer cells (Rangarajan et al., 2001; Sriuranpong et al., 2001), suggesting that the effect of Notch signalling on cell proliferation is highly context dependent and related to its normal function in a specific tissue.

1.4.7 Overview of the Wnt/β-catenin signalling pathway

Signalling by the Wnt family of secreted glycoproteins is one of the fundamental mechanisms that control cell proliferation and cell fate determination during embryonic

development (Logan and Nusse, 2004). Wnts are conserved in all metazoan animals. In mammals, the complexity and specificity of Wnt signalling is achieved by the actions of 19 different Wnts. β-catenin is central to Wnt signalling. In the absence of Wnt, cytoplasmic β-catenin is constantly degraded by the action of the Axin complex, which consists of the scaffolding protein Axin, the tumour suppressor APC, casein kinase 1 (CK1), and glycogen synthase 3 (GSK3) (Chen et al., 2000; Dale, 1998) (Figure 1.13). Following phosphorylation at its N-terminus by CK1 and GSK3, β-catenin is ubiquitinated and targeted for degradation. These events prevent β-catenin from reaching the nucleus, binding to the TCF/LEF family of proteins and activating the transcription of Wnt target genes. The binding of a Wnt ligand to the transmembrane receptor Frizzled (Fz) and its co-receptor, low-density lipoprotein receptor related protein6 (LRP6), results in the recruitment of the scaffold protein Dishevelled (Dvl), followed by LRP6 phosphorylation and recruitment of the Axin complex to the receptor (Logan and Nusse, 2004). These events lead to the inhibition of Axin-mediated β-catenin degradation and, thus, stabilisation of β-catenin, which travels to the nucleus and activates Wnt target genes. The ability of β-catenin to activate genes involved in proliferation, differentiation and apoptosis results in a vast array of biological responses, such as the activation of genes like cyclin D1 and c-myc, which has been implicated in cancer development.

1.4.7.1 Wnt/β-catenin signalling affects the proliferation and differentiation of neural precursor cells

The Wnt/β-catenin signalling pathway plays a key role in modulating proliferation and differentiation in the CNS. A study conducted by Zechner et al., revealed that constitutive

expression of β-catenin in neural crest stem cells (NCSCs) causes an expansion of the neural tube, while ablation of β-catenin results in a decrease of tissue mass accompanied by a reduction in the neuronal precursor population (Zechner et al., 2003). Gain and loss of function studies have shown that β-catenin regulates the size of the cerebral cortex in mice by promoting the proliferation of neural stem cells without affecting neuronal differentiation (Chenn and Walsh, 2002; Woodhead et al., 2006). Similarly, Wnt3a signaling seems to be involved in hippocampal development by regulating the maintenance of the progenitor pool (Lee et al., 2000). Analysis of the effect of Wnt signalling in cortical neurogenesis suggests that the outcome of Wnt activation strictly depends on the time and location of the signal. In contrast to the effect of constitutive expression of β-catenin, Wnt7a promotes differentiation and reduces the self-renewal capacity of cortical precursor cells (Viti et al., 2003). Moreover, in neurospheres obtained from E11.5 telencephala, treatments with Wnt3a also promote differentiation (Muroyama et al., 2004).

Wnt signalling has been shown to be involved in multiple cancer types (Reya and Clevers, 2005). Aberrant Wnt signalling was first implicated in brain tumours through the study of patients with Turcot syndrome, which involves APC mutations linked to high incidence of colorectal cancers and brain tumours, particularly medulloblastoma (Hamilton et al., 1995). Mutation of Axin, β-catenin and APC were also found in sporadic medulloblastoma (Baeza et al., 2003; Dahmen et al., 2001; Huang et al., 2000; Koch et al., 2001), and nuclear localisation of β-catenin has been found in medulloblastoma, suggesting constitutive activation of Wnt signalling (Eberhart et al., 2000).

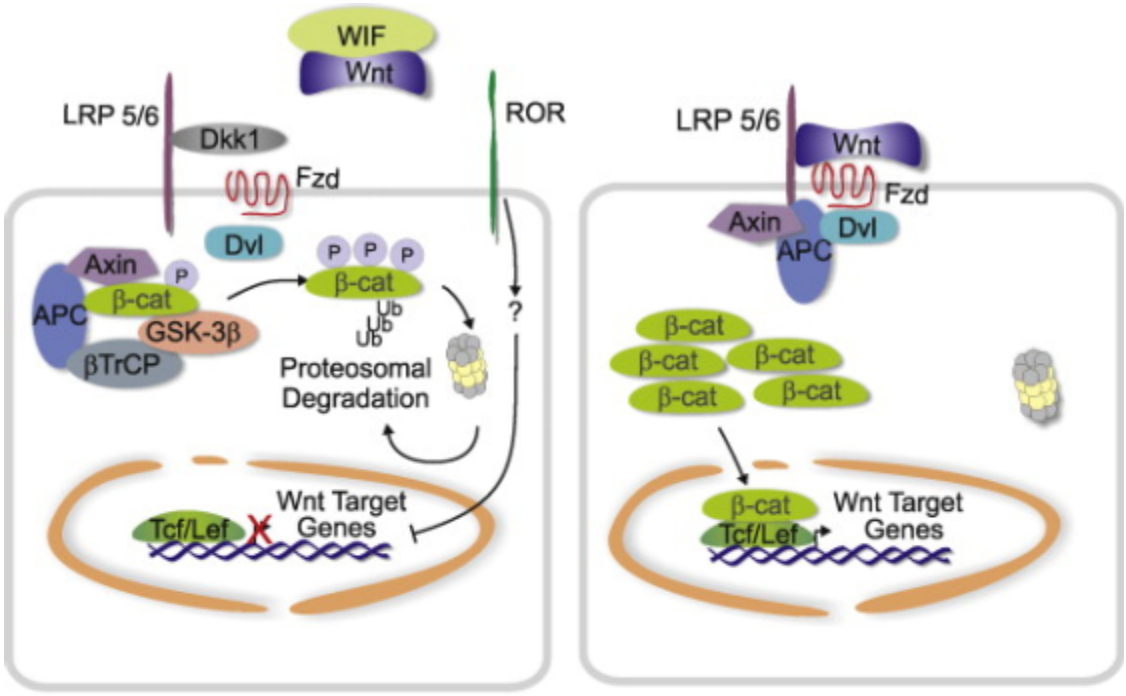


Figure 1.13: The Wnt signaling pathway. In the absence of a Wnt protein (Left Panel), the destruction complex is active and β-catenin molecules are phosphorylated by GSK-3β and degraded by the ubiquitin pathway. In the presence of a Wnt protein (Right Panel) Fzd receptor, along with the LDL-related receptor protein LRP5/6, transduces its signal by activating Dvl protein, which in turn inactivates the destruction complex. β-Catenin molecules are stabilised and cytosolic levels rise, thus allowing β-catenin to translocate in the nucleus, bind to TCF/LEF transcription factors and activate the expression of Wnt-target genes. Reproduced, with permission, from Toledo et al., 2008 © 2008, Elsevier.

1.5 Mechanisms involved in the development of hydrocephalus

Hydrocephalus is a multifactorial brain disorder that occurs when there is an increase in pressure in the cerebral ventricles due to accumulation of the cerebrospinal fluid (CSF). Untreated hydrocephalus leads to progressive enlargement of the ventricles accompanied by disruption of the brain tissue and mental retardation. Hydrocephalus can be divided in two types: communicating and non-communicating. The former is characterised by an increase of the overall CSF level due to an increase in production or reduced readsorbotion

into the blood or lymphatic system, while the latter develops as a result of an obstruction above the spinal cord (Crews et al., 2004). Several factors can be involved in the pathology of the disorder, including genetic alterations like X-linked recessive mutations in the L1 neural cell adhesion molecule (CAM) gene (Van Camp et al., 1993). Although the precise mechanisms that lead to the development of hydrocephalus are still unclear, several studies using transgenic mice have enabled the identification of two CNS structures as possible candidates: the ependymal cells and the choroid plexus (CP).

1.5.1 Morphological features and functions of ependymal cells

The ependyma is a simple ciliated epithelium that lines the ventricular surface of the CNS (Del Bigio). Ependymal cells are cuboidal, and are characterised by the presence of microvilli and cilia at the apical pole which contribute to the circulation of the cerebrospinal fluid (CSF) within the brain ventricles. Ependymal cells are connected to each other by well-developed AJs at the apical-lateral interface, while they lack continuous TJs. Ependymal cells are absent in the immature neural tubes and only appear when the VZ regresses. In the mouse spinal cord, ependymal cells are derived from Nkx6.1-expressing neuroepithelial cells, while mouse forebrain ependymal cells are derived from RGCs (Fu et al., 2003; Spassky et al., 2005). A study conducted by Johansonn *et al.* showed that ependymal cells are able to proliferate and differentiate in neurons and astrocytes in response to injury, thus proposing that ependymal cells are neural stem cells (Johansson et al., 1999). In contrast, other studies have suggested that SVZ but not ependymal cells posses stem cell characteristics (Chiasson et al., 1999). Some studies have proposed that ependymal cells can also affect processes like neuronal migration from the SVZ, by producing specific extracellular matrix and adhesion

molecules like PSA-NCAM (Hauwel et al., 2005). This notion is supported by the observation that, in human fetal brains, ependymal denudation may be associated with a neuronal migration disorder known as subependymal heterotopia (Dominguez-Pinos et al., 2005).

1.5.2 Structure and function of the choroid plexus

The choroid plexus (CP) is a highly vascularised structure, suspended in each of the four cerebral ventricles. The CP of the fourth ventricle is the first to emerge from the neuroepithelium during embryogenesis in the mouse at E12.5, and is also the largest. Although it has been reported that the CP expresses several morphogens like bone morphogenic proteins (BMPs), fibroblast-like growth factors (FGFs), transforming growth factor b (TGF bs) and Sonic hedgehog (Shh), little is known about the molecular pathways or developmental mechanisms that affect CP biogenesis (Bitgood and McMahon, 1995; Redzic et al., 2005).

The CP comprises a central stroma with numerous blood vessels, covered by a single layer of specialised ependymal cells (the choroidal epithelium) resting on a basement membrane (Figure 1.14). At the apical pole, CP epithelial cells display abundant microvilli and long cilia, with unknown function. The blood vessels of the CP are highly fenestrated and therefore highly permeable. In order to prevent the diffusion of substances from the blood into the ventricles, CP epithelial cells are equipped with TJs which are responsible for the blood-CSF barrier properties of the CP (Figure 1.14) (Brightman and Reese, 1969; Castel et al., 1974). Choroidal cells are rich in mitocondria and subcellular organelles, such as the endoplasmic reticulum and Golgi apparatus, which are typical features of cells engaged in active transport (Segal and Burgess, 1974). The presence of

several transporters and ion channels along the apical and basolateral sides of the CP epithelium ensures the active secretion of CSF by the CP. The driving force for fluid secretion across the epithelium is the osmotic gradient generated by the flux of ions from one side to the epithelium to the other, which is followed by the movement of water into the ventricular cavities. Recent studies have suggested that the CP synthesises and secretes a number of active molecules like polypeptide and growth factors into the CSF. For instance, Insulin-like growth factor II (IGF II) and transforming growth factor (TGF-b1) are secreted by the CP and play an important role in recovery after brain injury (Flanders et al., 1998; Nilsson et al., 1996). In addition to its role in the active secretion of CSF and several molecules, the CP is also engaged in the active transport of nutrients like glucose and amino acids from the CSF to the blood (Bradbury and Brondsted, 1973).

1.5.3 Involvement of the choroid plexus and ependyma in the pathogenesis of hydrocephalus

One of the main causes of increased intracranial pressure is the excessive production of CSF by the CP. Mice lacking the transcription factor E2F5 display hydrocephalus, associated with increased secretory activity of the CP (Lindeman et al., 1998). The E2F family of transcription factors has mainly been implicated in the regulation of the cell cycle to date, and the mechanisms by which E2F5 controls CSF secretion are still unknown. Alterations of the CP basal membrane in mice deficient for collagen type XVIII have also been associated with hydrocephalus (Utriainen et al., 2004). It has been hypothesised that the structural abnormalities in the basal membrane lead to altered filtration of fluid from the CP capillaries to the epithelial cells, resulting in altered production of CSF. Severe underdevelopment of the CP has been observed in mice null

for p73, which display severe hydrocephalus (Cabrera-Socorro et al., 2006). Several transgenic mice carrying mutations or deletion of proteins involved in cilia structure or function exhibit hydrocephalus. Cilia of the CP seem to be mainly involved in secretion of the CSF. Mice mutant for the cilia protein polaris display loss of cilia on the CP, associated with an increase in concentration of chloride in the CSF (Banizs et al., 2005). Interference with the function of ependymal cilia, the beating of which is believed to aid the flow of fluid through the ventricular system, can also cause hydrocephalus. For instance, disruption of the axonemal dynein protein Mdnah5 results in impaired cilia motility and hydrocephalus (Ibanez-Tallon et al., 2004). Cilia functions in the ventricular system are also important in humans, as shown by the incidence of hydrocephalus in patients with primary cilia diskenesia (Bush, 2000).

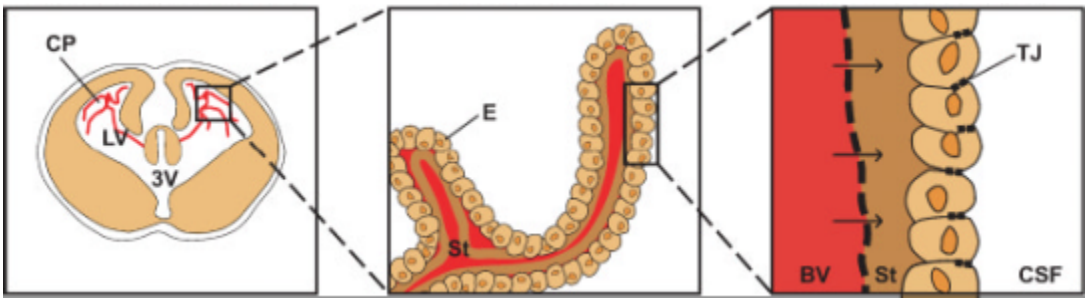


Figure 1.14: Schematic representation of the choroid plexus structure and blood-cerebrospinal fluid barrier property. CP: choroid plexus; LV: lateral ventricles; 3V: third ventricle; E: epithelium; St: stroma; BV: blood vessels; TJ: tight junctions; CSF: cerebrospinal fluid. Reproduced, with permission, from Johansson et al., 2008. © 2008 WILEY Periodicals, Inc.

1.6 Aim of the study

ASPP2 belongs to the ASPP family of proteins, which consists of three members: ASPP1, ASPP2 and iASPP. The ASPPs are characterised by a conserved C-terminal sequence, which contains **A**nkyrin repeats, an **S**H3 domain and a **P**roline rich region (Sullivan and Lu, 2007). The best characterized function of ASPP2 is its binding to p53, p63 and p73, which results in the stimulation of their apoptotic functions by selectively enhancing their DNA binding and transactivation activities on pro-apoptotic genes such as *BAX* and *PIG3* (Bergamaschi et al., 2004; Sullivan and Lu, 2007). The deletion of *ASPP2 in vivo* leads to a higher incidence of spontaneous and induced tumours as well as the development of severe hydrocephalus. Therefore, ASPP2 not only functions as a tumour suppressor in mice, but also play an important role during the development of the CNS. The aim of this study is to investigate the role of ASPP2 during CNS development by a detailed analysis of *ASPP2* mutant mice from embryonic to postnatal life. A better understanding of the developmental role of ASPP2 is vital to identify the mechanisms by which ASPP2 functions as a tumour suppressor.

2. Material and methods

2.1 Cell cultures

2.1.1 MCF7 and U373 cells

The human breast adenocarcinoma cell line MCF7 and the human glioblastoma-astrocytoma cell line U373 were obtained from American Type Culture Collection (ATCC). Both cell lines were grown at 37ºC -10% CO2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2mM L-glutamine and penicillin/streptomycin.

2.1.2 Mouse neural stem cells

Neural stem cells (NSCs) were isolated from the foetal central nervous system (CNS) as described in Conti et al., 2005. Embryos derived from *ASPP2* Δexon3 heterozygous mating were collected at E11.5 on ice in PBS. The telencephalon was isolated from the whole brain; the meninges and olfactory bulb were than removed on ice-cold PBS. The remaining tissue constituted by cortex and ganglionic eminence (GE) was mechanically dissociated into single cells, centrifuged at 2000 rpm for 3 minutes and re suspended in RHB-A media (Stem Cell Science) supplemented with 10 ng/ml of both mouse EGF and human FGF-2 (Peprotech). Cells were subsequently allowed to attach on 3 cm plastic dishes previously coated with a solution of 0.01 mg/ml laminin (Sigma) in PBS at 37ºC for two hours. Outgrowing cells were than expanded on laminin using the supplemented RHB-A media. For passaging established NSCs lines, cells were detached using Accutase (Sigma) and split 1:3 to 1:5 every two-three days.

2.2 Cell-based assays

2.2.1 Cell transfection

U373 and MCF7 cells were plated 24 hours before transfection. Fugene 6® transfection reagent (Roche) was used following the manufacture's protocol.

2.2.2 Transactivation assay

Twenty-four hours after transfection transactivation assay was carried out in U373 cells with the Dual-Luciferase® reporter assay system (Promega) following the manufacture's protocol.

2.2.3 Immunocytochemistry

MCF-7 cells were seeded on coverslip, while NSCs were seeded in Lab-Tek™ Chamber slides (Thermo Scientific) previously coated with laminin. Once experimental procedures were carried out, cells were fixed with 4% paraformaldehyde in PBS for 10 minutes then permeabilizied with 0.1% Triton X-100 in PBS for 4 minutes on ice. After 1 hour of incubation with a blocking solution of 0.1% of cold fish skin gelatine in PBS, primary antibodies were applied to the cells for 1 hour at room temperature. Protein expression was detected by using Alexa Fluor® (Molecular Probes) for 45 minutes. TO-PRO (Invitrogen) was used to stain nucleic acids.

Table 2.1: Plasmids

Plasmid	Source	Relevant information
Hes5 Luciferase	R.Kageyama, University of Kyoto	Hes5 promoter linked to luciferase reporter
Hes1 Luciferase	R. Kageyama, University of Kyoto	Hes1 promoter linked to luciferase reporter
pcDNA3.1/V5-His-	Susana Llanos	V5 tagged human ASPP2
pcDNA3.1/Notch intracellular domain(NICD)	T.Kadesh, University of Pennsylvania	Myc tagged human NICD
pFLAG-CMV2 Lgl1	T.Pawson, Mount Sinai Hospital, Toronto	Flag tagged mouse Lgl1
TA p73	Gerry Melino, University of Leicester	HA tagged human TAp73
pcDNA3 Hes5	R.Kageyama, University of Kyoto	pcDNA3 containing a fragment of Hes5 cDNA

Table 2.2: Antibodies used in immunocytochemistry

Antigen	Catalogue Number	Source	Dilution
PAR3	07-330	Upstate	1:50
ZO-1	617300	Invitrogen	1:50
β-catenin	610154	BD Transduction	1:100
βIII-tubulin	MRB-435P	Convance	1:500
RC2	Mouse mAb	Developmental Studies	1:50
Nestin (Rat-401)	Mouse mAb	Developmental Studies	1:500

2.3 DNA-RNA techniques

2.3.1 Large scale preparation of plasmid DNA

A single colony was used to inoculate 5 ml of LB medium supplemented with Ampicillin and shaken at 37°C for 8 hours. The resulting bacterial suspension was used to inoculate 250 ml of LB/ampicillin medium and shaken for further 16 hours at 37°C.

The cells were centrifuged at 4°C for 15 minutes at 5,000g. The large scale DNA preparation was carried out according to Qiagen Qiafilter Maxi DNA kit protocol.

2.3.2 RNA extraction and reverse transcription

RNA was extracted from confluent T75 flasks of NSCs according to the Qiagen RNeasy mini kit protocol. Extracted RNA was run on agarose gel to exclude degradation and verify the presence of contaminating genomic DNA. 1 mg of total RNA was used to generate cDNA with the SuperScript® First Stand Synthesis System (Invitrogen) following the manufacture's protocol.

2.3.3 Real-time PCR

Real-time quantitative PCR was performed on the 7500 real time PCR system (Applied Biosystem) using the QuantiTect SYBR Green PCR kit (Qiagen). Each reaction was performed in triplicate using 0.5 μl of cDNA in a final volume of 25 μl. The following thermal cycle was used for all the samples: 10 min-95°C; 40 cycles of 30s-95°C, 40s-primer specific annealing temperatures, 40s-72°C. For each experiment, the threshold was set to cross a point at which real-time PCR amplification was linear. Previously published primer for Hes1 and Hes5 were used (Niranjan et al., 2008). For each sample the transcript

level of the reporter gene and of the endogenous GAPDH were measured. The expression of GAPDH, an housekeeping gene, was used as internal control to normalize differences in the efficiency of the reverse transcription reaction. The expression level of each target gene was measured as a fold relative to GAPDH, according to the following formula: $2^{[C(t)\ GAPDH-C(t)\ target]}$.

2.4 *In vivo* work

2.4.1 Mice colony and genotyping

ASPP2 Δexon3 mutant mice were generated on a mixed C57BL/6Jx129SvJ background as described in Vives *et al.*, 2006 and backcrossed in a Balb/c background for 9 generations. *ASPP2* Δexon3 mutant mice were genotyped as described in Vives et al., 2006. p73 knock out mice in p73 background were obtained from Dr. Frank Mckeon and genotyped as previously described (Yang et al., 2000).

2.4.2 Histology and immunohistochemistry

Embryos at various stages were fixed in 10% buffered formalin overnight, or Boins fixative, dehydrated in an ethanol series and embedded in paraffin wax. For frozen sections, embryos were fixed overnight in 4% PFA at 4°C, cryoprotected in 30% sucrose, embedded in OCT medium and snap frozen in liquid nitrogen. Pup brains were treated with the same procedure after perfusion with 4% PFA. Sections were cut at 4μm thickness for paraffin and 12μm for frozen sections. Sections were used for hematoxilyn and eosin staining or immunohistochemistry. Prior to immunostaining, rehydrated paraffin-

embedded sections were microwaved in 10mM sodium citrate buffer (pH 6) to unmask the antigen, blocked with 10% goat serum in PBS for 1 hour and incubated overnight with the primary antibody at 4°C, followed by Alexa Fluor® (Molecular Probes) secondary antibodies for 30 minutes at room temperature. TO-PRO has been used as a counterstain.

2.4.3 BrdU injection

Pregnant females were injected with a dose of 50 mg/Kg of BrdU i.p. and sacrificed after 30 minutes for the analysis of the cell cycle length or 3 and 6 hours for the analyses of the interkinetic nuclear migration. Embryos were dissected and processed as described above for paraffin and cryostat sections.

2.4.4 Quantification of apoptosis, mitoses and cell cycle lenght

For the quantification of data obtained using immunofluorescences staining identical area of the cortex and ganglionic eminence of the ASPP2 mutant and wild type littermate were selected and the total number of cells and cells stained positive with the indicated antibodies were counted. Analysis of cell cycle length was performed as described in Chenn and Walsh (2002). For each genotype in each experiment at least three mice were analysed.

2.4.5 Transmission Electron Microscopy

Brains were dissected and fixed with 4% gluteraldehyde in Cacodylate buffer, at 4°C overnight. After fixation, the brains were sliced coronaly. The slices obtained were postfixed in 1% Osmomium tetrodoxide in cacodylate buffer for 1 hour, dehydrated in a

gradient of alcohol and embedded in resin. Sections of 1mm were cut and analysed by toluidine blue staining. The area of interest was trimmed, sections were cut at 0.1mm

Table 2.3: Antibodies used in immunohistochemistry

Antigen	Catalogue number	Source	Dilution
aPKC	610-176	BD Transduction Laboratories	1:500
BrdU	B2531	Sigma	1:500
Rhodopsin	R5403	Sigma	1:100
Neurofilament160	N2787	Sigma	1:100
Cralbp	ab65460	Abcam	1:100
Calbindin	C2724	Sigma	1:100
Ki67	RTU-Ki67-MM1	Novocastra	1:500
Pospho-Histone H3	07-081	Upstate	1:500
Active caspase 3	9661	Cell Signalling	1:500
ASPP2 S32	Rabbit pAb	Serum	1:100
ASPP2 LX116	Mouse mAb	Hybridoma	1:100
Tbr2	Rabbit pAb	Zoltan Molnar, University of Oxford	1:1000
Foxp2	Rabbit pAb	Zoltan Molnar, University of Oxford	1:1000
Er81	Rabbit pAb	Zoltan Molnar, University of Oxford	1:1000
SMI32	Rabbit pAb	Zoltan Molnar, University of Oxford	1:500
PAX6	Mouse mAb	Developmental Studies Hybridoma Bank	1:100

thickness and mounted on a TEM grid. One hundred sections from three different mice each genotype were examined to determine the percentage of open junctions.

2.4.6 *In vitro* transcription of labelled probe for in situ hybridazation

The plasmid containing a fragment of the Hes5 cDNA was linearized with HindIII (New England Biolabs) at 37 ºC overnight, run on a 1% agarose gel and purified using the Qiaquick gel extraction kit (Qiagen). 1 mg of linearized DNA were incubated with 1X transcription buffer (Roche), 1X DIG RNA labelling mix (Roche), 0.75 mM DTT and 20U of T3 RNA polymerase (Roche) at 37 ºC for 2 hours.

2.4.7 *In situ* hybridization

Buffers:

All the buffers were treated with diethylpyrocarbonate (DEPC) at the final concentration of 0.1% and autoclave to inactivate RNAses.

10x salts

2M NaCl

50mM EDTA

100 mM Tris-HCl pH 7.5

50mM NaH2PO4 $2H_2O$

50mM NaH2PO4

Hybridization buffer

1x salts

50% deionized formamide (Sigma)

0.1mg/ml yeast tRNA (Roche Diagnostic)

10% dextran sulphate (Sigma)

1x denhardt's solution (Sigma)

MABT

100mM maleic acid

150 mM NaCl

0.1% Tween 20

pH to 7.5

Wash buffer

1x SSC

50% formamide

0.1% Tween 20

20x SSC

NaCl 175.3g

Na Citrate 88.22g

ddH2O to1000

Blocking solution

MABT

2% blocking reagent (Roche Diagnostic)

10% heat inactivated sheep serum

2x Developing buffer

200 mM Tris-Hcl pH 9.8

200 mM naCl

100 mM MgCl2

5% Polyvinyl alcohol (PVA)

0.11 mM nitroblue tetrazolium chloride (NBT) (Roche Diagnostics).

0.11 mM 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Roche Diagnostics).

PVA, NBT and BCIP were freshly added before incubation of the slides.

Procedure:

E12.5 embryos were fixed overnight in diethylpyrocarbonate (DEPC)-treated 4% PFA and processed as described in section 2.4.2 for frozen sections. Labelled probe was diluited in 1:1000 in hybridazation buffer and add to the slides which were than layed with a coverslip to spread the solution over the sections. The slides were incubated at 65°C overnight in a humidified chamber. After removing the coverslip, slides were washed twice for 5 minutes in MABT at room temperature followed by two washes of 30 minutes with wash buffer at 65°C. Slides were incubated with the blocking solution for 1 hour and subsequently with anti- digoxygenin antibody (Roche Diagnostics) overnight at 4°C. Sites of hybridisation were revealed by incubation with developing buffer in the presence of NBT and BCIP (Roche Diagnostics).When the blue colour reached the required intensity slides were washed in water, dehydrated and mounted.

3. ASPP2 plays a pivotal role in the development of hydrocephalus

To further understand the functions of ASPP2 *in vivo*, a knock-out mouse was previously generated in our laboratory. The murine *ASPP2* gene was inactivated in 129Sv IT2 embryonic stem (ES) cells using a targeting vector in which exon 3 was replaced by a neomycin resistance gene cassette (Vives et al., 2006). Neomycin-resistant ES clones, in which the targeting vector was integrated by homologous recombination, were selected for injection into C57BL6/6J blastocysts. Germline transmitting chimeric mice were then crossed onto a C57BL6/6J background to produce *ASPP2* Δexon3 heterozygous mice, which were crossed to generate homozygous *ASPP2* Δexon3 mutants. Deletion of the ASPP2 protein was confirmed in *ASPP2* Δexon3 embryos using a polyclonal antibody raised against the N-terminal fragment of ASPP2 (residues 59-383) (Vives et al., 2006). Only around 6.4% of the *ASPP2* mutant pups survived birth in a mixed 129SvxC57BL/6J background, well below the expected Mendelian segregation of 25%. Gross and histological analysis of *ASPP2* mutant pups revealed a 100% incidence of hydrocephalus (Vives et al., 2006). The work described in this chapter therefore aims to investigate the underlying causes of the hydrocephalus observed in *ASPP2* mutant mice. Since the increase in intracranial pressure that characterises hydrocephalus can cause secondary abnormalities in the brain, only *ASPP2* mutant pups in a Balb/c background were used in this study, as they exhibit a less severe phenotype compared to those in a J129/C57BL6 background.

3.1 *ASPP2* mutant pups develop hydrocephalus independently of the genetic backgrounds

Since *ASPP2* mutant pups in the mixed 129SvxC57BL/6J background were born at a significantly reduced rate and all died before weaning, *ASPP2* mutant mice were generated in a Balb/c background through backcrossing for 9 generations in order to obtain a pure background. Unlike the mixed 129SvxC57BL/6J background, *ASPP2* mutant mice in the Balb/c background were born according to the expected Mendelian segregation of 25% (Figure 3.1), and the majority survived after weaning. Interestingly, as in the 129SvxC57BL/6J background, all of the *ASPP2* mutant mice born in the Balb/c background developed hydrocephalus similarly to the 129SvxC57BL/6J., This suggests that ASPP2 plays an important role in the development of the central nervous system (CNS), regardless of the genetic background.

Hydrocephalus is a condition in which the cerebrospinal fluid (CSF) accumulates in the ventricles, generating abnormal pressure. As a consequence of the increased pressure generated by the CSF in the brain, ventricles of the newborn *ASPP2* mutant animals appeared to be drastically dilated (Figure 3.2B) compared to those of wild-type mice (Figure 3.2A). In 10% of *ASPP2* mutant pups with severe hydrocephalus the cortices were remarkably thinner than the one of the wild-type, and the anatomy of the brain was severely compromised, as shown by the shift of the hippocampus along the midline of the brain. Although the thinning of the cortex may be a consequence of the increased intracranial pressure associated with the hydrocephalus, other causes like an increase in neural apoptosis cannot be ruled out. The majority of *ASPP2* mutant mice displayed mild hydrocephalus (Figure 3.2 C-D) and survive up to sixth month of age. No blockage of the Aqueduct of Sylvius, which allows the flow of CSF between the brain ventricles, was

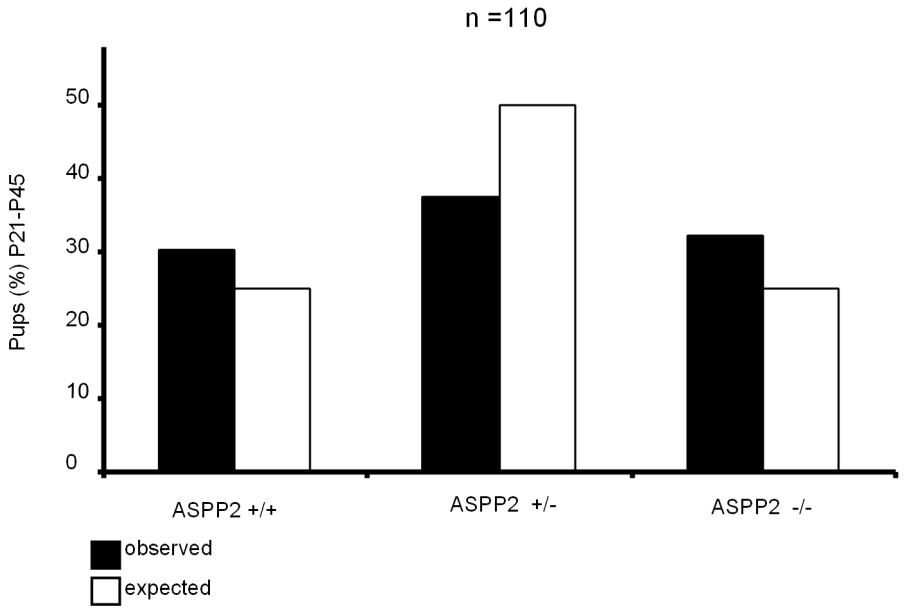


Figure 3.1: ASPP2 deletion is not embryonic lethal in Balb/c background. Genotype distributions obtained from *ASPP2* heterozygous mating between postnatal day 21 (P21) and P45 in a cohort of 110 pups. The Mendelian segregation frequency (expected) and the observed frequency (observed) are shown.

detected in *ASPP2* mutant pups (Figure 3.2 E-F). Therefore, the hydrocephalus observed was not due to an obstruction of CSF's in *ASPP2* mutant mice.

In 50% of the *ASPP2* mutant mice, hydrocephalus was associated with massive brain haemorrhage. Thus, the ultra structure of blood vessels was analysed by transmission electron microscopy (TEM) in wild-type and *ASPP2* mutant pups. The blood vessels did not display any morphological abnormalities in *ASPP2* mutant pups (Figure 3.3). Tight junctions (TJs) between endothelial cells, which maintain the integrity of the blood vessels, were detected in *ASPP2* mutant pups. (Therefore, the intracranial bleeding observed in *ASPP2* mutant pups was most probably secondary to the compression of tissues generated by the elevated intracranial pressure. It will be deleted in agreement with point 17)

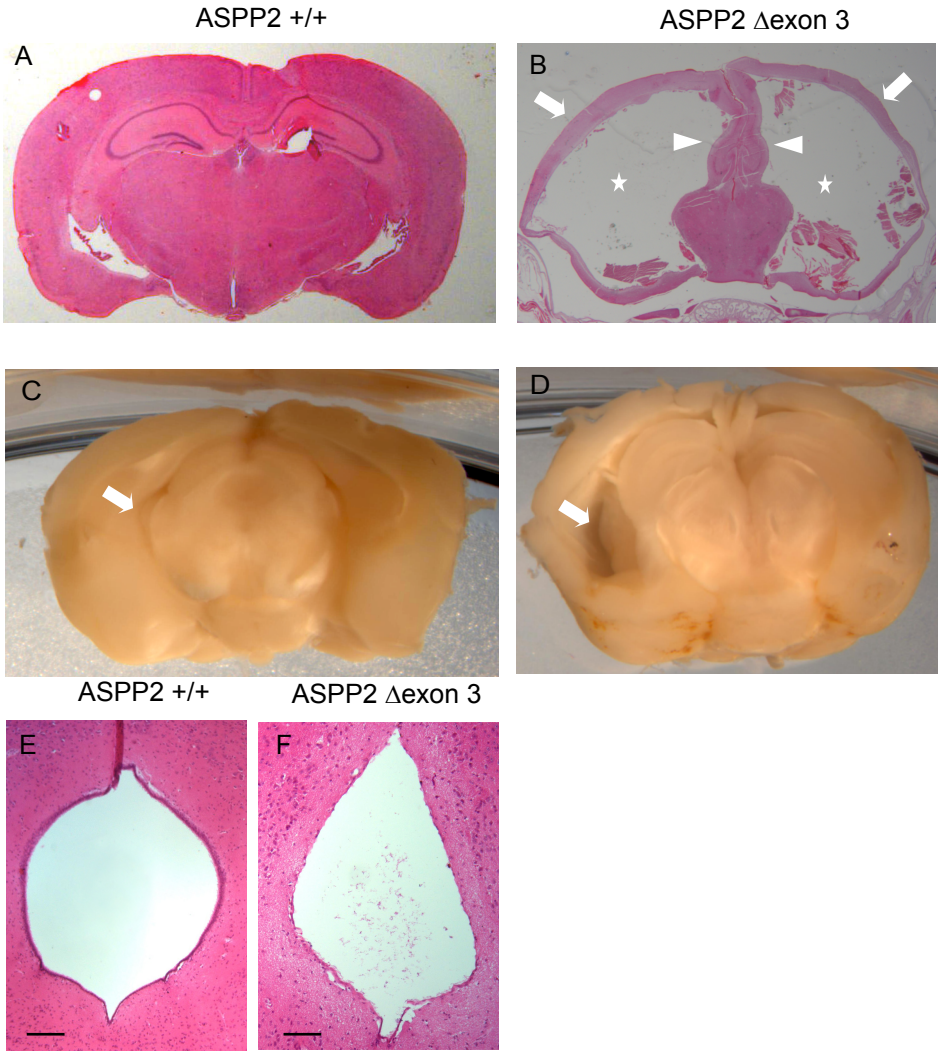


Figure 3.2: *ASPP2* mutant mice display hydrocephalus in Balb/c background without obstruction of the acqueduct of Sylvius. (A-B) Haematoxilyn and eosin (H&E) staining of coronal sections from P21 wild-type (A) and *ASPP2* mutant (B) littermates. The stars in (B) indicate the dilated ventricles in *ASPP2* mutant brain. The arrows in (B) indicate the cortex of the *ASPP2* mutant pups, while the arrowheads indicate the shifted hippocampus. Brains from wild-type and *ASPP2* mutant pups at P30 with mild hydrocephalus. The arrows in C and D indicate the ventricles. (E-F) H&E staining of coronal sections from P21 wild-type (C) and *ASPP2* mutant (D) littermates at the level of the aqueduct of Silvius. Scale bar 10 μm.

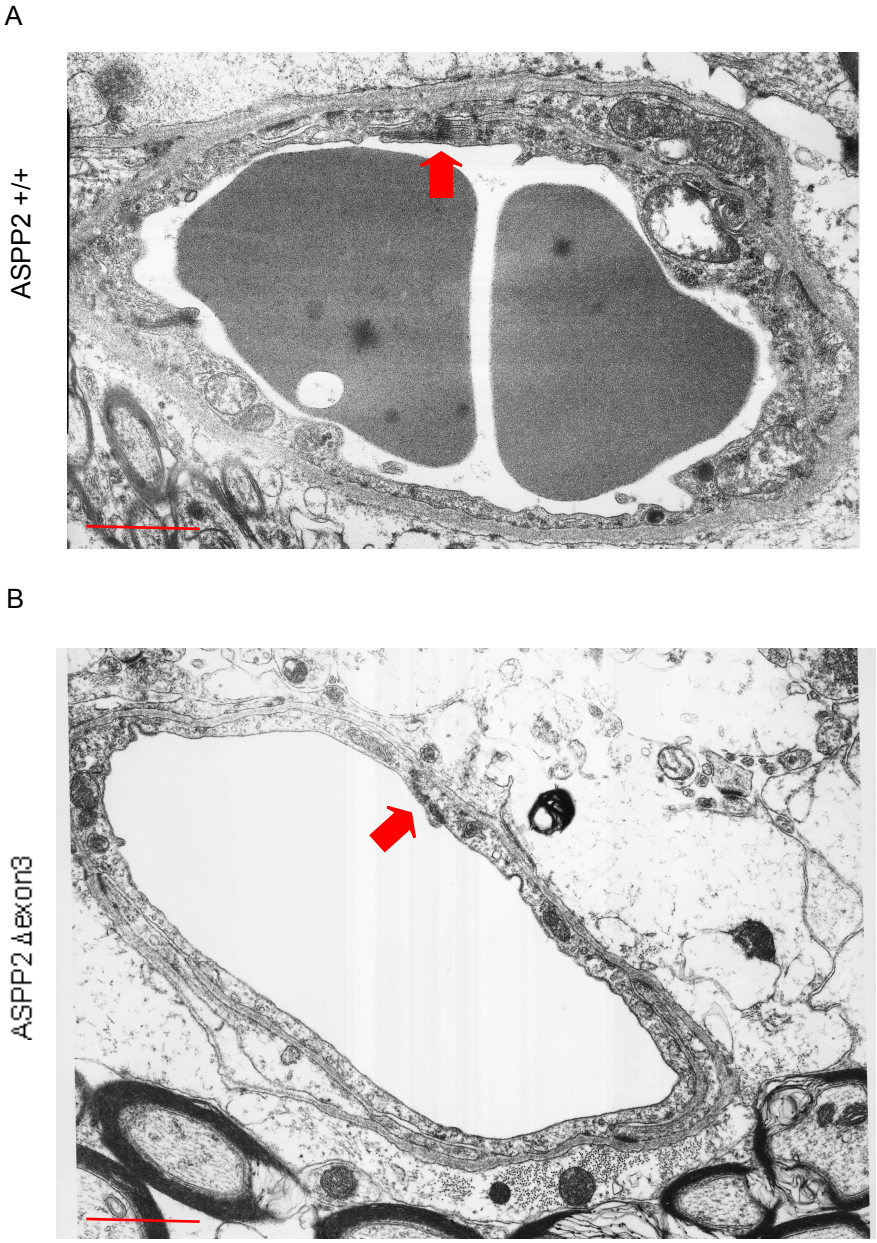


Figure 3.3: ASSP2 deletion does not cause ultra-structure abnormalities in the brain blood vessels. TEM of brain blood vessels of the cerebral cortex in wild-type (A) and *ASPP2* mutant (B) pups at P45. Arrows indicate TJs between adjacent endothelial cells. Scale bar 500 nm.

3.2 Developmental abnormalities in the choroid plexus of *ASPP2* mutant mice

The choroid plexus (CP) is a vascular structure that arises from the wall of the ventricles, and is responsible for the production of CSF. The CP epithelium forms the blood-CSF barrier which prevents the diffusion of molecules from the blood to the brain cavities. The barrier function of the CP is ensured by the presence of TJs between adjacent epithelial cells (see section 1.5.2). Since several abnormalities in the CP have been linked to the development of hydrocephalus (see section 1.5.3), the structure of the CP was analysed in *ASPP2* mutant pups. Only mice with very mild hydrocephalus were used to avoid secondary changes due to increased intracranial pressure. Analysis by haematoxylin and eosin (H&E) staining and light microscopy revealed a smaller CP in *ASPP2* mutant mice compared to wild-type littermates (Figure 3.4A-B). A significant dilation between the two layers of epithelial cells was also observed (Figure 3.4 C-D).

A detailed analysis of the wild-type and mutant CP by TEM did not reveal any abnormalities in the number and structure of the cilia (Figure 3.5A, D), or in the basal membrane epithelium (Figure 3.5B, E), the alteration of which is known to cause hydrocephalus (see section 1.5.3). A normal 9+2 structure of CP cilia was detected in *ASPP2* mutant mice (Figure 3.5A' and D'). TEM also revealed that cells of the CP were round instead of cuboidal in *ASPP2* mutants, with individual cells forming protrusion into the ventricles (Figure 3.5C, F). In addition, the tight/adherens junctions between cells were less electron-dense in *ASPP2* mutant pups when compared to the wild-type (Figure 3.6 A-B), and many of the TJs at the apical poles of the CP cells failed to close tightly (Figure 3.6 C). As shown in Figure 3.6D, a higher percentage of open TJs was detected in the *ASPP2* mutant CP. Disruption of the TJ marker ZO-1 (Figure 3.7 C-D), which

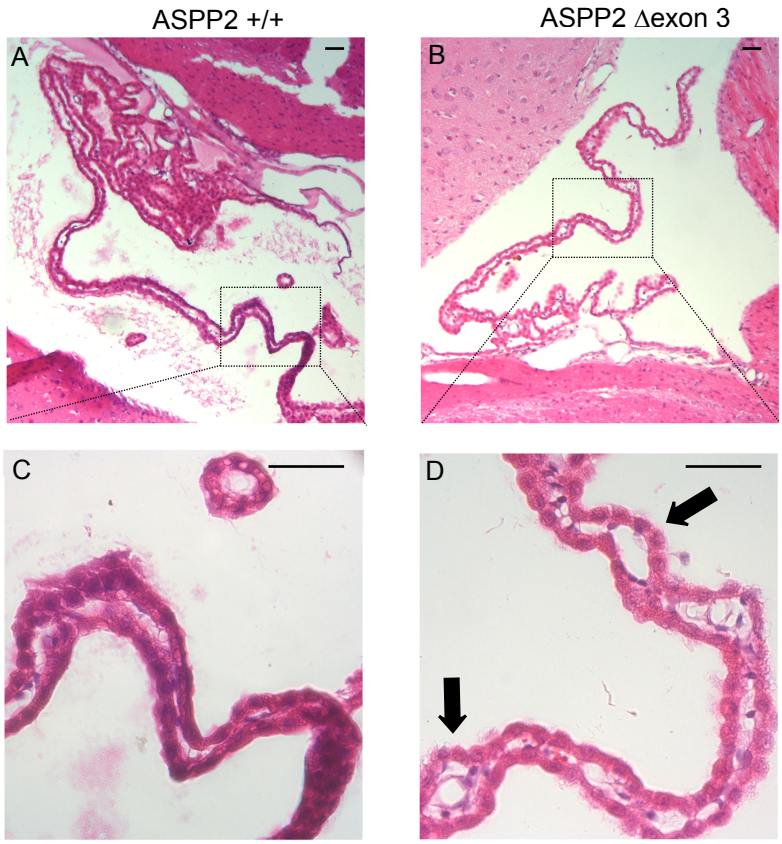


Figure 3.4: *ASPP2* mutant pups exhibit developmental and structural abnormalities in the choroid plexus. H&E staining of a representative coronal sections from P45 wild-type (A, C) and *ASPP2* mutant choroid plexus (B, D) at the level of the fourth ventricle. The boxed regions in (A) and (B) are shown at higher magnification in (C) and (D). Arrows in (D) indicate the dilatation between the two layers of epithelial cells. Scale bar 10 μm. For each genotype five animals were examined.

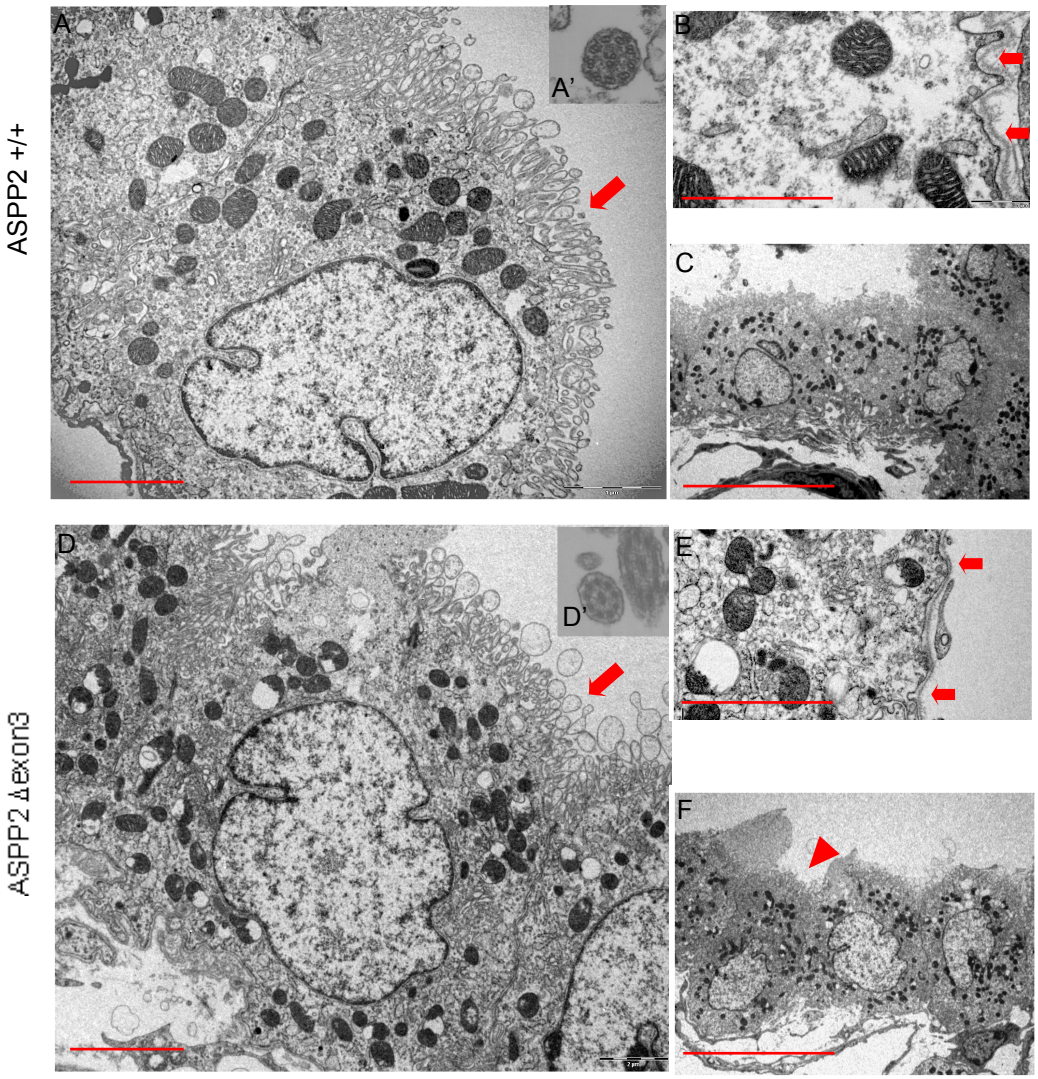


Figure 3.5: Ultra-structure alterations in the choroid plexus of *ASPP2* mutant mice. Transmission electron microscopy of the fourth ventricle choroid plexus from wild-type (A-C) and *ASPP2* mutant littermates (D-F) at P60. Arrows in (A) and (D) indicate microvilli and cilia at the apical cell pole. (A') and (D') represent transverse sections of cilia. Arrows in (B) and (E) indicate the basal membrane. Arrowheads in (F) indicates round-shaped cells in the choroid plexus of *ASPP2* mutant mice. (A, D) Scale bar 1µm; (B, E) Scale bar 500 nm; (C, F) Scale bar 5 µm. For each genotype three animals were analysed.

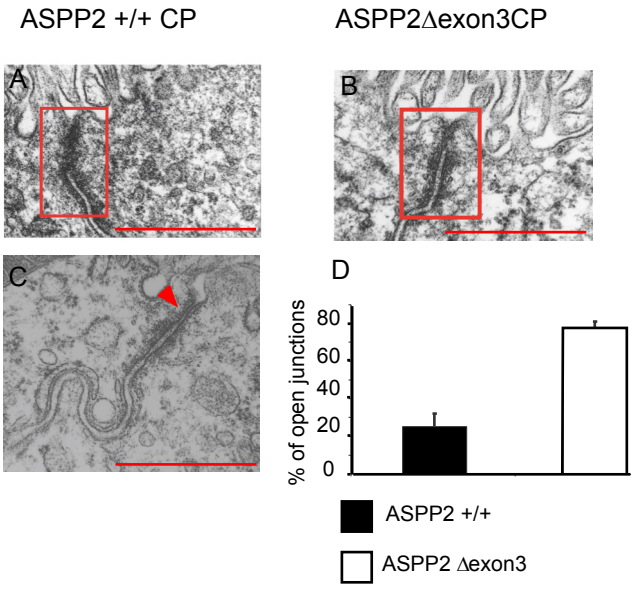


Figure 3.6: ASPP2 maintains the integrity of tight junctions *in vivo.* Transmission electron microscopy of the fourth ventricle choroid plexus from wild-type (A) and *ASPP2* mutant (B) littermates at P60. Boxed region in (A) and (B) represent the tight/adherens junction at the apical pole of the cell. The arrowhead in (C) indicates an open tight junction in *ASPP2* mutant choroid plexus. Histogram in (D) shows the percentage of open junctions in the wild-type and *ASPP2* mutant choroid plexus. n indicates the number of mice included in the quantification for each genotype. Scale bar in (A-B), 1 µm; Scale bar in C-F, 500 nm. One hundred sections from three different mice each genotype were examined to determine the percentage of open junctions.

extensively colocalises with ASPP2 (Figure 3.7E), was also observed in the *ASPP2* mutant CP.

Molecular aggregates stained by secondary anti-mouse, but not anti-rabbit, antibody were detected in the ventricles of *ASPP2* mutant mice during examination of histological sections (Figure 3.8). These aggregates may be constituted by proteins, therefore indicating a leakage at the blood-CSF barrier. Since the aggregates may also be cell debris, further experiments will be needed to conclude whether the deletion of ASPP2 compromises the function of the blood-CSF barrier.

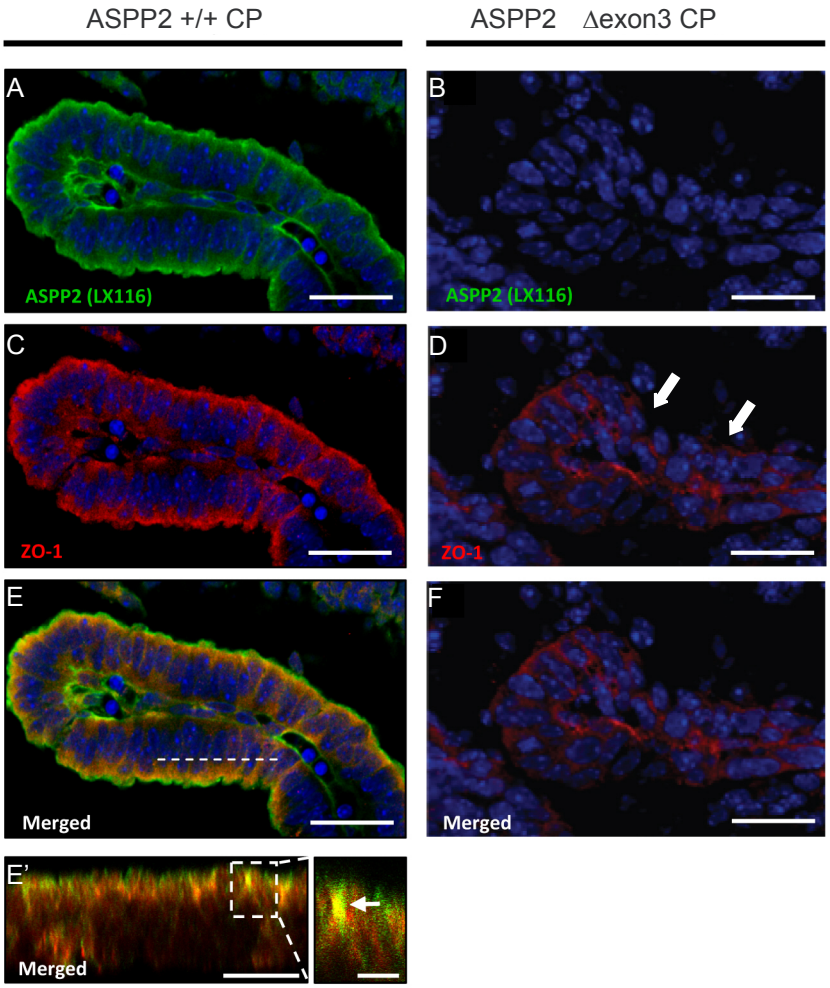


Figure 3.7: Disruption of ZO-1 staining pattern in *ASPP2* mutant choroid plexus. Coronal sections from wild-type (A, C, E) and *ASPP2* mutant (B, D, F) choroid plexus co-immunostained with anti-ZO-1 and anti-ASPP2 antibodies. Arrows in (D) indicate the distruption of ZO-1 staining in *ASPP2* mutant choroid plexus. A cross-section (location indicated by broken line) of the *ASPP2* mutant choroid plexus is indicated in (E'). Arrow in (E') indicates the colocalisation between ASPP2 and ZO-1 at the apical domain. Staining patterns and corresponding antibodies are colour-coded. Topro (blue) was used as a counterstain. Scale bars 28 µm, 10 µm, and 2.5 µm (A–F).

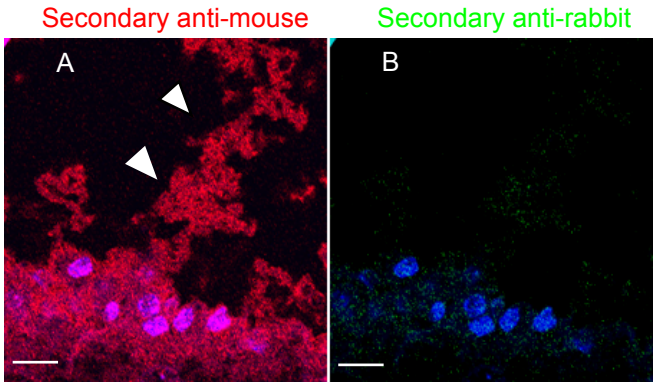


Figure 3.8: Molecular aggregates detected in the brain cavities of *ASPP2* mutant mice. Immunostaining of coronal sections of the ventricular surface from P60 *ASPP2* mutants with secondary anti-mouse (A) and anti-rabbit (B) antibodies. Arrowheads in (A) indicate molecular aggregates within the *ASPP2* mutant ventricles. Scale bar 10 μm.

3.3 ASPP2 and p73 share overlapping functions during development

A study conducted in our laboratory identified p73 as an activator of ASPP2 *in vitro* (Bergamaschi et al., 2004). Moreover, p73 knock-out mice develop non-obstructive hydrocephalus accompanied by a severely underdeveloped CP (Meyer et al., 2004; Yang et al., 2000), similar to *ASPP2* mutant mice. This similarity suggests that p73 and ASPP2 may functionally interact *in vivo*. In order to test this hypothesis, mice double heterozygous for *p73* and *ASPP2* were inter-crossed. In a cohort of 180 pups the expected combinations of genotypes were obtained, except for the *p73-ASPP2* double knock-out which was never observed post-natally. Moreover, the percentage of *ASPP2*-null pups obtained was reduced in the *p73* +/- background (Figure 3.9). In order to understand the impact of *p73* and *ASPP2* deletion during development, embryos were obtained from a double *p73-ASPP2* heterozygous mating and collected at embryonic day 13.5 (E13.5). Since *p73-ASPP2* double knock-out embryos were still viable at this stage, embryos of all the nine genotypes were analysed by light microscopy (Figure 3.10). *ASPP2*-null embryos in *p73* +/- or -/- backgrounds were runty and did not seem to be properly developed, which could explain the lethality observed at later developmental stages.

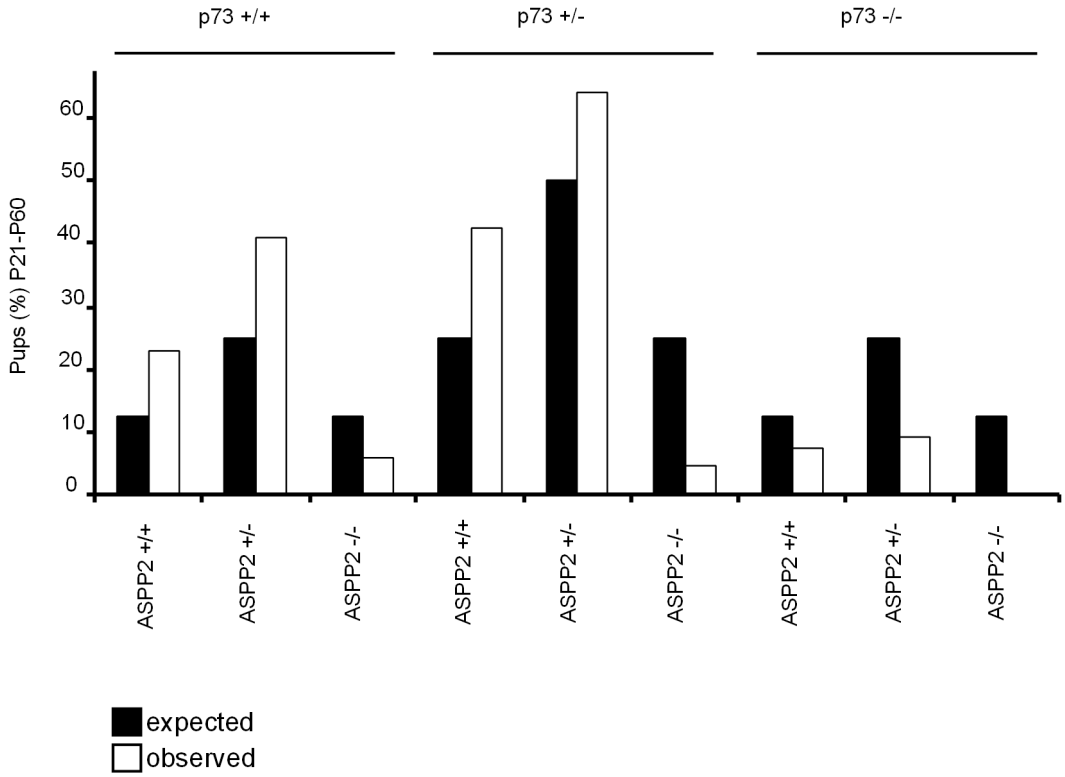


Figure 3.9: The double deletion of *p73* and *ASPP2* is lethal. Comparison of the expected and observed Mendelian segregation frequency in a cohort of 180 pups generated from crossing of *p73* and *ASPP2* mutant mice.

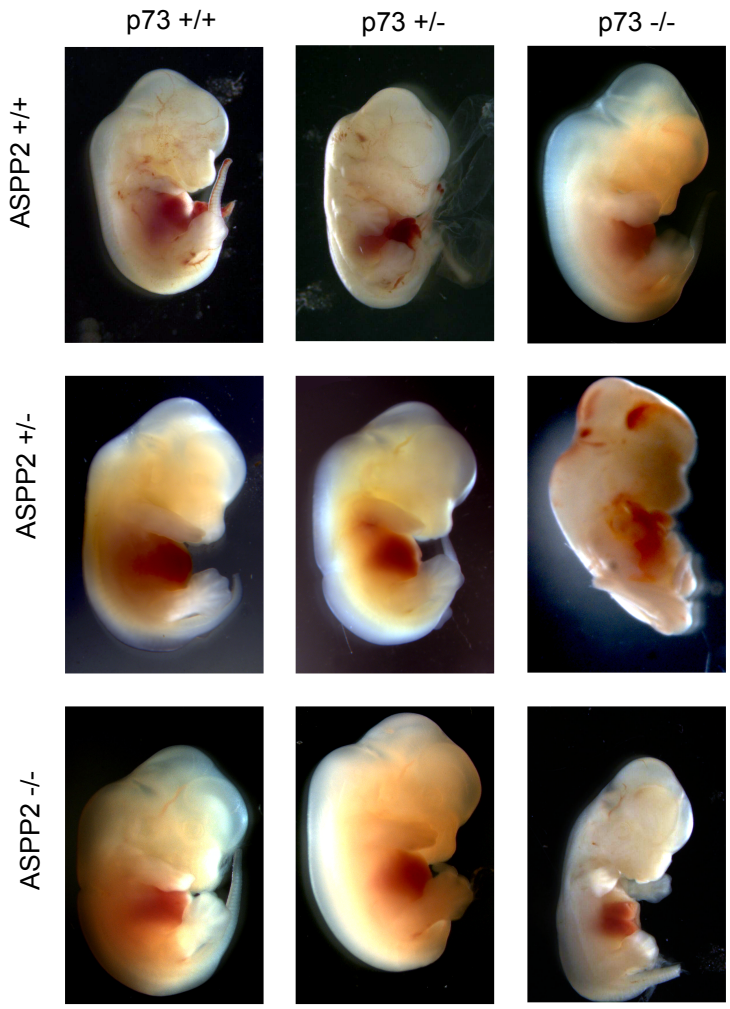


Figure 3.10: Developmental abnormalities of *ASPP2* +/- and *ASPP2* -/- embryos in *p73* null background. Light microscopy analysis of embryos obtained from p73-ASPP2 heterozygous mating at E13.5.

3.4 p73 and ASPP2 cooperate in the development of the CP

Since *ASPP2* mutant mice display hydrocephalus accompanied by an underdeveloped CP, and p73 interacts with ASPP2 *in vivo* and *in vitro*, it can be hypothesised that p73 and ASPP2 cooperate during the development of the CP. To validate this theory, the CP of embryos generated from a p73-ASPP2 heterozygous mating were analysed in matching sections by H&E staining and light microscopy. As shown in Figure 3.11 A-B, the sizes of the forebrain and hindbrain CPs were reduced by the single deletion of *p73* or *ASPP2*. The double deletion of *p73* and *ASPP2* caused a further reduction in the size of the CPs, in particular in the hindbrain.

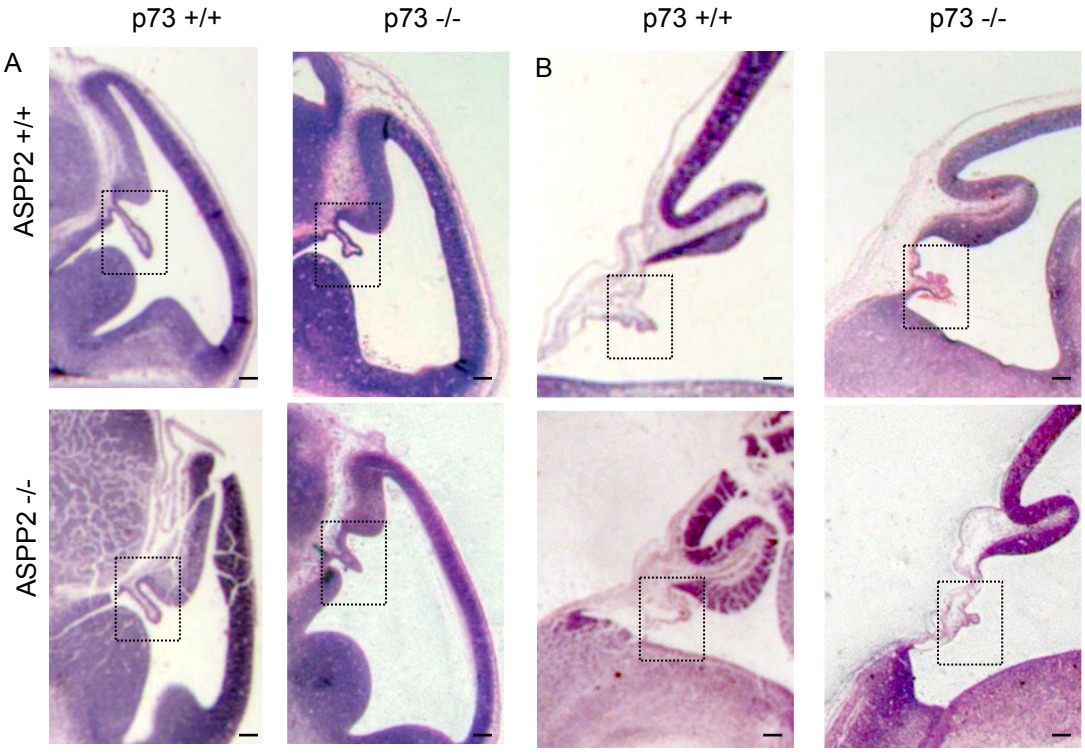


Figure 3.11: p73 and ASPP2 cooperate during choroid plexus development. H&E stained sagittal sections of E13.5 forebrain (A) and hindbrain (B) in the indicated genotype. The boxed regions show the choroid plexus. Scale bar 10 μm.

3.5 Hydrocephalus in *ASPP2* mutant mice is associated with ependymal denudation

Ependymal cells form a continuous layer of multiciliated cells lining the ventricles. Loss of ependymal cells, or denudation, has been associated with hydrocephalus (Sarnat, 1995). In order to study the ependymal cells in *ASPP2* mutant mice, the ventricular surface of P21 pups was analysed by histology and light microscopy. While the lateral ventricles of wild-type pups had a continous ependymal lining (Figure 3.12A), in large areas of the *ASPP2* mutant ventricles ependymal cells were not present (Figure 3.12B). Foci of accumulated abnormal cells were detected in *ASPP2* mutant brains, randomly distributed along the ventricular surface (Figure 3.12B-C). In order to verify whether these clusters of cells were constituted by mislocalised ependymal cells, immunostaining with the ependymal cell marker S100 was performed. Ependymal cells in the wild-type brain stained positive for S100, while no staining was detected in the *ASPP2* mutant brain (Figure 3.12D-E). This result indicates that the cells in the clusters were not ependymal. Since accumulation of clusters of β-III tubulin-positive neurons has been reported in hydrocephalus (Dominguez-Pinos et al., 2005), immunostaining with β-III tubulin was performed in *ASPP2* mutant brains. As expected, the ependymal cells in the wild-type ventricles were β-III tubulin negative (Figure 3.13A), whereas the cells in the foci of mutant mice brains were positive for this marker (Figure 3.13B), thus confirming that they are (post-mitotic will be removed in agreement with point 23) neurons. Only projections of neurons located in deeper layers of the cortex were detected underneath the ependymal layer in the wild-type brain (Figure 3.13B).

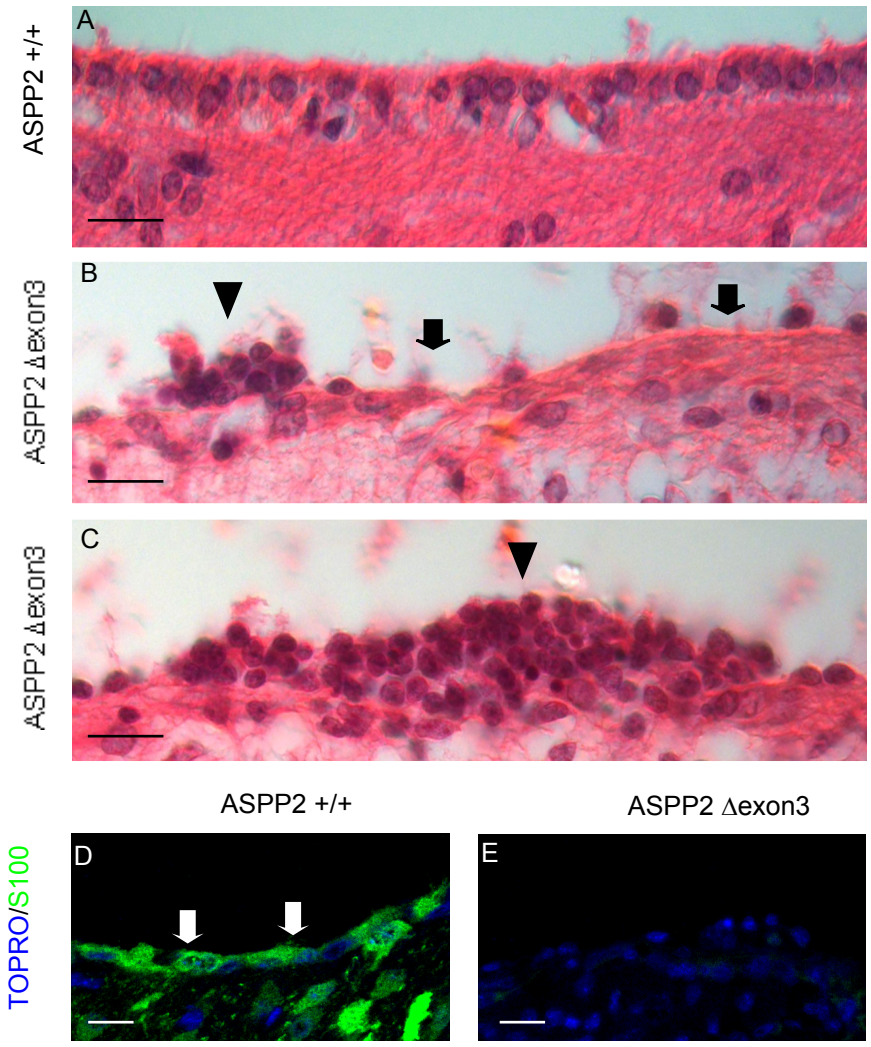


Figure 3.12: Ependymal denudation and accumulation of abnormal cell clusters in *ASPP2* mutant brain. Lateral ventricular surface of P21 wild-type (A) and *ASPP2* mutant (B, C) brains stained with H&E (A-C) and S100 (D-E). Arrows in (B) indicate the ares of ependymal denudation in the ASPP2 mutant brain. Arrowheads in (B) and (C) indicate foci of abnormal cells on the ventricular surface of *ASPP2* mutant brain. Arrows in (D) indicate the S100 positive ependymal cells. Topro (blue) was used as a counterstain. Scale bar 10 μm.

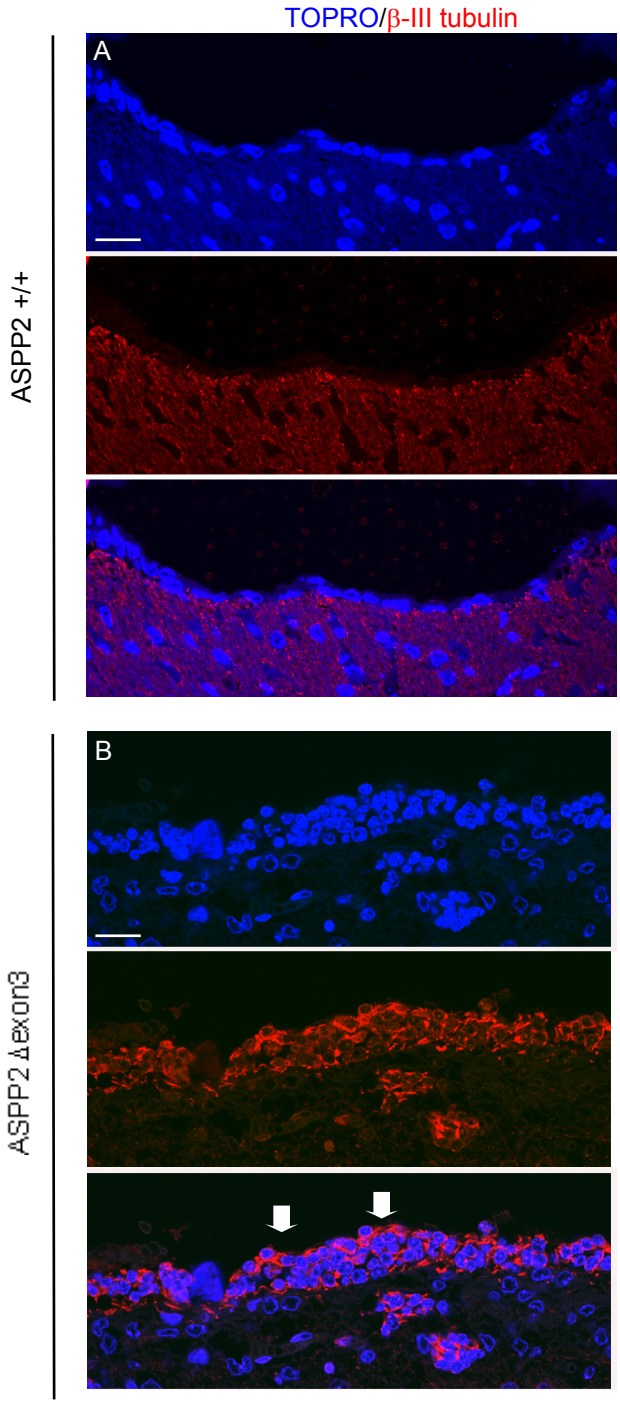


Figure 3.13: Accumulation of β – III tubulin positive neurons on the *ASPP2* mutant ventricular surface. Lateral ventricular surface from wild-type (A) and *ASPP2* mutant brain (B) at P21 stained with β–III tubulin. Arrows in B represent a cluster of β–III tubulin positive neurons abnormally accumulating on on the ventricular surface. Scale bar 10 μm.

3.5 Summary

The aim of the work described in this chapter was to identify the mechanism responsible for the development of hydrocephalus in *ASPP2* mutant mice. The two main structures implicated in the pathogenesis of hydrocephalus are the CP and the ependymal cells. These structures were analysed by histology and TEM in *ASPP2* mutant pups with mild hydrocephalus. The CP of *ASPP2* mutant pups appeared less developed in comparison to that of wild-type pups and displayed several abnormalities. The epithelium of the CP was severely disorganised in *ASPP2* mutants and composed of round, instead of cuboidal, cells. Moreover, TJs at the apical pole fail to close properly in the *ASPP2* mutant CP, suggesting that ASPP2 is required for proper TJ assembly or maintenance *in vivo.* TJs between epithelial cells are responsible for the blood-CSF barrier property of the CP, which prevents the leakage of blood-borne molecules into the CSF. (A leakage of the blood-CSF barrier was detected in *ASPP2* mutant pups, as indicated by the accumulation of aggregates in the ventricles will be deleted in agreement to point 22). The defects observed in TJs could be responsible for a leakage of the blood-CSF barrier which may cause an increase in osmotic pressure, with consequent accumulation of water therefore resulting in hydrocephalus. Since the molecular aggregates detected in the ASPP2 mutant ventricles could represent protein or cell debris from ependymal denudation, further experiments will be needed to determine whether the blood-CSF barrier is defective in ASPP2 mutant mice.

Mice deficient for *p73*, which is a known ASPP2 binding partner, exhibit hydrocephalus associated with underdevelopment of the CP, similar to that seen here in *ASPP2* mutant mice. The analysis of the genotype distribution of pups derived from a *p73-ASPP2* heterozygous mating revealed that the numbers of *ASPP2* null pups obtained

was dependent on *p73* gene dosage. Double knock-out for *p73* and *ASPP2* was never detected post-natally, and the percentage of *ASPP2* null pups obtained was also reduced in a *p73* +/- background. In line with the lethality of these combinations, embryos null for *ASPP2* were runty and underdeveloped in a *p73* +/- or -/- background. Moreover, analysis of embryonic CP histology revealed that double deletion of *p73* and *ASPP2* caused a further reduction in the size of the CP, suggesting that p73 and ASPP2 may cooperate during the development of the CP. Since double knock-out mice for *p73* and *ASPP2* all died *in utero,* it is not possible to conclude whether underdevelopment of the CP can have an impact on the pathogenesis of hydrocephalus. However, these data do suggest that p73 and ASPP2 may functionally interact during development *in vivo*, even if non-overlapping functions of these two proteins can still contribute to the lethality of the *p73-ASPP2* deletion.

Finally, analysis of the ependyma by histology and light microscopy revealed a lack of ependymal cells in the ventricular surface of *ASPP2* mutant pups, suggesting that ASPP2 may play a role in the formation of ependymal cells or may prevent the denudation of the ependymal layer. The beating of ependymal cilia ensures the movement of CSF within the ventricles. The overall reduction in the number of ependymal cells lining the ventricles observed in *ASPP2* mutant brains may, therefore, disturb the flow of CSF and further contribute to the hydrocephalus. In addition to the ependymal loss observed, clusters of post-mitotic neurons were found on the ventricular surface in *ASPP2* mutant brains, indicating that the loss of ependymal cells in *ASPP2* mutant mice is associated with an abnormal migration of neurons in the ventricular cavity.

4. ASPP2 is required for the maintenance of epithelial cell polarity

Tight junctions (TJs) and adherens junctions (AJs) form the apical-junctional complex (AJC), which plays a central role in the establishment and maintenance of epithelial cell polarity, ensuring the separation between the apical and basolateral domains (see section 1.3.1). As described in Chapter three, the deletion of *ASPP2* affects tight junctions (TJs) in the epithelium of the choroid plexus, raising the possibility that ASPP2 could also affect cell polarity. Genetic analysis of model organisms such as *C. elegans* and *D. melanogaster* has demonstrated that three polarity complexes also play a major role in cell polarity. Two of these polarity complexes, Crumb/Pals1/Patj (CRB) and Par3/Par6/aPKC (PAR), localise at the apical membrane domain, while the Scrib/Dlg/Lgl (SCRIB) complex localised basolaterally (see section 1.3.2-1.3.4).

Studies in *Drosophila* and transgenic mice have demonstrated that components of polarity complexes and AJCs play key roles in neurogenesis, neuronal migration and axon extension during the development of the central nervous system (CNS). In particular, mice null for components of AJ, such as N-cadherin, β–catenin or the polarity protein aPKC, exhibit in fact disordered neuroepithelial architecture and impaired neuronal migration in the cortex (Imai et al., 2006; Kadowaki et al., 2007; Machon et al., 2003).

The work described in this chapter aims to investigate the role of *ASPP2* in the maintenance of epithelial cell polarity by a detailed analysis of the CNS of *ASPP2* mutant embryos. The embryonic cortex was also analysed as a model in which cell polarity plays a pivotal role during development.

4.1 Loss of cell polarity in *ASPP2* mutant radial glial cells

In order to establish whether the disruption of TJs observed in *ASPP2* mutant mice could also affect cell polarity in the CNS, wild-type and *ASPP2* mutant embryos were analysed at E13.5. Loss of tissue organisation in several areas of the developing CNS was observed in *ASPP2* mutant embryos in both J129/C57BL6 and Balb/c backgrounds. In the wild-type retina, retinal precursor cells were arranged radially along the apical basal axis (Figure 4.1A). In contrast, the retinal precursor cells in the *ASPP2* mutant retina had lost their normal orientation and formed rosette-like structures (Figure 4.1B-C), which resembled the ones described in primary neuroectodermal tumours of the CNS (Pfister et al., 2009). In addition, normal radial orientation of radial glial cells (RGCs) was lost in the ganglionic eminence (GE), cortex and spinal canal of *ASPP2* mutant embryos. In the control embryos, the ventricular zone (VZ) of these regions was columnar and pseudostratified (Figure 4.1D, F, H). In contrast, cells in the VZ in the *ASPP2* mutant embryos were disorganised, less columnar and protruded into the ventricles (Figure 4.1E, G, I). Immunostaining for cell polarity proteins and components of the TJs and AJs was performed at E13.5 in wild-type and *ASPP2* mutant embryos. In normal neuroepithelia, aPKC and Par3 localised apically (Figure 4.2A, C). In contrast, the distribution of these proteins was markedly disorganised in *ASPP2* mutant neuroepithelia in the GE (Figure 4.2B, D). Similarly, the apical localisation of β-catenin and ZO-1 was impaired in *ASPP2* mutant neuroepithelia (Figure 4.2E-H). Loss of cell polarity was also observed in the cortex (Figure 4.2J-K) of *ASPP2* mutant embryos, as suggested by mis-localisation of β–catenin staining. Although the mis-localisation of polarity proteins is a marker of tissue

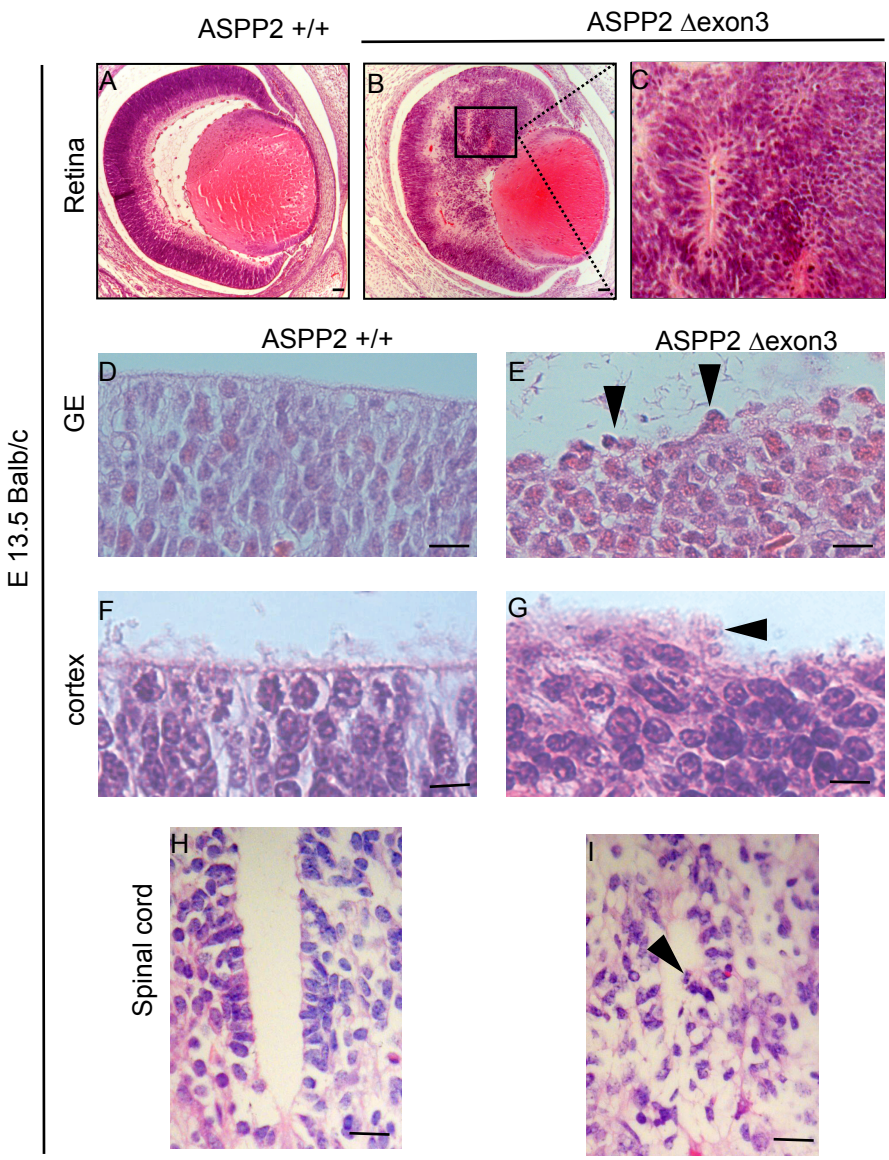


Figure 4.1: Loss of neuroepithelial organisation in the central nervous system of *ASPP2* mutant embryos. H&E staining of the retinas (A-C), GE (D-E), cortex (F-G) and spinal cord (H-I) from wild-type and *ASPP2* mutant embryos at E13.5. The square in (B) is enlarged in (C) and represents a rosette. Arrowheads indicate cells protruding in the ventricles in *ASPP2* mutant embryos. Scale bar 10 μm.

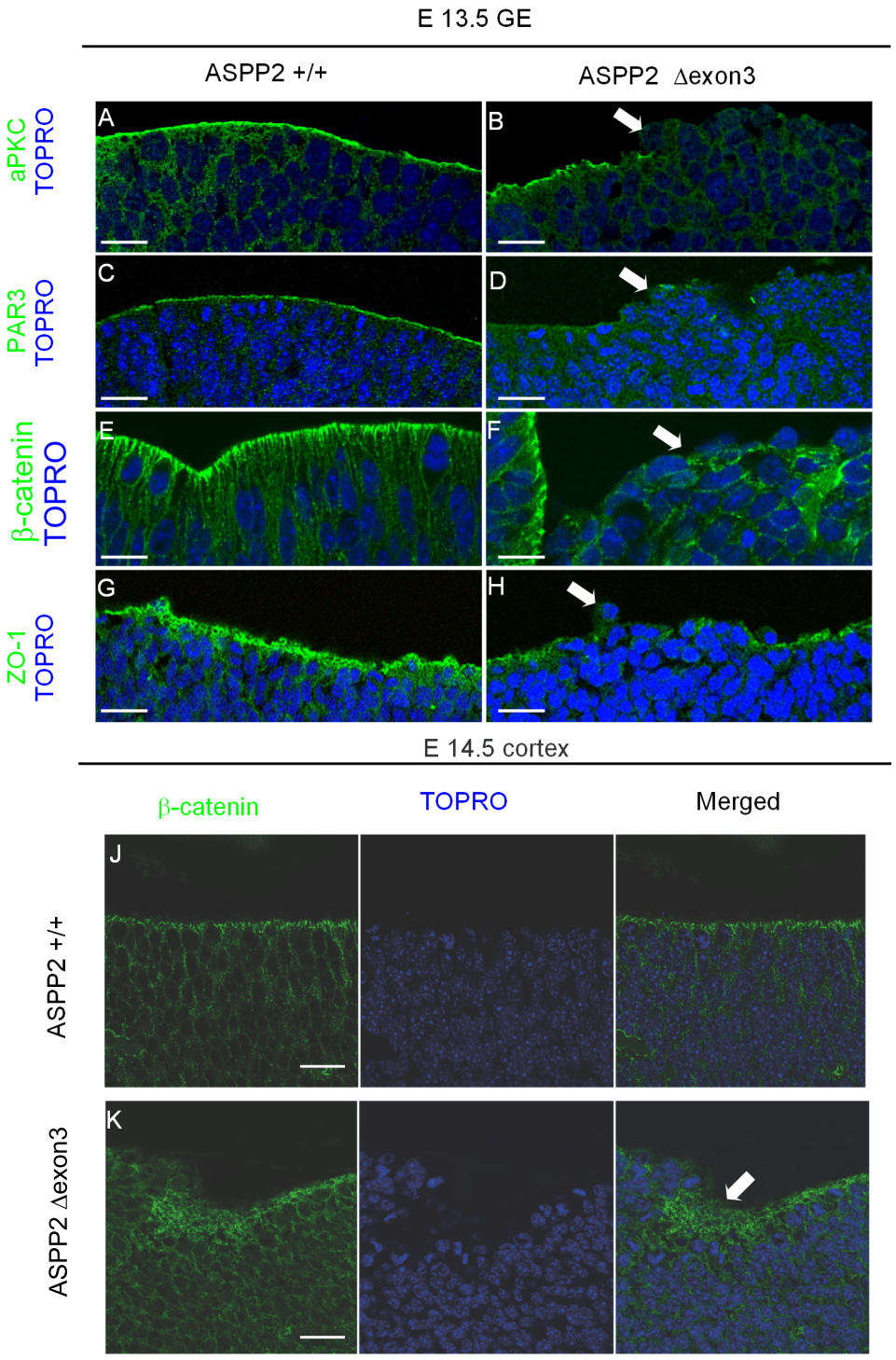


Figure 4.2: Loss of cell polarity in *ASPP2* mutant neuroepithelia. (A-H) Coronal sections from wild-type and *ASPP2* mutant GE at E13.5 stained with the following antibodies: (A-B) aPKC, (C-D) Par-3, (E-F) β-catenin and (G-H) ZO-1. (I-J) β-catenin immunostaining at E14.5 of coronal sections from wild-type and *ASPP2* mutant cortex. Arrows indicate the loss of cell polarity and mis-localisation of apical junctional complex proteins in *ASPP2* mutant progenitors. Topro (blue) has been used as a counterstain. Scale bar 10 μm.

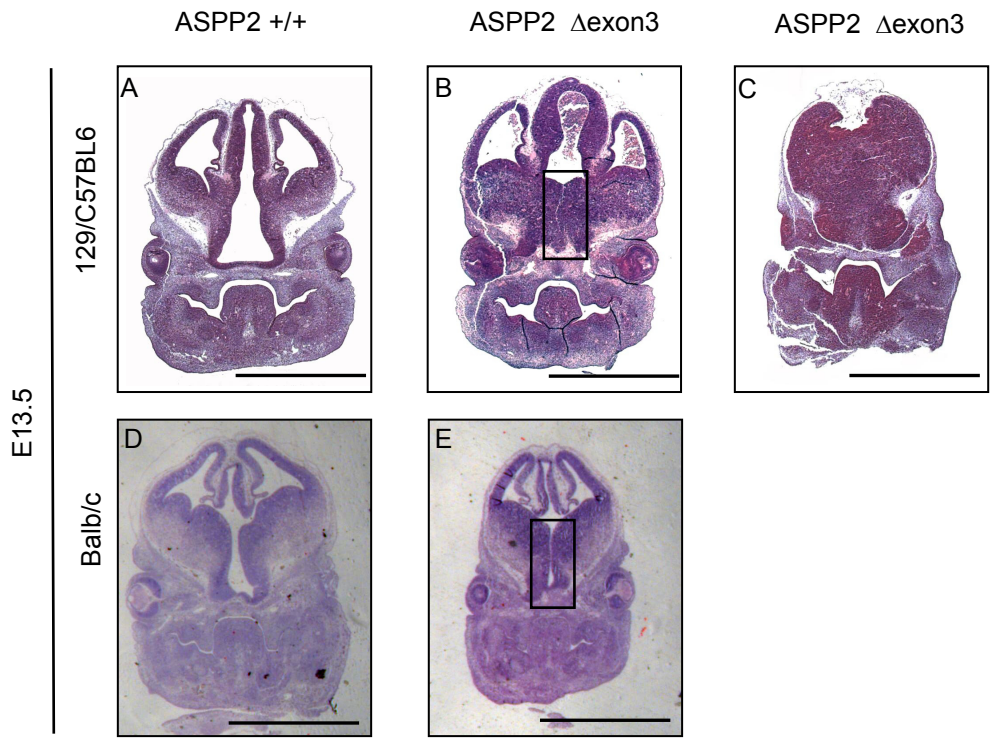


Figure 4.3: Expansion of the neuroepithelia in *ASPP2* mutant embryos. H&E staining of coronal sections from wild-type and *ASPP2* mutant embryos at E 13.5 in J129/C57BL6 (A-C) and Balb/c (D-E) backgrounds. The boxed regions indicate the abnormal growth of the neuroepithelia in *ASPP2* mutant embryos at the level of the third ventricle. Scale bar 1 mm.

disorganisation, it is still not clear whether this mis-localisation is the primary event or vice-versa.

Expansion of the neuroepithelium was observed in *ASPP2* mutant embryos, either in the ganglionic eminence (GE) at the level of the third ventricle (Figure 4.3B) or throughout the brain (Figure 4.3C), when compared to wild-type embryos (Figure 4.3A). Rosette-like structures were also present in the over-proliferating neuroepithelia of the *ASPP2* mutants. Expansion of the neuroepithelia was also detected in Balb/c embryos, although the overgrowth was less dramatic than that seen in the mixed 129/C57BL6

background. The majority of *ASPP2* mutant embryos in the Balb/c background only exhibited overgrowth in the third ventricle (Figure 4.3E) compared to the wild-type (Figure 4.3D).

4.2 ASPP2 colocalises with components of the Par polarity complex and AJC *in vivo* and *in vitro*

To better understand how ASPP2 controls cell polarity, its expression pattern in the GE and cortical neuroepithelium was analysed using an antibody against the N-terminal domain of ASPP2 (residues 291-300) for immunohistochemistry (IHC) on paraffin sections. ASPP2 is expressed in the cytoplasm of neuroepithelial cells in both the GE and cortex. ASPP2 staining was particularly strong at the apical side of the GE and cortex, where the Par polarity complex and AJC are localised (Figure 4.4A-B). No signal was detected in the *ASPP2* mutant mice, confirming the specificity of the antibody (Figure 4.4C). Since the SCRIB complex localises basolaterally, and the deletion of Crumbs in mice does not phenocopy ASPP2 deletion, the following co-localisation analysis was focused on the PAR complex and the AJC. Co-localisation of ASPP2 with Par-3, ZO1 and β–catenin was analysed in the GE at E13.5. Interestingly, ASPP2 extensively co-localises with PAR3 and ZO-1 (Figure 4.4D-E), but only partially with β–catenin (Figure 4.4E) at the apical side of the neuroepithelium. Similar results were obtained when the expression pattern of ASPP2 was analysed in the breast cancer cell line MCF-7, an epithelial cell line (Figure 4.5). Interestingly, in MCF-7 cells ASPP2 was also found in the perinuclear region, although the expression pattern and the intensity of the signal seems to vary in different cells.

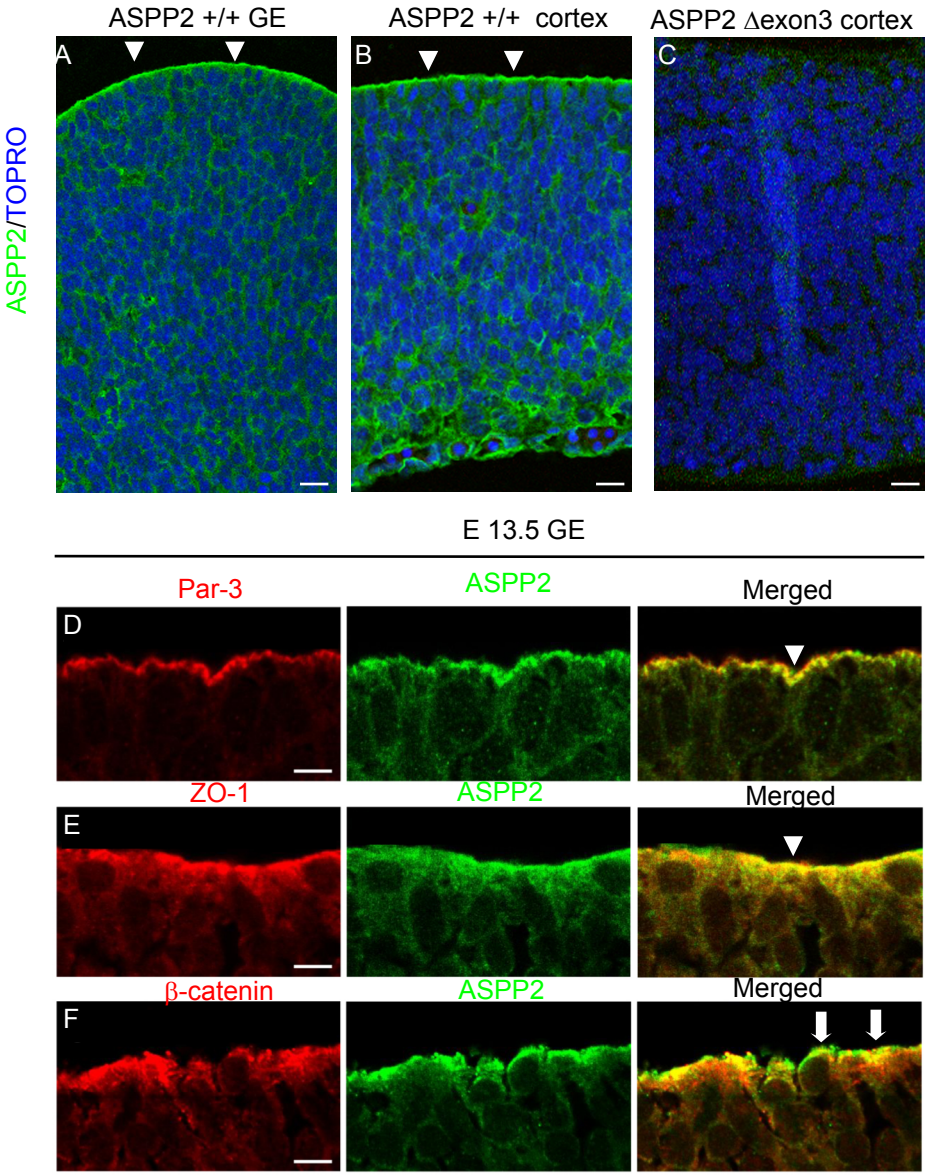


Figure 4.4: ASPP2 colocalises with the PAR complex and apical junctional complex components *in vivo.* Coronal sections from E 13.5 wild-type GE (A) and cortex (B) stained with anti-ASPP2 antibody (S32). Arrows in (A) and (B) indicate the apical localization of ASPP2 in the neuroepithelium. Coronal sections from E13.5 wild-type GE stained with the following antibodies: (E) Par-3, (F) ZO-1 and (G) β–catenin. Arrowheads in (D) and (E) indicate the colocalisation between ASPP2 and Par3/ZO-1, while arrows in F indicates the partial colocalisation between ASPP2 and β–catenin. Staining patterns and corresponding antibodies are colour-coded. Topro (blue) has been used as a counterstain. Scale bar 10 μm.

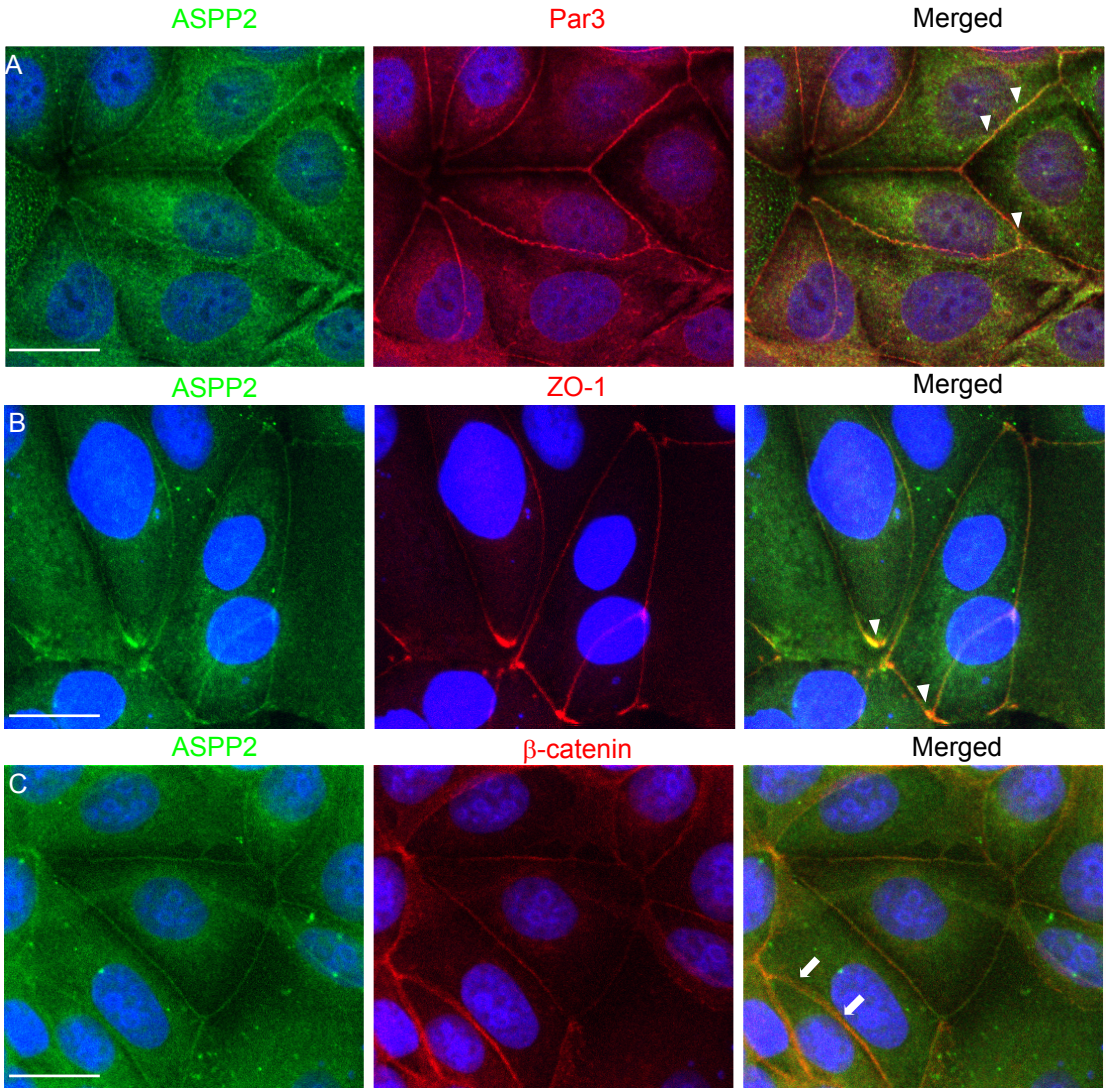


Figure 4.5: ASPP2 colocalises with the PAR complex and apical junctional complex components *in vitro*. Immunostaining of ASPP2 and Par3 (A), ZO-1 (B) and β–catenin (C) in MCF-7 cells. Arrowheads in (A) and (B) indicate the colocalisation between ASPP2-PAR3 and ASPP2-ZO-1. Arrows in (C) indicate the partial colocalisation between ASPP2 and β–catenin. Staining patterns and corresponding antibodies are colour-coded. Topro (blue) has been used as a counterstain. Scale bar 10 μm.

4.3 ASPP2 maintains the architecture of the cortex

To establish whether the disorganisation of the neuroepithelium observed in *ASPP2* mutant mice has an impact on the organisation of highly laminated structures, the cortices of *ASPP2* mutant mice were analysed. As severe expansion of neuroepithelia throughout the brain is often observed in *ASPP2* mutant embryos in a mixed 129/C57BL6 background, preventing the identification of different structures in the forebrain, the cortex could only be analysed in the Balb/c background. To examine the effect of ASPP2 deletion on the gross morphology of the neocortical region, histological analysis was first performed by light microscopy at E13.5. In control embryos, RGCs were uniformly packed and radially oriented (Figure 4.6A), while groups of cells were randomly oriented and protruded into the ventricles in *ASPP2* mutant embryos (Figure 4.6B). Gross histological analysis of cortical lamination at E14.5 also revealed abnormalities. While clearly visible in the control embryos (Figure 4.6C), the ventricular zone (VZ), subventricular zone (SVZ) and intermediate zone (IZ) were severely disorganised and difficult to distinguish in *ASPP2* mutant embryos (Figure 4.6D). The abnormalities were more evident at E14.5, therefore subsequent analyses were conducted at this stage of development.

Since the organisation of the neocortex was severely disrupted in *ASPP2* mutant embryos, the position of mitotic cells labelled with an anti-phosphohistone H3 antibody was analysed. During each cell cycle, RGCs undergo a distinctive pattern of oscillation in the VZ termed interkinetic nuclear migration (INM). Cells go through S phase at the basal surface of the VZ, and mitosis at the apical surface (see section 1.4.2). As expected, in control embryos, mitotic cells were mainly found at the ventricular surface while only a small minority were found in the non-surface area of the VZ and SVZ (Figure 4.7A). By

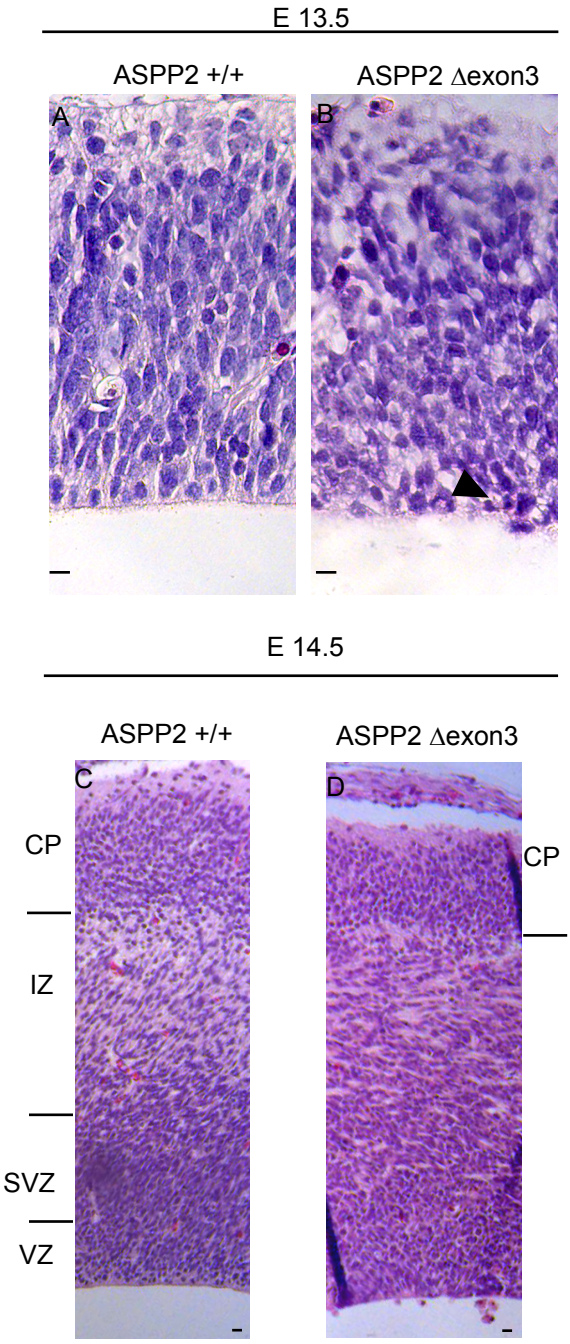


Figure 4.6: Histological abnormalities in *ASPP2* mutant neocortex. (A-B) H&E staining of coronal sections from wild-type and *ASPP2* mutant cortices at E13.5. Arrowhead in (B) points at a group of cells protruding into the ventricle. (C-D) H&E staining of coronal sections from wild-type and *ASPP2* mutant cortices at E14.5. The distribution of VZ, SVZ, IZ and CP is indicated. VZ: ventricular zone; SVZ: subventricular zone; IZ: intermediate zone; CP: cortical plate. Scale bar 10 μm.

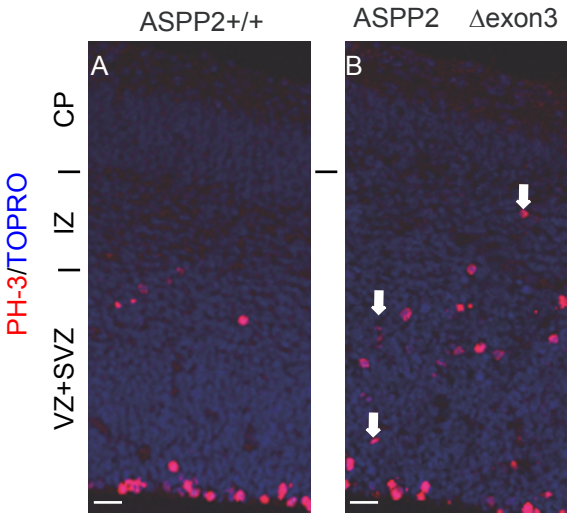


Figure 4.7: Mis-localisation of mitotic cells in *ASPP2* mutant neocortex. Phospho-histone H3 immunostaining of coronal sections from wild-type (A) and *ASPP2* mutant cortices (B) at E14.5. Arrows in (B) indicate mis-localised mitotic cells in *ASPP2* mutant cortex. VZ: ventricular zone; SVZ: subventricular zone; IZ: intermediate zone; CP: cortical plate; PH-3: Pospho-Histone 3. Topro (blue) has been used as a counterstain. Scale bar: 10 μm.

contrast, only a small proportion of mitotic cells were found on the ventricular surface of *ASPP2* mutant embryo neocortices, with the majority scattered throughout the cortex (Figure 4.7B). The mis-localisation of mitotic cells observed in *ASPP2* mutant embryos suggests that ASPP2 may play a role in controlling INM. In order to analyse INM in the *ASPP2* mutant cortex, pregnant females from an *ASPP2* heterozygous mating at E14.5 were injected with a single pulse of BrdU. BrdU is a synthetic thymidine analogue that is incorporated into newly synthesised DNA during S-phase of the cell cycle. Cells were labelled with BrdU and their position determined after 30 min (Figure 4.8A-B), 3 hours (Figure 4.8C-D) or 6 hours (Figure 4.8E-F). The number of BrdU-positive nuclei at the apical side of the VZ was then divided by the total number of BrdU-positive cells, identified in a 10 μM high area after a 3 or 6 hour pulse (Figure 4.8C-F), obtaining a

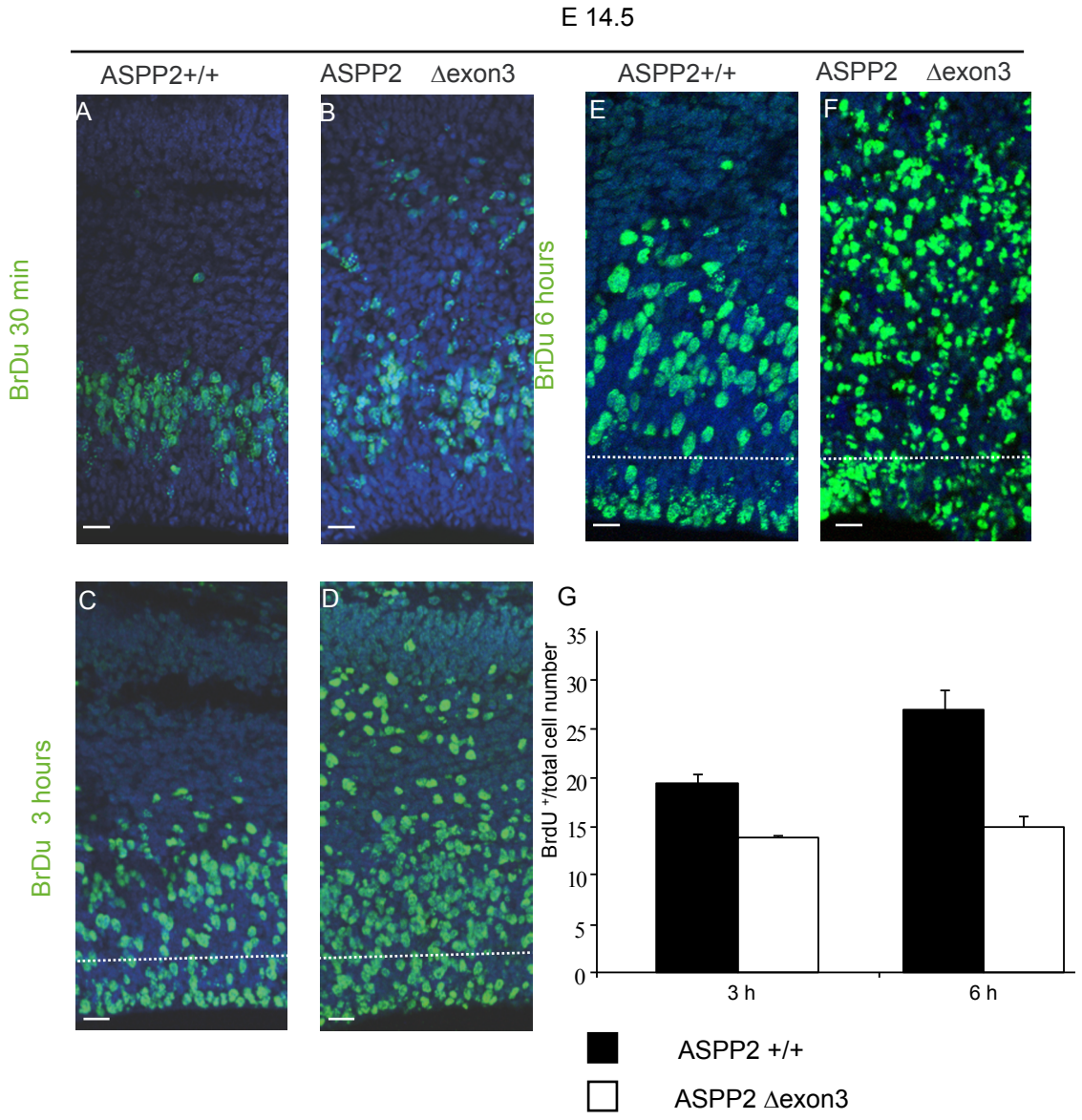


Figure 4.8: Expansion of ventricular and subventricular zone in ASPP2 mutant neocortex. Distribution of BrdU positive cells after a 30 min (A-B), 3 hour (C-D) and 6 hours (E-F), BrdU single pulse in wild-type and *ASPP2* mutant cortices at E14.5. (G) Analysis of Interkinetic Nuclear Migration (INM) as percentage of BrDu positive nuclei at the apical position identified in a 10 μm high area (dashed line in F-I). Error bars represent the standard deviation of three independent experiments. Topro (blue) has been used as a counterstain. Scale bar 10 μm.

percentage of BrdU-positive nuclei that was used to estimate the INM. The percentage of cells detected at the apical side was 14 ±0.08% and 15±1% in *ASPP2* Δexon3 cortices compared to 19±0.8% and 27±2% in *ASPP2* wild-type cortices after 3 and 6 hour BrdU pulses, respectively (Figure 4.8G). The clear reduction in the percentage of BrdU-positive cells detected at the apical side of *ASPP2* mutant cortices, in comparison to those in the wild-type, may indicate a cortical defect in INM in *ASPP2* mutant embryos. In order to fully test this hypothesis in vivo time laps microscopy in brain slices, which allows to follow the cell movement in the VZ, will be needed.

4.4 Interkinetic nuclear migration is not affected *in vitro*

INM is a complex process regulated by cell autonomous and non-autonomous mechanisms (see sections 1.4.2 and 1.4.5). To determine whether *ASPP2* deletion affects nuclear movement, neural stem cells (NSCs) were isolated from the telencephalon of wild-type and *ASPP2* mutant mice as described by Conti and colleagues (Conti et al., 2005). NSCs were grown as an adherent culture on laminin, supported by human epidermal growth factor (EGF) and human fibroblast growth factor-2 (FGF-2), which have been shown to mediate cell proliferation in the embryonic telencephalon (Drago et al., 1991; Gensburger et al., 1987; Kilpatrick and Bartlett, 1993). Adherent NSCs uniformly display morphological, biological and molecular features of RGCs and can therefore be used to study INM *in vitro* (Conti et al., 2005). In order to assess the identity of the cells isolated from wild-type and *ASPP2* mutant telencephala, immunocytochemistry was performed with antibodies against nestin and RC2, markers specific for RGCs, and the neuronal marker βIIItubulin. As shown in Figure 4.9, the cells isolated uniformly expressed nestin and RC2, while failing to express βIII-tubulin, confirming that they are RGCs.

To examine the nuclear movement, the nucleokinesis of long bipolar cells with characteristic RGC morphology was followed by time-lapse microscopy over a period of 8 hours. Nuclear movements were observed in both wild-type and *ASPP2* mutant cells (Fig.4.10 A-B). In order to quantify the speed of migration of the nuclei, only cells that had reached a similar level of confluency were analysed. As shown in Figure 4.10C, no difference was detected between wild-type and ASPP2 mutant RGCs in term of speed of nuclear migration, indicating that INM is not affected *in vitro* following *ASPP2* deletion.

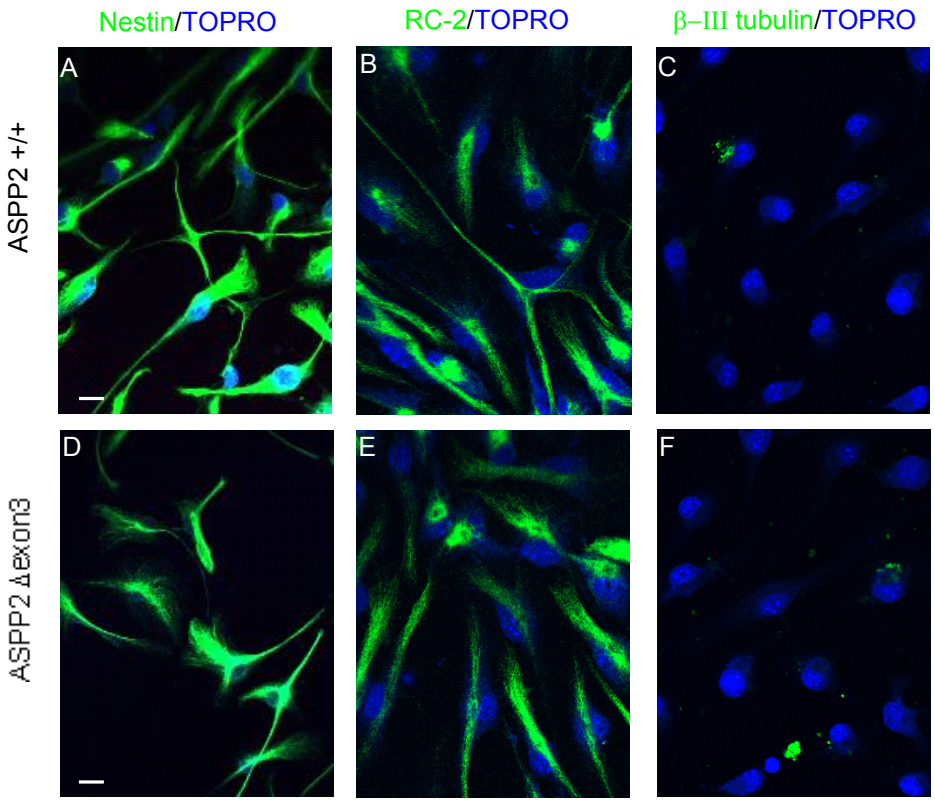
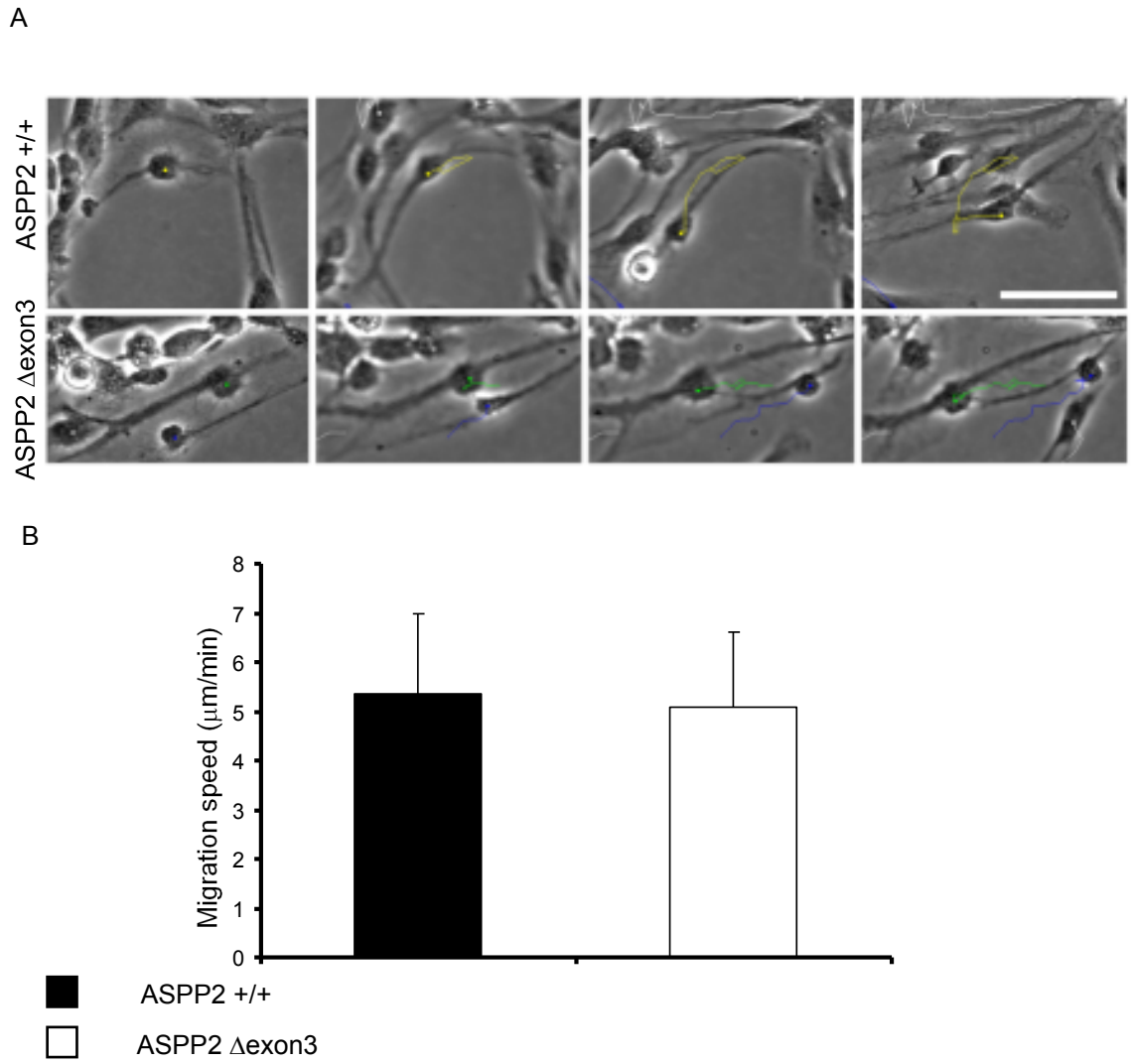


Figure 4.9: Isolation of radial glial cells from E12.5 wild-type and *ASPP2* mutant telencephala. Immunostaining of wild-type and *ASPP2* mutant radial glial cells with antibodies against nestin (A, D), RC2 (B, E) and βIII–tubulin (C, F). Scale bar 10 μm.



4.10: ASPP2 deletion does not affect Interkinetic Nuclear Migration *in vitro.* (A) Frames from time lapse experiments. The snapshots were taken at two hours intervals. Yellow lines show the movement of the indicated nuclei. Scale bar 100 μm. (B) Quantification of the nuclear migration speed in wild-type and *ASPP2* Δexon3 NSC. 10 cells per each field of view were included in the quantification in two separate experiments.

4.5 ASPP2 controls lamination and differentiation in the cortex and retina

To further characterise the role of ASPP2 in the development of the neocortex, the distribution of two laminar markers Pax6 and Tbr2 was analysed. Pax6 is a homeodomain transcription factor expressed in apical progenitors, and Pax6-positive nuclei accumulate in the VZ of the normal cortex. In contrast, Tbr2 (T-box brain 2) is a transcription factor expressed by intermediate progenitor cells (IPCs) in the SVZ during development (see section 1.4.1). While Pax6 and Tbr2-positive cells were only found in the VZ or SVZ respectively in wild-type embryos (Figure 4.11A-C), they were scattered outside those regions in *ASPP2* mutant neocortices (Figure 4.11B-D).

As progenitor cells were displaced in *ASPP2* mutant neocortices, the distribution of bIII-tubulin-expressing post-mitotic neurons was also analysed. Cortical neurons arise from the proliferative pseudostratified epithelium, at the margin of embryonic cerebral ventricles. As cortical development proceeds, progenitor cells exit the cell cycle, begin to differentiate into neurons and migrate out of the VZ towards their final resting place in the developing cortex in a process known as radial migration (see section 1.4.3). In wild-type embryos, bIII-tubulin-expressing postmitotic neurons were found at the outer margin of the cortex (Figure 4.10E). In the VZ, only neuronal projections were identified (Figure 4.11F). However, in *ASPP2* mutant embryos, bIII-tubulin-positive neuronal bodies were found in the VZ (Figure 4.11F-H). Quantification of the percentage of βIII-tubulin-positive cells in wild-type and *ASPP2* mutant cortices (Figure 4.11I) showed an overall reduction in the number of differentiated neurons in the *ASPP2* mutant cortex.

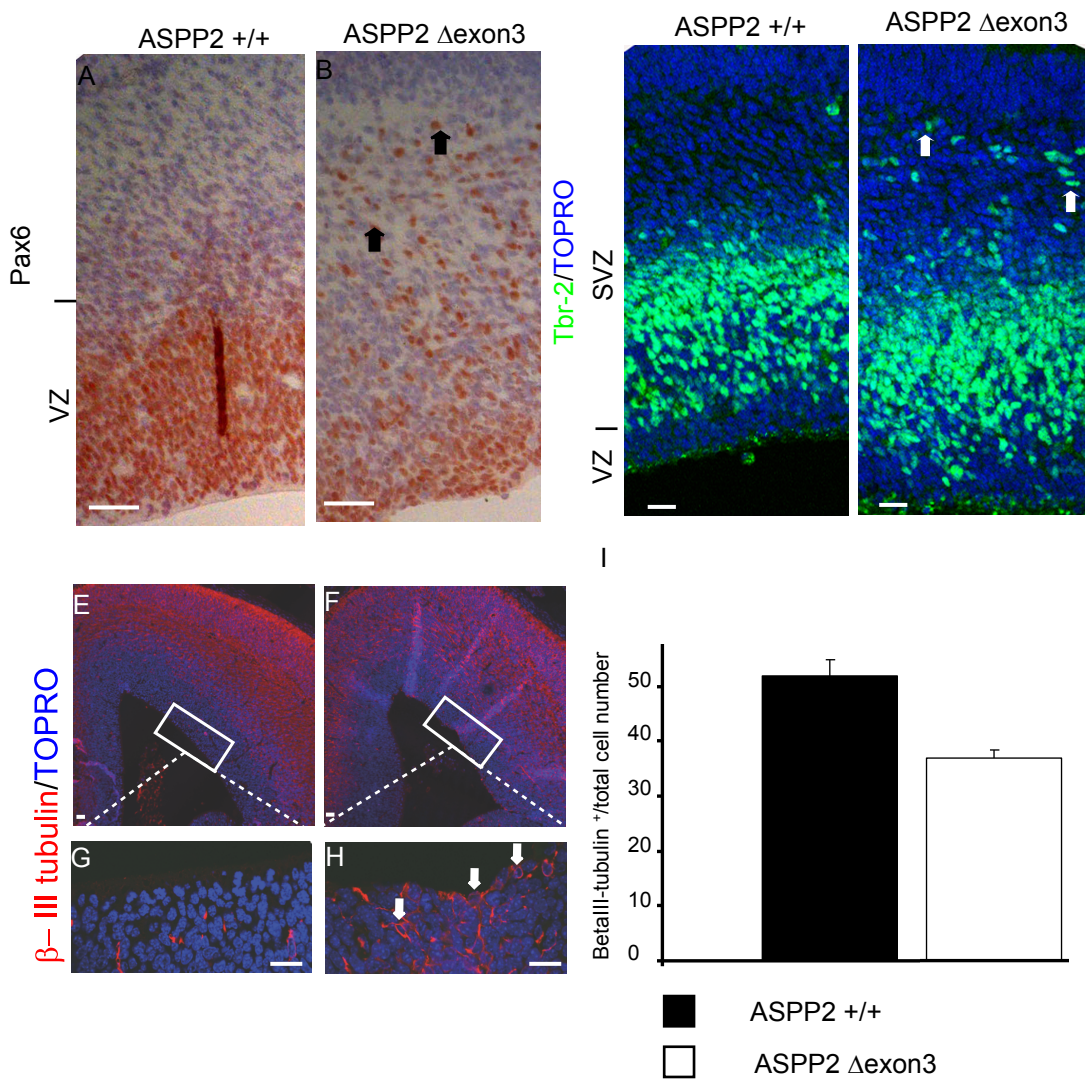


Figure 4.11: Lamination and differentiation are impaired in *ASPP2* mutant neocortex. (A-B) Pax6 and (C-D) Tbr2 immunostaining of coronal sections from wild-type and *ASPP2* mutant neocortices at E14.5. Black arrows in (B) and white arrows in (D) point at mislocalised Pax6 and Tbr2 positive cells respectively. βIII-tubulin immunostaining of coronal sections from wild-type (E,G) and *ASPP2* mutant (F, H) neocortices at E14.5. The boxed regions in (E) and (F) are shown at higher magnification in (G) and (H). Arrows (H) point at the somata of neurons ectopically localized in the VZ of *ASPP2* mutant cortices. (I) Percentage of βIII-tubulin positive cells in wild-type and *ASPP2* mutant cortices. Error bars represent the standard deviation of three independent experiments. VZ: ventricular zone; SVZ: subventricular zone; IZ: intermediate zone; CP: cortical plate. Topro (blue) has been used as a counterstain. Scale bar 10 μm.

To further analyse the role that ASPP2 plays in cortical development, markers of early (Foxp2 and Er81) and late (SMI32)-born neurons were used to analyse the lamination of postnatal P15 wild-type and *ASPP2* mutant cortices. While Er81 and Foxp2-expressing early-born neurons were detected in the correct layers in wild-type and mutant cortices (Figure 4.11A-D), SMI32-expressing late-born neurons were mis-localised in the mutant cortex (Figure 4.12E-F). Moreover, the number of SMI-32 positive cells appeared to be reduced in the cortices of *ASPP2* mutant mice (Figure 4.12E-F).

The effect of ASPP2 on lamination was further analysed in the retina, another highly laminated structure, using several markers for different retinal cell types. Although all cell types were present in the *ASPP2* mutant retina, they were mis-localised (Figure 4.13A-H). Partial lamination was observed in rosette-like structures that displayed a heavy staining of rhodopsin at the centre (Figure 4.13B).

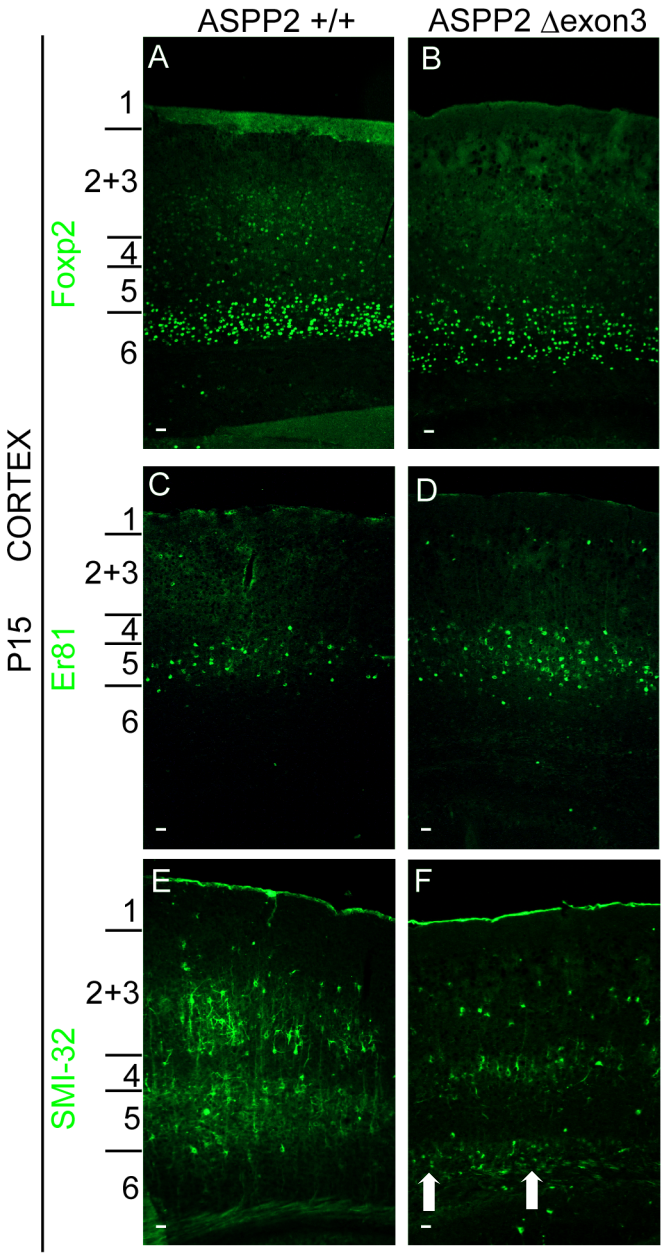


Figure 4.12: Lamination is impaired in *ASPP2* mutant cortex. (A-B) FoxP2, (C-D) Er81 and (E-F) SMI32 immunostaining of coronal sections from wild-type and *ASPP2* mutant P15 cortices. Arrows in F indicate mislocalised SMI32 positive cells in layer 6 of *ASPP2* mutant cortex. Scale bar 10μm.

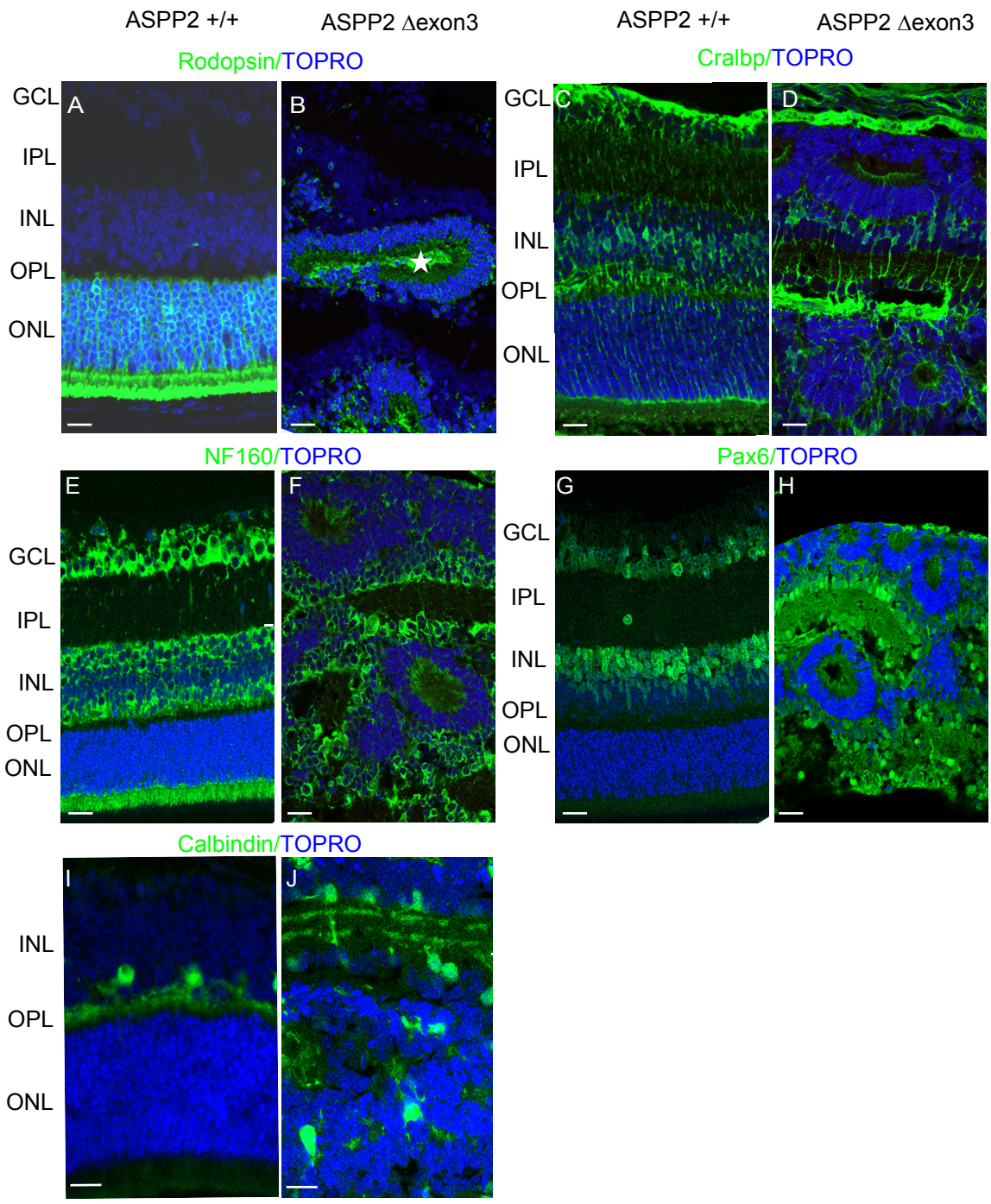


Figure 4.13: Lamination is impaired in *ASPP2* mutant retina. Coronal sections from P15 wild-type and *ASPP2* mutant retina stained with antibodies against different retina cell type markers: Rhodopsin (A-B) rods; CRALBP (C-D) Muller glia; Neurofilament 160 (E-F) Horizontal and Retinal ganglionic cells; Pax6 (G-H) Amacrine and Retinal ganglionic cells; Calbindin (I-J) cones. Star in (B) indicates the heavy staining of rhodopsin at the center of a rosette-like structure. GCL: ganglionic cell layer; IPL: inner plexiform later; INL: inner nuclear layer; ONL: outer plexiform layer; ONL: outer nuclear layer. Topro (blue) has been used as a counterstain. Scale bar: 20 μm.

4.6 Summary

The detailed analysis of the embryonic CNS of *ASPP2* mutant mice described in this chapter reveals a new function of ASPP2 in controlling epithelial cell polarity. RGCs showed a complete loss of radial orientation and a randomised arrangement in the developing neuroepithelium, in several area of the CNS. The mis-localisation of proteins that are normally distributed apically in the neuroepithelium, like components of the PAR complex and TJ/AJ molecules, indicates a loss of apical-basal cell polarity. Interestingly, ASPP2 co-localised extensively with PAR3 and ZO-1, but only partially with β–catenin, at the apical side of neuroepithelium, suggesting that ASPP2 mainly associates with the PAR polarity complex and TJs in the neuroepithelium.

The lack of tissue organisation observed in *ASPP2* mutant embryos severely impacted upon the architecture of the neo- and postnatal cortex and retina. Cortical layers were not clearly defined in *ASPP2* mutant embryos and mitotic cells were found to be dispersed throughout the cerebral wall. The lamination of the embryonic neocortex was also affected in *ASPP2* mutant embryos, as suggested by the random distribution of apical (Pax6 positive), basal (Tbr2 positive) progenitor and post-mitotic neurons. Mis-localisation of late-born neurons in deeper layers of the cortex was observed postnatally, while early-born neurons were unaffected. The number of early-born neurons seemed to decrease in *ASPP2* mutant postnatal cortices, suggesting that ASPP2 deletion may specifically affect the differentiation and migration of specific populations of neurons.

Although all of the retinal cell types were detected, defective lamination was also observed in the postnatal retina, suggesting a broader role for ASSP2 in controlling lamination in the developing CNS. Due to the massive disorganisation of the retina, it was

not possible to quantify the different retinal cell types. Therefore, further studies are needed to elucidate the role of ASPP2 in the differentiation of the retina. Finally, analysis of *ASPP2* mutant embryos also revealed a massive expansion of the neuroepithelium, raising the possibility that ASPP2 has functions in the neuroepithelium of the developing CNS, in addition to its role in controlling cell polarity.

5. ASPP2 negatively regulates proliferation during the development of the central nervous system

The expansion of the neuroepithelium in *ASPP2* mutant embryos described in Chapter 4 suggests that ASPP2 could control not only the polarity, but also the proliferation, of neuroepithelial and radial glial cells (also called NSCs). NSCs of the mammalian CNS form a polarised neuroepithelium, the VZ, which delimits the lumen of the developing neural tube. These cells have the ability to expand, generating additional NSCs, and to differentiate, giving rise to more committed progenitors or post-mitotic cells. NSCs can either divide symmetrically to expand the NSC population, or asymmetrically to generate a new stem cell and a cell with a more restricted differentiation potential, which will eventually give rise to a terminally differentiated cell. The process of asymmetric division relies on the establishment of polarity in the cell, which enables the asymmetric segregation of cell-fate determinants (see section 1.4.5).

Two main pathways are known to play a pivotal role in the regulation of NSC proliferation and differentiation: Notch and β-catenin/Wnt signalling. The up-regulation of the *Hes* and *Herp* genes by Notch signalling antagonises proneural genes like *Mash1*, inhibiting neuronal differentiation and maintaining the NSC pool. Over-expression of *Hes* genes in mouse embryos has been shown to inhibit neurogenesis and promotes NSC maintenance (Ohtsuka et al., 2001). Notch signalling has also been shown to directly regulate the proliferation of radial glial cells and cerebellar granule neuron precursor cells (Chambers et al., 2001; Solecki et al., 2001).

The expression of stabilised β-catenin, which is the central component of the Wnt signalling cascade, leads to the expansion of the progenitor pool in several areas of the

CNS (Chenn and Walsh, 2002; Zechner et al., 2003), underlying the importance of β-catenin/Wnt signalling in the regulation of NSC proliferation. Moreover, perturbation of β-catenin/Wnt signalling, following the disruption of epithelial cell polarity, has also been associated with an increase in cell proliferation (see section 1.3.6).

The aim of the work described in this Chapter is, therefore, to investigate the role played by ASPP2 in controlling NSC proliferation *in vivo* and *in vitro*. The relationship between ASPP2 and the Notch/Wnt signalling pathways, in the context of embryonic CNS development, will also be addressed. Finally, a possible link between ASPP2 and asymmetric cell division will be explored.

5.1 ASPP2 inhibits neural stem cells proliferation

It was possible that the expansion of the neuroepithelium that characterize *ASPP2* mutant embryos could be caused by an increase in cell proliferation. In order to test this hypothesis, mitotic cells were labelled in wild-type and mutant embryos using an antibody against phosphorylated histone H3 (anti P-H3) at two different time points. Since histone H3 is phosphorilated at Serine 10 only when chromosomes condensates during mitosis, the anti P-H3 antibody can be used as a marker for dividing cells. The mitotic index, defined as the ratio of mitotic cells (P-H3+) to the total cell number, revealed an increase in the percentage of mitotic cells in the *ASPP2* mutant ganglionic eminence (GE), in comparison to that seen in wild-type embryos (Figure 5.1A). The increase in cell proliferation was more severe at E13.5, although the mitotic index was already higher in *ASPP2* mutant embryos at E11.5 when compared to the one of wild-type embryos. Similar results were obtained in the neocortex of *ASPP2* mutant embryos (Figure 5.1B). Since the

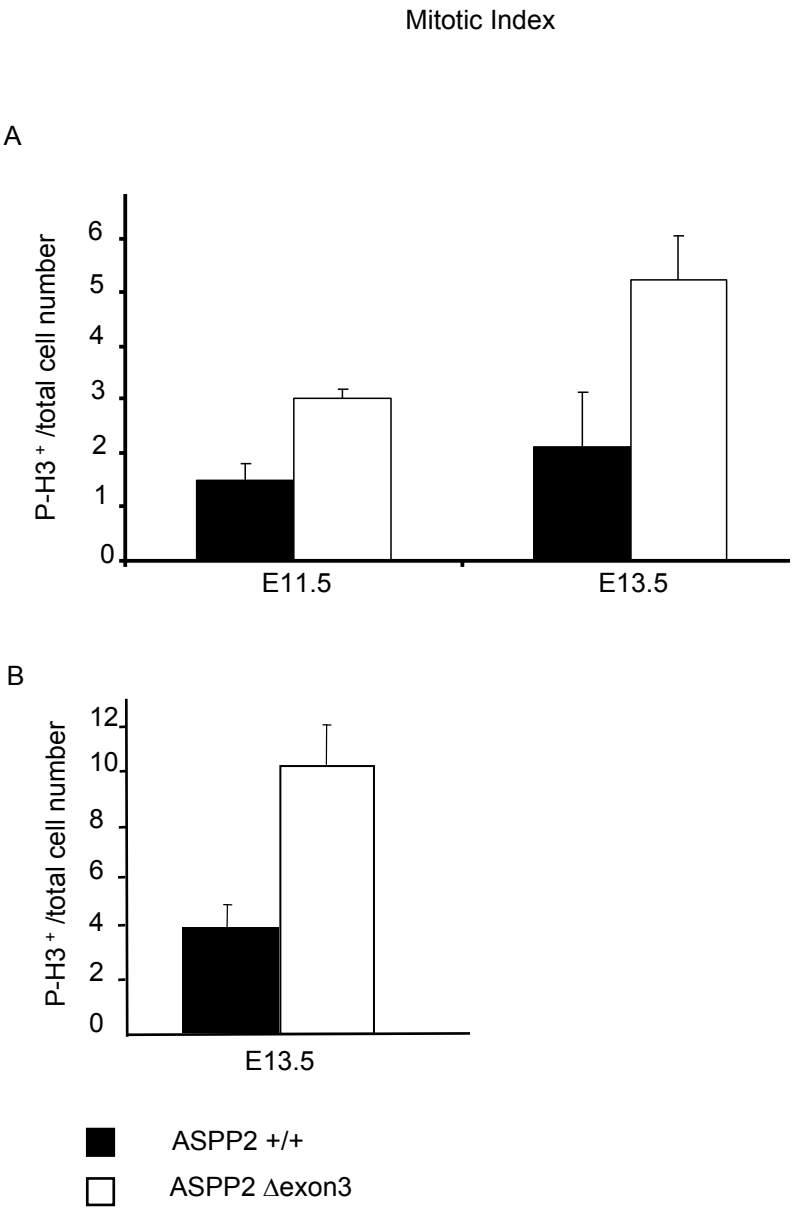


Figure 5.1: Activation of cell proliferation in *ASPP2* mutant embryos. Quantification of the mit otic index defined as the ratio of mitotic cells (P-H3 +) to total cell number in the GE (A) and cortex (B) of wild-type and *ASPP2* mutant embryos. Errors bar represent the standard deviation of three independent experiments.

observed over-proliferation was more evident after E11.5,. *ASPP2* mutant embryos were mainly analysed between E12.5 and E13.5.

In order to establish the identity of the overgrown cells in the *ASPP2* Δexon3 homozygous neuroepithelium, immunohistochemistry (IHC) was performed with cell-type specific markers at E12.5. Ki67 is a cell-cycle related nuclear protein, expressed by proliferating cells in all phases of the active cell cycle (G1, S, G2 and M phase), but absent in resting (G0) cells. NSCs are highly proliferative and express Ki67, which can be used as a marker for this particular cell type (Hartfuss et al., 2001) in the CNS. *Hes5* is a downstream target of the Notch signalling pathway and its expression can similarly be used to identifies NSCs (Basak and Taylor, 2007). Ki67 and Hes5 expression, only visible expressed in the VZ of wild-type embryos (Figure 5.2A-C), was found to be scattered throughout the whole of the *ASPP2* mutant brain (Figure 5.2B-D). Cells present in the rosette-like structures were also positive for the progenitor marker Hes5 (Figure 5.2D).

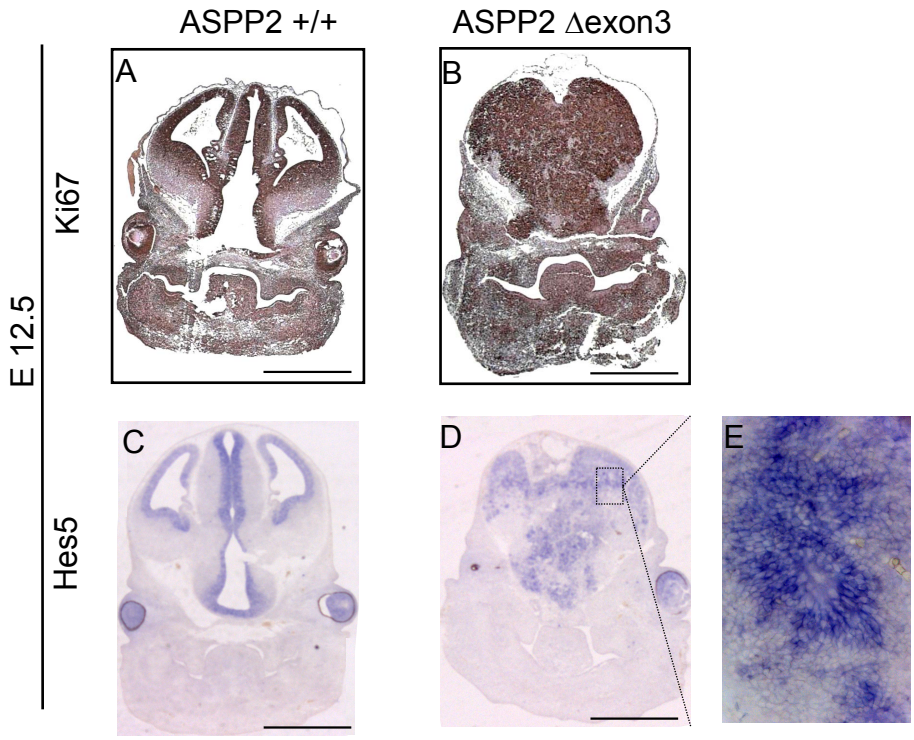


Figure 5.2: Expansion of the neural precursor pool in the *ASPP2* mutant embryos. Ki67 immunostaining of coronal sections from wild-type (A) and *ASPP2* mutant embryos (B) at E12.5. Hes5 *in situ* hybridazation of coronal sections from wild-type (C) and *ASPP2* mutant (D) at E13.5. The boxed region in D is shown at higher magnification in (E) and contains a rosette-like structure. Scale bar 1 mm.

5.2 Shortening of the cell cycle, but not reduction of apoptosis, in *ASPP2* mutant neuroepithelium

The enlargement of the neural progenitor pool observed in the *ASPP2* mutant embryos can be caused by a shortening of the cell cycle, reduced apoptosis or a combination of both. To identify any changes in cell cycle length, pregnant females from an *ASPP2* heterozygous mating were injected with BrdU, which labels cells in the S phase of the cell cycle, and sacrificed after 30 minutes. The proportion of NSCs, identified by Ki67 immunoreactivity, labeled by BrdU was then counted in the wild-type and *ASPP2* mutant GE to provide an estimate of cell-cycle length, as described by Chenn and Walsh (Chenn and Walsh, 2002) (Figure 5.3 A-B). A 2-fold increase in the percentage of BrdU-positive progenitors was observed in *ASPP2* mutant over wild-type brains (Figure 5.3C). Since the length of S phase in mammalian cells is constant (DiSalvo et al., 1995), the increase in the fraction of neural stem cells labeled by BrdU, in the short time frame of the experiment, indicates a shortening of the cell cycle.

Apoptosis occurs during normal development of the CNS (Kuan et al., 2000) and decreased apoptosis may be one of the mechanisms underlying the expansion of the neural precursor pool observed in *ASPP2* mutant brains. Given that ASPP2 enhances p53-mediated apoptosis, its deletion should in theory result in a decreased apoptotic rate. Activation of Caspase-3 is the final step in the apoptotic cascade, thus IHC using anti-active Caspase-3 antibodies can identify cells undergoing apoptosis. The number of apoptotic cells was, thus, compared in the GE between wild-type and *ASPP2* mutant embryos in areas of 10000 μM^2. Unexpectedly, the number of apoptotic cells detected in *ASPP2* mutant brains was higher than that in the wild-type (Figure 5.4A-B).

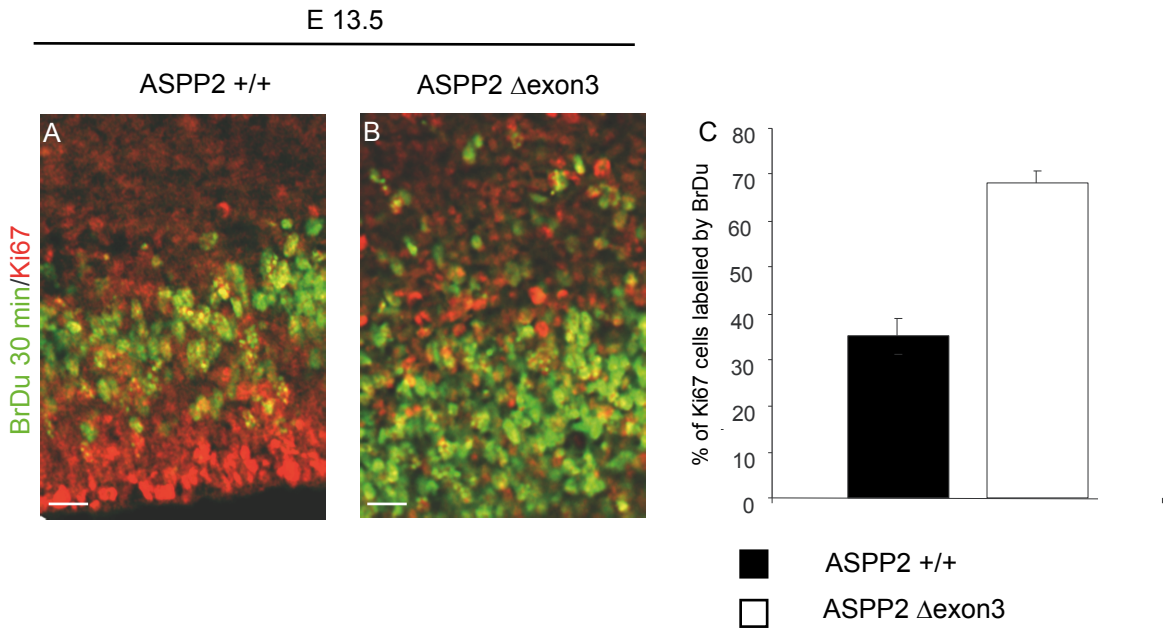


Figure 5.3: Increase in proliferation in *ASPP2* mutant brain is due to shortening of the cell cycle. Double staining of BrdU and Ki67 of coronal sections from wild-type (A) and *ASPP2* mutant (B) GE after a single 30 minute BrdU pulse at E13.5. (C) Estimation of cell cycle length as a percentage of Ki67 cells labelled by BrdU, after a 30 minute single pulse, in wild-type and *ASPP2* mutants at E13.5. Error bars represent the standard deviation of three independent experiments. Staining patterns and corresponding antibodies are colour-coded. Scale bar 10 μm.

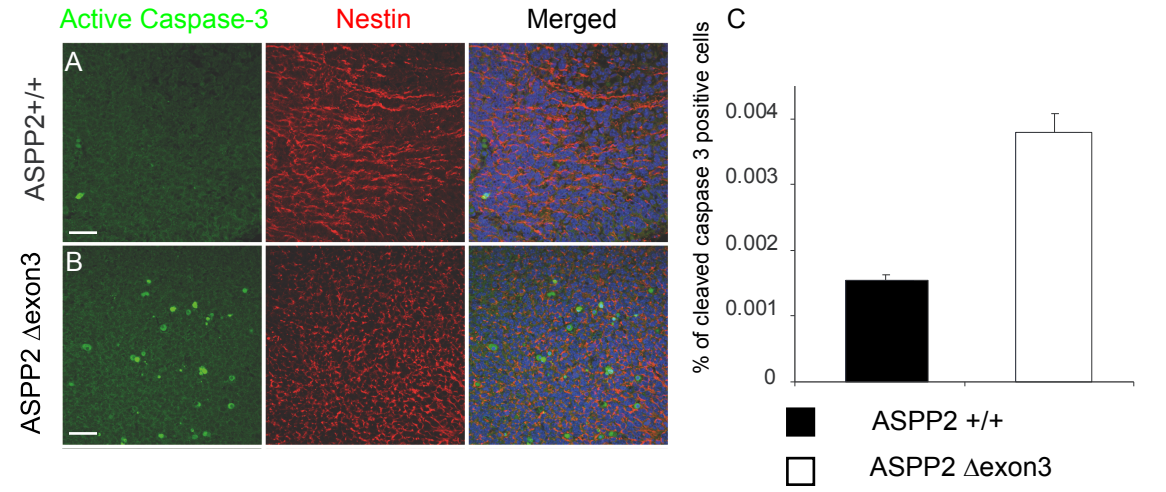


Figure 5.4: Apoptosis is not reduced in *ASPP2* mutant embryos. Cleaved caspase-3 and nestin immunostaining of wild-type (A) and *ASPP2* mutant (B) GE at E13.5. Staining patterns and corresponding antibodies are colour-coded. Topro (blue) has been used as a counterstain. Scale bar 10μm. (C) % of cleaved caspase 3 positive cells in the GE of wild-type and ASPP2 mutant mice. Three mice each genotype were included in the quantification

5.3 Notch signalling is up-regulated in neural stem cells derived from *ASPP2* mutant telencephala

The expansion of the progenitor pools which characterise *ASPP2* mutant mice could be explained by increased Notch signalling. *In situ* hybridisation (ISH) with a probe against the Notch signalling target *Hes5* revealed an expansion of cells with active Notch signalling. Since ISH does not reveal changes in mRNA expression unless of several order of magnitudes due to the qualitative nature of the detection system , the levels of *Hes5* and *Hes1*, another Notch target, were measured in NSCs derived from wild-type and *ASPP2* mutant mice by quantitative real-time PCR (qPCR) to asses the activity of Notch signalling. NSCs were isolated at E11.5 when over-proliferation was first observed in *ASPP2* mutant neuroepithelium. The expression of *Hes5* was significantly up-regulated in NSCs derived from *ASPP2* mutant mice (Figure 5.5A), indicating increased Notch signalling activity, while the expression level of *Hes1* was unchanged (Figure 5.5B).

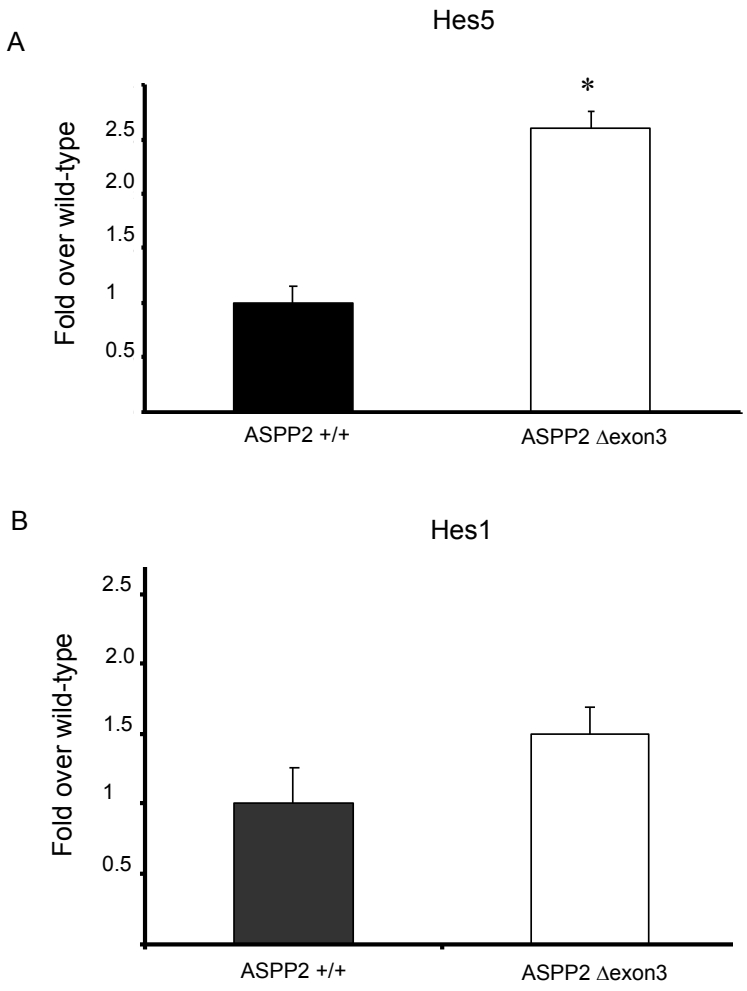


Figure 5.5: Notch signalling is up-regulated in neural stem cells derived from *ASPP2* mutant telencephala. qPCR analysis of Hes5 (A) and Hes1 (B) transcripts in wild-type and *ASPP2* mutant NSCs. The levels of expression are represented by arbitrary unit with wild-type level adjusted to one. Error bars represent the standard deviation of three independent experiments. $P<0.01$ (paired student's *t*-test).

5.4 ASPP2 selectively antagonises the transcription of the Notch target gene *Hes5,* independently of TAp73

In order to verify whether ASPP2 modulates Notch signalling at the level of gene transcription, a transactivation assay using *Hes1* and *Hes5* reporter constructs was performed. Since NSCs cannot be easily transfected, the human glioblastoma-astrocytoma epithelial-like cell line U373 was used in this assay. Hes1 and Hes5 reporter constructs were transfected singly, or in combination with DNA encoding the Notch intracellular domain (NICD) and increasing amounts of ASPP2. While ASSP2 was able to antagonise NICD-induced Hes5-Luc activity by up to 40%, it had no effect on Hes1-Luc activity (Figure 5.6A-B). This result is in agreement with the selective upregulation of the Hes5 promoter detected in *ASPP2* mutant NSCs.

The transcription factor TAp73, previously identified to be a binding partner of ASPP2 (Bergamaschi et al., 2004), plays a pivotal role in mammalian neural development (Abraham et al., 2004; Meyer et al., 2002) and has recently been shown to antagonise Notch signalling in neuroblastomas and primary neurons (Hooper et al., 2006). In order to test whether ASPP2 cooperates with TAp73 in repressing Notch signalling, the *Hes5* reporter construct was transfected into U373 cells in combination with DNA encoding the NICD, ASPP2 and TAp73. While ASPP2 and TAp73 were equally capable of inhibiting NICD-induced Hes5-Luc activity, no synergistic effect was observed when the DNA encoding ASPP2 and TAp73 were co-transfected into U373 cells (Figure 5.6C).

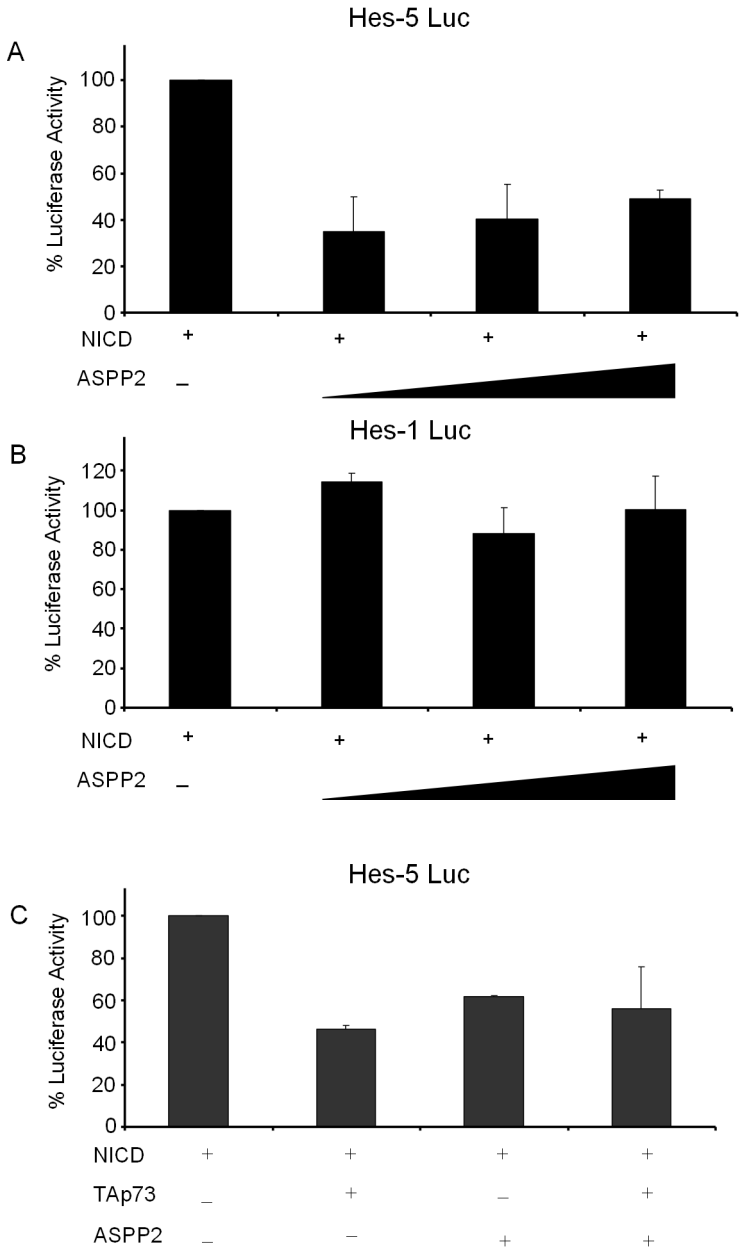


Figure 5.6: ASPP2 antagonises Notch signalling in U373 cells independently of p73. U373 cells were transfected with Hes5 (A) and Hes1 (B) reporter constructs, NICD alone or in combination with different amount of ASPP2. (C) U373 cells were transfected with Hes5 reporter constructs, NICD alone or in combination TAp73 and/or ASPP2 as indicated . Cells were lysed and luciferase activity was determined 24 hours after transfection. Renilla luciferase activity was determined and used to normalise for transfection efficiency. Results are expressed as percentage of luciferase activity of cells transfected with NICD alone. Error bars represent the standard deviation of three independent experiments.

5.5 *ASSP2* deletion does not lead to ectopic activation of β-catenin/Wnt signalling

Alteration of β-catenin/Wnt signalling represented another mechanism that could explain the phenotype of *ASPP2* mutant mice. Although β-catenin/Wnt signalling is no longer active in the GE at E12.5, where over-proliferation is observed, the loss of cell polarity caused by *ASPP2* deletion could in theory cause ectopic activation of Wnt signalling, and therefore an increase in cell proliferation. In order to test this hypothesis, the presence of nuclear β-catenin was analysed in the over-proliferative area of the neuroepithelium by ISH, with a probe against Axin2 at E12.5. Axin2 is up-regulated in response to an increase in the nuclear β-catenin concentration, and functions to limit the duration and intensity of Wnt signalling (Kikuchi and Yamamoto, 2008; Lustig et al., 2002). Thus, detection of Axin2 mRNA indicates the activation of Wnt signalling. According previously published observations (Machon et al., 2007), β-catenin/Wnt signalling is active in the hippocampal primordium of the telencephalon of wild-type mice at E12.5, where a strong signal was detected in this study (Figure 5.7A). In the *ASPP2* mutant telencephalon, the hippocampal primordium could not be clearly identified. This was due to the massive disorganisation caused by over-proliferation of cells, rather than a lack of hybridisation, as a positive signal was detected in the mutant brain (Figure 5.6B). No hybridasation was detected in the over-proliferating neuroepithelium of the GE (Figure 5.7B), indicating that *ASPP2* deletion does not cause ectopic activation of β-catenin/Wnt signalling.

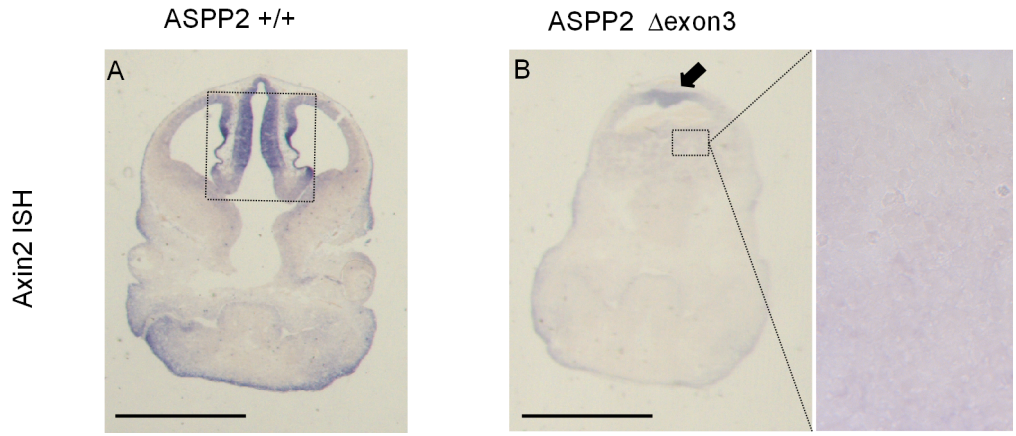


Figure 5.7: Wnt signalling is not activated following ASPP2 deletion. *In situ* analysis of Axin2 expression in wild-type (A) and *ASPP2* mutant (B) embryos at E12.5. The boxed region in (A)indicates the hippocampal primordium. The arrow in (B) points at an area of positive Axin2 hybridazation. The boxed region in (B) is shown at higher magnification in C and indicates an overproliferating area of the neuroepithelium which is negative for Axin2 expression. Scale bar 1mm.

5.6 ASPP2 co-localises with the tumour suppressor Lgl1

Failure of cells to divide asymmetrically can lead to an expansion of the NSC pool, causing an increase in cell proliferation. Given that asymmetric cell division relies on the establishment of cellular polarity, a defect in this process might explain how ASPP2 can control cell polarity and also affect proliferation. Lgl1, a tumour suppressor in *Drosophila* as well as in mammals, is involved in the regulation of asymmetric cell division. It is essential for the asymmetric distribution of the cell fate determinant Numb, which inhibits Notch signalling in cells undergoing differentiation (Vasioukhin, 2006). Mice deficient for Lgl1 display cell polarity defects accompanied by disruption of the AJC. Lgl1-null progenitors fail to asymmetrically localise Numb and, therefore, continue to divide symmetrically generating more progenitors (Klezovitch et al., 2004). This phenotype of Lgl1-null mice is remarkably similar to that of ASPP2 mutant mice, suggesting that ASPP2 and Lgl1 may function in the same genetic pathway.

In order to determine whether ASPP2 and Lgl1 interact, MCF7 cells were transfected with plasmid expressing V5-tagged ASPP2 and Flag-tagged Lgl1. The two transfected proteins were found to co-localise strongly at the cell membrane (Figure 5.8). However, the lack of a specific Lgl1 antibody did not allow any further characterisation of the interaction between Lgl1 and ASPP2.

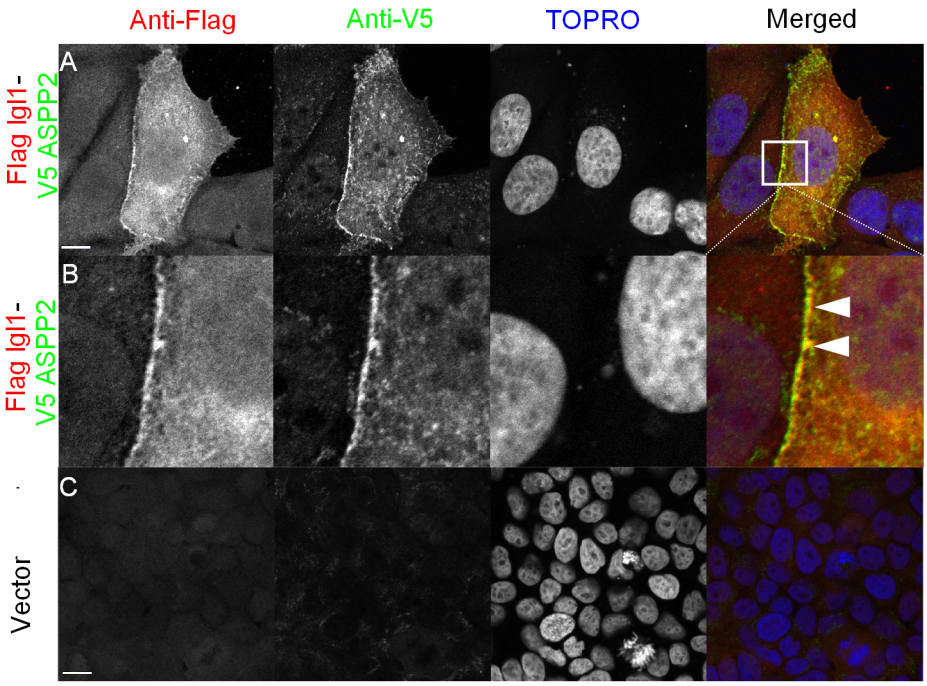


Figure 5.8: ASPP2 colocalises with Lgl1 in MCF-7 cells. (A-B) Double immunostaining of transfected Flag-Lgl1 and V5-ASPP2 in MCF7 cells. The boxed region in (A) is enlarged in (B). Arrows in B indicate colocalisation between ASPP2 and Lgl1 at the cell membrane. (C) Double immunostaining of MCF7 cells transfected with the empty vectors. Staining patterns and corresponding antibodies are colour-coded. Individual channels are shown in grey scale. Topro (blue) has been used as a counterstain. Scale bar 10 µm.

5.7 Summary

The work described in this Chapter reveals that ASPP2 negatively regulates the proliferation of NSCs. The mitotic index was significantly increased in *ASPP2* mutant telencephala in both the cortex and GE, with a consequent expansion of the NSC pool, as identified by markers like Ki67 and Hes5. Analysis of cell-cycle length by BrDu labelling revealed a shortening of the cell cycle in *ASPP2* mutant NSCs. The apoptotic rate was also increased in *ASPP2* mutant neuroepithelia, indicating that cell death occurs in the absence of ASPP2. Taken together, these results suggest that the observed increase in cell proliferation in *ASPP2* mutant neuroepithelia is due to a shortening of the cell cycle, but not a lack of apoptosis.

The relationship between ASPP2 and the two main signalling pathways involved in NSC maintenance was analysed via *in vivo* and *in vitro* approaches. Up-regulation of *Hes5* expression in NSCs isolated from the *ASPP2* mutant telencephalon, revealed increased Notch signalling activity which could result in augmented cell proliferation. Since NSCs were isolated from the telencephalon at E11.5, when proliferation started to increase, the observed upregulation of Notch signalling was likely to be a primary effect resulting from the deletion of ASPP2. Interestingly, no difference was observed between wild-type and mutant NSCs in the level of *Hes1* expression. Similarly, ASPP2 was only able to suppress NICD induced *Hes5* transcription, without affecting *Hes1*, in reporter assays. This result suggests that ASPP2 is able to select and differently regulate these promoters, consistent with its already known ability to discriminate between p53-related apoptotic and cell cycle arrest genes. In order to finally conclude that ASPP2 selectively binds to the promoter of *Hes5* further approaches, like Chromatin immunoprecipitation (Chip), will be needed. Alternatively, the selective up-regulation of *Hes5* may be explained by the fact that *Hes1*

expression, in contrast to that of *Hes5*, is regulated by other signalling pathways (see section 1.4.6.1). *Hes5* expression starts at the same point as Notch1, whereas *Hes1* mRNA is expressed in the neural ectoderm before *Notch1* or *Hes5* are detected and remains expressed in the absence of Notch1 (de la Pompa et al., 1997). When a transactivation assay was used to evaluate whether ASPP2 and TAp73 are able to cooperate in inhibiting Notch signalling, no synergistic effect was observed on Hes5-Luc activity when TAp73 and ASPP2 were co-expressed. This result suggests that, in this context, ASPP2 acts independently of p73.

Ectopic activation of β-catenin/Wnt signalling, caused by a loss of cell polarity, is unlikely to contribute to the over-proliferation of the neuroepithelium in *ASPP2* mutant embryos. ISH with Axin2 failed to detect active β-catenin/Wnt signalling in the over-proliferating neuroepithelia of *ASPP2* mutant embryos, indicating that ASPP2 deletion does not have an impact on β-catenin/Wnt signalling.

Finally, the similarity between the phenotypes of *ASPP2* and *Lgl1* mutant mice, together with the observed co-localisation of the two transfected proteins, suggests that ASPP2 may be involved in the process of asymmetric cell division. A failure in this process may also be one of the underlying mechanisms behind the increase in cell proliferation caused by the deletion of ASPP2.

6. Discussion

6.1 ASPP2 plays a new role in the development of the central nervous system.

ASPP2 has been identified as a haplo-insufficient tumour suppressor in mice (Kampa et al., 2009; Vives et al., 2006) and belongs to the ASPP family of proteins. To date, the best known function of ASPP2 is its binding to p53, p63 and p73, and the stimulation of their apoptotic functions by selectively enhancing their DNA binding and transactivation activities on pro-apoptotic genes such as *BAX* and *PIG3* (Bergamaschi et al., 2004; Samuels-Lev et al., 2001). However, in the past decade, ASPP2 has also been identified as a binding partner of a number of other proteins (Sullivan and Lu, 2007). ASPP2 may, therefore, integrate different signalling pathways and functions independently of its ability to mediate p53-dependent apoptosis. The work described in this thesis sheds new light on the biological functions of ASPP2, showing that it is able to control multiple aspects of the CNS. The deletion of ASPP2 *in vivo* leads to the hydrocephalus, independently of the genetic background (chapter 3). Electron microscopy analysis of the choroid plexus (CP) revealed that tight junctions (TJs) at the apical pole fail to close properly in the *ASPP2* mutant CP, suggesting that ASPP2 is required for proper TJ assembly or maintenance *in vivo.* TJs between epithelial cells are responsible for the blood-CSF barrier property of the CP, which prevents the leakage of blood-borne molecules into the CSF. The defects observed in TJs could be responsible for a leakage of the blood-CSF barrier which may cause an increase in osmotic pressure, with consequent accumulation of water therefore

resulting in hydrocephalus. Since the beating of ependymal cilia ensures the movement of CSF within the ventricles, the lack of ependymal cells on the ventricular surface of ASPP2 mutant brain, could also contribute to the development of hydrocephalus. It will be interesting to further investigate the involvement of ASPP2 in the formation of ependymal cells by analysing wild-type and ASPP2 mutant embryos at E16 and E18 when majority of the ependymal cells are born (Spassky et al., 2005).

In addition to the development of hydrocephalus, *ASPP2* deletion profoundly affected the lamination of the cerebral cortex and retina (Chapter 4). Apical and basal progenitors were randomly distributed in the ASPP2 mutant cerebral cortex, intermingled with post mitotic cells. This phenotype may be caused by an impaired cell migration, which heavily contributes to the shaping of highly laminated structure in the CNS, or may be a consequence of the massive tissue disorganization observed in the ASPP2 mutant neuroepithelium (chapter 4). Interestingly, interkinetic nuclear migration (INM) was not impaired *in vitro* when RGCs were grown on a support of laminin, indicating that *ASPP2* deletion does not directly affect nucleokinesis. The mislocalization of progenitor cells in the ASPP2 mutant cortex, is likely to be a consequence of the tissue disruption caused by *ASPP2*'s deletion. The nuclei of RGCs need an apical contact in order to exert a pulling force and migrate. *In vitro*, where anchorage is provided by the culture substrate, nuclei of *ASPP2* mutant cells were able to migrate normally. In contrast, *in vivo* RGCs lost their apical contacts due to massive tissue disorganisation, and some of their nuclei failed to migrate up to the apical side, and instead remained dispersed throughout in the cerebral wall. Since RGCs provide the scaffold along which post-mitotic neurons migrate (see section 1.4.3), loss of the radial orientation of RGCs could account for the impaired radial migration observed in the *ASPP2* mutant cortex.

It will be interesting to investigate whether ASPP2 directly influences cell migration by *in vivo* microscopy.

6.2 ASPP2's role in cell polarity and proliferation during the development of the central nervous system

The analysis of the choroid plexus of *ASPP2* mutant mice revealed that ASPP2 is required for TJ formation or maintenance *in vivo* (Chapter 3). Moreover, severe disorganisation, accompanied by loss of apical-basal cell polarity in the neuroepithelium, was observed in several areas of the developing CNS of *ASPP2* mutant embryos (Chapter 4), suggesting that ASPP2 plays a pivotal role in the maintenance of cell polarity. Given that the integrity of the AJC, constituted by TJs and AJs, is required for the maintenance of cell polarity, ASPP2 could owe its newly identified function to its ability to affect TJs. Interestingly, ASPP2 was found to extensively co-localise with ZO-1 and PAR3, but only partially with β–catenin, in the neuroepithelium and also in epithelial cell lines, indicating that ASPP2 preferentially interacts with TJs rather than AJs (Chapter 4 and Sottocornola et al. 2010). A study conducted recently by our group (Sottocornola et al. 2010) has shown that ASPP2 is recruited *in vitro* with Par-3, but not β–catenin, at primordial cell/cell contacts and is also required for apical localisation of PAR3. In addition, primary kidney epithelial cells (PKECs) of *ASPP2* mutant mice show delayed kinetics in the re-establishment of cell-cell junctions after disruption by calcium depletion. Taken together, these results suggest that ASPP2 could affect TJ formation and, therefore, cell polarity through its ability to control PAR3 localisation.

In addition to its ability to control cell polarity, ASPP2 plays an important role in inhibiting cell proliferation in the developing mammalian brain, as increased cell proliferation and expansion of the NSC pool are also prominent features of *ASPP2* mutant embryos (Chapter 5). Despite the massive overproliferation observed in the developing embryos, the postnatal cortex of ASPP2 mutant mice appears thinner than the one of wild-type mice. Although the hydrocephalus could contribute to the thinning of the cortex, this phenotype could be explained by an increase in neuronal apoptosis at later stages. Since a lack of differentiation was observed in the neo- and post-natal cerebral cortices of *ASPP2* mutant mice, as indicated by the decrease in the percentage of βIII-tubulin positive neurons in the embryo and early born neurons postnatally (Chapter 4), ASPP2 may also be required for proper neuronal differentiation. Although the precise mechanism by which ASPP2 controls proliferation and differentiation still needs to be elucidated, the work described in this thesis suggests that ASPP2 exerts these functions by repressing Notch signalling, which is known to activate proliferation and inhibit differentiation in the CNS (see sections 1.4.6.1 and 1.4.6.3). Quantification of the expression levels of Notch target genes, by quantitative real time PCR in NSCs, revealed that *ASPP2* deletion leads to up-regulation of Notch signalling (Chapter 5). Transcription assays performed using reporter constructs for the Notch target gene Hes5 in combination with ASPP2, indicate that ASPP2 is able to repress Notch signalling by directly inhibiting the transcription of Notch targets. The intracellular domain of Notch (NICD) activates gene transcription by interacting with CBF-1 through its Ankyrin repeat domain (Lubman et al., 2004). An ongoing study conducted by our group has shown that ASPP2, which also has an Ankyrin repeat domain, physically interacts with CBF1 (L.Tordella, personal communication).

ASPP2 may, therefore, compete with the NICD for binding to CBF1, thereby repressing Notch signalling.

In addition to the up-regulation of Notch signalling, the increased proliferation observed in *ASPP2* mutant embryos is also compatible with a failure in asymmetric division. NSCs divide asymmetrically to generate a new progenitor, and another cell that has a more restricted differentiation potential and will eventually give rise to a terminally differentiated cell. Failure of cells to divide asymmetrically can result in an expansion of the NSC pool and, thus, in an increase in cell proliferation. Interestingly, ASPP2 co-localises with the *Drosophila* tumour suppressor Lgl1 (Chapter 5), which plays a pivotal role in controlling asymmetric cell division by regulating the segregation of cell fate determinants in both *Drosophila* and mammals (Vasioukhin, 2006). This observation, together with the remarkable similarity between the phenotypes of *ASPP2* and *Lgl1* mutant mice, raises the possibility that ASPP2 and Lgl1 may act in the same pathway to control the segregation of cell fate determinants and, thus, asymmetric division. Interestingly, a recent study suggests that PAR3 may function as a cell fate determinant in cortical NSCs (Bultje et al., 2009). PAR3 is, in fact, asymmetrically distributed in dividing NSCs and the ectopic expression of PAR3, results in it being present in both daughter cells, promotes NSC fate. As ASPP2 controls the localisation of PAR3 in epithelial cells, it is possible that deletion of *ASPP2* would lead to a failure in the asymmetric distribution of PAR3 in dividing NSCs, thereby causing an expansion of the NSC pool and increased proliferation. In order to verify this possibility, further *in vivo* and *in vitro* approaches are needed to monitor the distribution of PAR3 in dividing wild-type versus *ASPP2* mutant cells, and evaluate the outcome of *ASPP2* deletion on the mode of NSC division.

As p53 negatively regulates the proliferation of NSCs and ASPP2 is a p53-binding protein, its regulation of cell proliferation could, in theory, involve p53. Down-regulation of p21, the expression of which is normally induced by p53, is thought to be responsible for the increased proliferation of p53-null NSCs. As ASPP2 enhances the ability of p53 to activate apoptotic, but not cell cycle-related genes like p21, it is likely that ASPP2 controls cell proliferation independently of its function as an activator of p53-mediated gene transcription. p73, which also binds ASPP2, has been shown to antagonise Notch signalling in neuroblastoma cell lines. Since ASPP2 also represses Notch signalling in NSCs, it could cooperate with p73 to exert this function. However, transcription assays, with a reporter construct for Notch targets in combination with p73 and ASPP2, did not shown any synergic effect of ASPP2 and p73 on Notch signalling, suggesting that ASPP2 acts independently of p73 in this context. Due to the embryonic lethality of the ASPP2/p53 (Vives et al., 2006) phenotype, studies with conditional knock-out mice are needed to fully determine whether or not the ASPP2's function in controlling cell proliferation, identified here, is mediated by p53 .

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